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DOTTORATO DI RICERCA IN

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Innovative approaches for molecular diagnosis of genetic diseases

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ABBREVIATIONS

AFP:	α-feto protein
ARMS-PCR:	amplification refractory mutation system-polymerase chain reaction
BAC:	bacterial artificial chromosome
BIA:	biomolecular interaction analysis
bp:	base pairs
CCD:	charge coupled device
ccffDNA:	circulating cell-free fetal DNA
cfDNA:	circulating cell-free DNA
CeNA:	cyclohexene nucleic acids
cenM-FISH:	centromere-specific multicolour fluorescence *in situ* hybridization
cfDNA:	circulating fetal DNA
CFTR:	cystic fibrosis transmembrane conductance regulator gene
CGH:	comparative genomic hybridization
CHMP:	committee for medicinal products for human use
CNV:	copy number variation
COBRA-FISH:	combined binary ratio labelling fluorescence *in situ* hybridization
COLD-PCR:	co-amplification at lower denaturation temperature-polymerase chain reaction
CRISPR:	clustered regularly interspaced short palindromic repeats
Ct:	threshold cycle value
Da:	Dalton
DAPI:	4'-6-diamidino-2-phenylindole
DAZ:	deleted in azoospermia
ddNTP:	dideoxynucleoside triphosphate

DHPLC:	denaturing high performance liquid chromatography
DMD:	Duchenne muscular dystrophy
DMPK:	dystrophia myotonica protein kinase gene
dPCR:	digital polymeration chain reaction
DSB:	double strand break
dUTP:	2'-deoxyuridine 5'-triphosphate
DYS14:	testis specific protein Y-linked
EMA:	european medicine agency
EPO:	erythropoietin
FACS:	fluorescence-activated cell sorting
FAM^{TM}:	carboxyfluorescein
FANA:	2'-deoxy-2'fluoro-β-D-arabino nucleic acid
FDA:	food and drug administration
FGFR3:	fibroblast growth factor receptor 3 gene
FISH:	fluorescence *in situ* hybridization
FITC:	fluorescein isothiocyanate
FMR1:	fragile X mental retardation 1
FMRP:	fragile X mental retardation protein
FRET:	fluorescence resonance energy transfer
FXS:	fragile X syndrome
GC-SPR:	grating coupled-surface plasmon resonance
GEq:	genome equivalence
GMO:	genetic modified organism
GVHD:	graft versus host disease
Hb:	hemoglobin
HbA:	adult hemoglobin

HBA:	alfa globin
HBB:	beta globin
HbF:	fetal haemoglobin
HBS-EP+:	HEPES-buffered saline-EP+
HD:	Huntington disease gene
HDR:	homology directed repair
HEX™:	carboxyhexachlorofluorescein
HIV:	human immunodeficiency virus
HIV:	human immunodeficiency virus
HPFH:	hereditary persistence of fetal haemoglobin
HPLC:	high performance liquid chromatography
HRM-PCR:	high resolution melt-polymerase chain reaction
HSPC:	hematopoietic stem and progenitor cell
IE:	ineffective erythropoiesis
IFC:	integrated microfluidic cartridge
IKBKG:	inhibitor of nuclear factor kappa B kinase gene
iPSC:	induced pluripotent stem cell
ISH:	*in situ* hybridization
IVS:	intervening sequence
Kb:	kilobase
KLF:	Krüppel like factor 1
LCR:	locus control region
LED:	light-emitting diode
LRSPR:	long-range surface plasmon resonance
LSPR:	localized surface plasmon resonance
MAA:	marketing authorisation application
MeDIP:	methylation DNA immunoprecipitation

MF:	morpholino phosphoroamidate
M-FISH:	multicolour fluorescence *in situ* hybridization
miRNA:	microRNA
MLPA:	multiplex ligation-dependent probe amplification
MNP:	multiple nucleotide polymorphism
mRNA:	messenger RNA
MSD:	matched sibling donor
MW:	molecular weight
NF-kB:	nuclear factor kappa B
NFQ:	non-fluorescent quencher
NGS:	next generation sequencing
NHEJ:	non-homologous end joining
NP:	N3'-P5' phosphoroamidate
NRBC:	nucleated red blood cell
NTDT:	non transfusion dependent thalassemia
PAC:	bacterial/P1-derived artificial chromosome
PAH:	phenylalanine hydroxylase gene
PAP:	pyrophosphorolysis-activated polymerization
PAPP-A:	pregnancy-associated plasma protein-A
PBS:	phosphate buffered saline
PCR:	polymerase chain reaction
PNA:	peptide nucleic acid
PNA:	peptide nucleic acid
pod-FISH:	parental origin determination fluorescence *in situ* hybridization
PPi:	pyrophosphate

PTC:	premature termination codon
Q-FISH:	quantitative fluorescence *in situ* hybridization
QF-PCR:	quantitative fluorescence polymerase chain reaction
qRT-PCR:	quantitative real time polymeration chain reaction
RASSF1:	Ras association domain family member 1
RBC:	red blood cell
RFLP-PCR:	restriction fragment length polymorphisms-polymerase chain reaction
RFU:	relative fluorescence unit
RhD:	rhesus D
ROX™:	rhodamine X
RU:	resonance unit
RUfin:	final resonance units
RUin:	initial resonance units
RUres:	residual resonance units
SA:	streptavidin
SCD:	sickle cell disease
SKY-FISH:	spectral karyotyping fluorescence *in situ* hybridization
SNP:	single nucleotide polymorphism
snRNA:	small nuclear RNA
SPR:	surface plasmon resonance
SPR-I:	surface plasmon resonance-imaging
SRY:	sex-determining region Y
STR:	short tandem repeat
TALEN:	transcription activator like effector nuclease
TAMRA™:	carboxytetramethylrhodamine

T-ARMS-PCR:	tetra-primer amplification refractory mutation system-polymerase chain reaction
tcDNA:	tricycle DNA
TDT:	transfusion dependent thalassemia
TEAA:	triethylammonium acetate
Tm:	melting temperature
UTR:	untranslated region
VIC®:	chlorophenyldichlorocarboxyfluorescein
wt:	wild type
ZFN:	zinc finger nuclease
ZFX:	zinc finger X-linked gene
ZFY:	zinc finger Y-chromosome gene
β-hCG:	β-human chorionic gonadotropin

ABSTRACT

Comunemente la diagnosi di patologie genetiche fetali viene effettuata a partire da materiale biologico prelevato tramite tecniche invasive come ad esempio l'amniocentesi o la villocentesi. La principale problematica associata a queste tecniche di prelievo è il rischio di aborto, pari circa all'1%. Inoltre, il prelievo non può essere effettuato prima dell'undicesima settimana di gestazione.

Per questo motivo, negli ultimi anni, la diagnosi prenatale non invasiva, essendo di grande interesse, ha avuto un'esponenziale crescita. Essa viene effettuata a partire da un semplice prelievo di sangue materno dal quale viene isolato il ccffDNA (DNA circolante fetale), senza alcun rischio per la madre e per il feto, diversamente dalle tecniche invasive. Dall'analisi del ccffDNA è possibile individuare patologie ad esso legate e monitorare lo stato della gravidanza.

Nel caso specifico, la diagnosi del sesso in età prenatale risulta di fondamentale importanza per definire il rischio di malattie legate al cromosoma X, tra le quali la distrofia muscolare di Duchenne e l'emofilia sono solo due esempi. Il sesso è predittivo del fenotipo patologico, infatti, in caso di malattie recessive legate al cromosoma X il rischio persiste solo per i soggetti di sesso maschile.

Una diagnosi precoce permette, in tempi brevi, l'inizio della terapia convenzionale prevista per la specifica malattia ed eventualmente una più specifica terapia personalizzata.

In questo scenario si colloca la prima parte della tesi di ricerca, che ha come obbiettivo lo sviluppo di approcci diagnostici molecolari non invasivi rapidi sensibili ed economici per la determinazione del sesso fetale, in particolare a settimane di gestazione precoci.

In uno studio precedentemente pubblicato dal nostro gruppo di ricerca, la Real Time PCR e la tecnologia Biacore™ (biosensore di affinità basato sul fenomeno della risonanza plasmonica di superficie) sono state impiegate per la determinazione del sesso fetale a partire da 26 campioni di ccffDNA, estratti da plasma materno, andando a verificare la presenza o meno nel gene SRY, presente sul cromosoma Y.

I risultati ottenuti hanno dimostrato che entrambe le tecniche sono in grado di diagnosticare efficientemente il sesso ma fino alla nona settimana di gestazione per la Real Time PCR e la settima settimana di gestazione per la tecnologia Biacore™, richiedendo per settimane di gestazione ancora più precoci un approccio molecolare maggiormente sensibile ed accurato che riesca ad identificare il gene SRY in presenza di quantità ridotte di DNA di origine fetale nel plasma materno.

Allo scopo di confermare il limite di rilevabilità della tecnica Real-Time PCR abbiamo, in primis, ampliato il numero di campioni a 139, selezionandoli con un'ampia variabilità gestazionale.
Ottenuta la conferma, la tecnologia di nuova generazione digital droplet PCR (ddPCR), nota per la sua sensibilità ed accuratezza, dovuta all'estrema diluizione del campione in gocce oleose, è stata applicata per la diagnosi non invasiva del sesso a settimane di gestazione precoci.
Dopo aver ottimizzato le condizioni sperimentali utilizzando DNA genomici e circolanti maschili e femminili, 29 campioni a settimane di gestazione comprese fra le 12 e le 4.5, estratti da plasma materno, sono stati analizzati, e per tutti la tecnica si è dimostrata accurata al 100% nel determinare correttamente il sesso fetale, la cui conferma è stata ottenuta, con il sequenziamento del DNA del neonato.
Siamo, così, riusciti a sviluppare, per la prima volta, un approccio per la diagnosi prenatale non invasiva del sesso fetale a settimane di gestazione precedenti alla settima mediante una tecnologia robusta ed efficiente come la ddPCR.

La β talassemia è una patologia autosomica recessiva causata da più di 300 mutazioni. Questa malattia del sangue, che ha una grande incidenza nell'area del Mediterraneo, è associata all'assenza o riduzione delle catene β globiniche dell'emoglobina adulta, che porta a importanti conseguenze patologiche tra cui una severa anemia. I soggetti che presentano la forma più severa della patologia (β thalassemia major) non possono sopravvivere senza un adeguato piano terapeutico trasfusionale associato a chelazione del ferro. Le terapie convenzionali sono solo di mantenimento, infatti, per questa condizione patologica, l'unico vero trattamento definitivo è il trapianto di midollo, il quale però è associato a forti rischi, fra i quali infezioni e malattia acuta da rigetto.
Per questo motivo, sono stati studiati approcci terapeutici innovativi con l'obbiettivo di una terapia personalizzata. Alcuni esempi di terapia personalizzata per la β thalassemia sono la strategia antisenso, applicata a mutazioni di splicing, e la strategia basata sul read-through, studiata per mutazioni che creano codoni prematuri di terminazione.
Per questa ragione, l'identificazione della specifica alterazione molecolare, che causa la patologia, è di fondamentale importanza al fine di scegliere la corretta strategia terapeutica. In maggior misura, la diagnosi in età prenatale è la chiave per l'attuazione di una tempestiva terapia personalizza.
Chiaramente, una diagnosi in tempi brevi è anche fondamentale per iniziare precocemente la terapia convenzionale e migliorare la qualità di vita dei pazienti.

Diverse tecniche molecolari sono state sviluppate negli anni per l'individuazione di mutazioni puntiformi, tra le quali il sequenziamento genomico è l'approccio d'elezione.
Tuttavia, queste tecniche hanno lo svantaggio di essere costose e laboriose.
In questo contesto si sviluppa la seconda parte della ricerca che ha come principale obbiettivo lo sviluppo di approcci molecolari diagnostici pre- e postnatali rapidi, sensibili e poco costosi per l'identificazione delle più frequenti mutazioni puntiformi talassemiche nel bacino del Mediterraneo ($\beta^0$39, β^+IVSI-110, β^+IVSI-6, β^0IVSI-1).
Attualmente, infatti, non esistono kit commerciali per la diagnosi prenatale di mutazioni puntiformi, i test di screening forniti alle donne in gravidanza sono in grado di identificare solo aneuploidie, piccole delezioni ed inserzioni.
Data la sua nota sensibilità, la prima tecnica selezionata, per la diagnosi postnatale delle quattro mutazioni talassemiche, è stata la tecnologia Biacore^{TM}. In seguito all'immobilizzazione di una sonda mutata e normale sul chip dello strumento, per ciascuna mutazione, le sonde sono state validate valutandone il legame con oligonucleotidi complementari. Infine, è stato estratto, da sangue e tamponi salivari, il DNA genomico di 71 tra soggetti sani e pazienti omozigoti ed eterozigoti per le mutazioni considerate, sul quale è stata effettuata l'analisi.
Per tutti i campioni analizzati è stato possibile discriminare il diverso genotipo, confermato in seguito dal sequenziamento, dimostrando in questo modo di aver sviluppato un efficiente rapido e riproducibile approccio molecolare per la diagnosi postnatale delle mutazioni talassemiche.
Allo scopo di sviluppare un secondo approccio diagnostico postnatale, sono stati disegnati saggi di genotipizzazione da utilizzare in Real-Time PCR per ciascuna delle mutazioni considerate. In seguito all'estrazione di DNA genomico di soggetti sani e pazienti eterozigoti e omozigoti, i saggi disegnati sono stati ottimizzati e validati. Infine, è stata effettuata la diagnosi di 25 pazienti β talassemici di genotipo non conosciuto. Per ciascun campione analizzato, il genotipo individuato è stato confermato tramite sequenziamento genico, dimostrando lo sviluppo di un efficiente e poco costoso approccio diagnostico postnatale basato sulla Real-Time PCR.
Dati questi presupposti, abbiamo pensato di applicare lo stesso approccio per la diagnosi prenatale delle stesse quattro mutazioni puntiformi a partire da ccffDNA. Ci siamo concentrati sulle mutazioni ereditate per via paterna: caso più semplice che non presuppone una quantificazione allelica, considerando che la mutazione non è presente nel DNA circolante materno.

Ventisei campioni di sangue sono stati prelevati da donne in gravidanza, a differenti settimane di gestazione, aventi partner portatori di una delle quattro mutazioni talassemiche considerate, e in seguito il DNA circolante è stato estratto dal plasma.
Considerata la bassa concentrazione del DNA fetale, i campioni sono stai preamplificati e analizzati utilizzando i saggi di genotipizzazione allo scopo di determinare se il feto avesse o meno ereditato la mutazione dal padre.
I risultati ottenuti hanno mostrato come questo approccio sia stato in grado di identificare efficientemente il genotipo fetale a partire dalla nona settimana di gestazione.
Quindi, per poter estendere la diagnosi prenatale non invasiva per mutazioni puntiformi talassemiche ereditate per via paterna, a settimane di gestazione precedenti alla nona abbiamo utilizzato, ancora una volta, la ddPCR come approccio molecolare. Inoltre, questa tecnologia è stata impiegata anche per la diagnosi prenatale non invasiva di mutazioni talassemiche ereditate sia per via materna che da entrambi i genitori: caso più complesso che necessita una precisa quantificazione degli alleli mutato e normale, considerato l'apporto allelico mutato della madre.
I saggi di genotipizzazione per le mutazioni $\beta^0$39 e β^+IVSI-110 sono stati ottimizzati in ddPCR, in termini di efficienza di amplificazione e specificità nell'ibridazione, usando DNA genomici, di differenti genotipi, non digeriti e digeriti enzimaticamente, per simulare la frammentarietà del DNA circolante. Successivamente, sono stati validati utilizzando miscele di due DNA genomici digeriti in diverse percentuali per simulare il diverso apporto allelico fetale a seconda delle settimane di gestazione.
Infine, sono stati collezionati e analizzati 36 campioni di sangue da donne gravide non portatrici o microcitemiche con partner non portatori o anche essi microcitemici, dopo estrazione di DNA circolante materno e fetale da plasma.
Per i campioni in cui la mutazione è ereditata per via paterna, la sola presenza dell'allele mutato è indice che il feto è eterozigote. Per tutti i campioni analizzati, compresi quelli a settimane di gestazioni più precoci, fino alla quinta, il genotipo fetale è stato correttamente individuato e confermato dal sequenziamento.
Per i campioni in cui solo la madre o entrambi i genitori sono portatori, è stata effettuata una precisa quantificazione degli alleli mutato e normale: presupposto fondamentale per l'identificazione del genotipo. Infatti, per i campioni in cui sia la madre che il feto erano eterozigoti, il rapporto allelico è risultato bilanciato, invece per i campioni in cui la mamma era eterozigote e il feto omozigote normale, il rapporto allelico è risultato sbilanciato verso l'allele normale, confermando quanto atteso. Sfortunatamente non avevamo campioni in cui il feto risultasse omozigote.

Analizzando i campioni, è stato possibile individuare due intervalli, sulla base del rapporto allelico, statisticamente separati, necessari per considerare il feto non portatore o eterozigote per le due mutazioni considerate, a partire dalla nona settimana di gestazione per mancanza di campioni a settimane precedenti.
In conclusione, abbiamo sviluppato un nuovo approccio diagnostico semplice e sensibile, basato sull'utilizzo di saggi di genotipizzazione applicati alla ddPCR, per la diagnosi prenatale non invasiva di mutazioni puntiformi ereditate sia per via paterna che materna e, caso più interessante, da entrambi i genitori, a settimane precoci di gestazione (dalla nona), confermando la grande sensibilità e accuratezza della tecnologia.

Una delle prospettive future sarà sicuramente ampliare il numero di campioni di ccffDNA, allo scopo di confermare l'effettiva efficienza ed applicabilità della tecnica, in modo particolare a settimane di gestazione inferiori alla nona. Inoltre si potrebbe estendere l'analisi anche alle altre mutazioni puntiformi talassemiche.
In aggiunta, in futuro, tutti gli approcci diagnostici molecolari sviluppati potrebbero essere estesi ad altre patologie, causate da mutazioni puntiformi, con l'obbiettivo di una stratificazione di pazienti e feti per lo sviluppo di una medicina di precisione personalizzata.

ABSTRACT

Commonly, the diagnosis of genetic diseases in fetal age is obtained using invasive procedures such as amniocentesis and chorionic villus sampling, but these procedures hide a risk of miscarriage of 1% and cannot be performed until 11 weeks of gestation.

For this reason, recently, non-invasive pre-natal diagnosis has become increasingly important and several techniques are widely under investigation for detecting pre-natal disorders and pregnancy monitoring.

Non-invasive pre-natal diagnosis is based on circulating cell-free fetal DNA (ccffDNA) analysis starting from a simple maternal peripheral blood sampling, without impairing the unborn child and the pregnant woman health, avoiding the risks associated with conventional invasive techniques.

In particular, the fetal sex determination is necessary for predicting the risk of X-linked disorders, such as hemophilia and Duchenne muscular dystrophy. The gender is predictive of the pathological phenotype, in fact, in case of recessive X-linked diseases, the risk persists for males.

An early diagnosis allows, as soon as possible, the beginning of the conventional therapy specific for the disease, and a possible personalized therapy.

Against this background, the principal purpose of the first part of the research proposed in this thesis, was the development of rapid, sensitive, and cost-effective non-invasive diagnostic methods for the fetal sex determination, in particular at early gestational ages.

In an already reported study, developed by our research group, the quantitative Real Time PCR (qRT-PCR) and the Biacore™ technology (affinity biosensor based on the surface plasmon resonance -SPR- phenomenon) were employed for the fetal sex determination of 26 ccffDNA samples, extracted from maternal plasma, detecting the SRY gene on Y chromosome.

The obtained results demonstrated that the fetal sex diagnosis can correctly performed by qRT-PCR starting from the 9th gestational week, and from the 7th using Biacore™ system.

Therefore, in order to detect low amount of fetal DNA, prior to the 7th gestational week, a new sensitive and accurate molecular approach was required.

With the aim to confirm the gestational limit of the technique, at first, we decided to extend the qRT-PCR analysis to 139 circulating cfDNA samples with a wide variability in term of gestational weeks.

Secondly, the innovative technology digital droplet PCR (ddPCR), known for its sensitivity and accuracy due to the extreme partition of template DNA, was applied to non-invasive sex diagnosis at early gestational ages.

After optimizing the ddPCR experimental conditions, using genomic and circulating DNAs belonging to male and female subjects, 29 ccffDNA samples at early gestational stages (12-4.5 weeks) were analyzed and for all of them the fetal gender was correctly determined even at earliest gestational age (4.5 weeks), achieving 100% accuracy.

For the first time, a non-invasive pre-natal diagnosis approach was developed for the fetal sex determination at early gestation ages (prior to 7^{th} weeks) through a robust, sensitive, efficient and reliable technology as ddPCR.

β thalassemia is an autosomal recessive inherited disease caused by nearly 300 mutations. This genetic blood disorder, with high incidence in the Mediterranean area, is associated with the absence (β^0) or reduction (β^+) of adult hemoglobin β chains, causing a severe clinical picture. β thalassemia major is the most severe form of the disease, in fact, patients are unable to survive into adulthood without a therapeutic transfusion plan associated with iron chelation. The conventional therapies are just for maintenance, the only definitive treatment, for this condition, is the bone marrow transplantation, which hides transplant-related complications such as infections, rejection and the graft-versus-host disease.

In this context, innovative therapeutic approaches are been investigated in order to employ a personalized therapy. The antisense approach, targeting splicing mutations, and the read-through approach, specifically studied for nonsense mutations, are some examples of β thalassemia personalized therapies.

For this reason, the detection of the specific pathogenic molecular alteration is crucial for the employment of the correct targeted and personalized therapy. Even more, a pre-natal β thalassemia diagnosis is the kay for the timely personalized therapy application. Clearly, an early diagnosis is also need to start a maintenance therapy as soon as possible, in order to improve the patient life quality.

Several molecular techniques have been developed, over the years, for the detection of point mutations, and today genomic DNA sequencing is the approach routinely employed to identify molecular alterations. However, all these techniques are costly, labour intensive and technically demanding.

Against this background, the aim of the second part of the research proposed in this thesis, was the development of rapid, sensitive and cost-effective diagnostic approaches for the identification of the four most common mutations causing β thalassemia in the

Mediterranean area ($\beta^0$39, β^+IVSI-110, β^0IVSI-1, β^+IVSI-6), from genomic DNA of patients (post-natal diagnosis) and circulating cfDNA extracted from plasma of pregnant women (pre-natal diagnosis).

Actually, no commercial assays are available to recognize point mutations; the non-invasive screening tests can detect only aneuploidies, small deletions or insertions.

The first technique employed for post-natal diagnosis of the four β thalassemic point mutations was Biacore^{TM} system, chosen for its sensitivity. After the immobilization of a normal and a mutated biotinylated oligonucleotide probes on the instrument chip, a pair for each mutation, they were validated evaluating the binding with normal and mutated complementary oligonucleotides. Finally, the diagnosis was performed from single-stranded PCR products obtained from genomic DNA of 71 subjects of different genotype, extracted from blood and buccal swabs. For all the specimens it was possible to correctly discriminate the different allelic conditions for each mutation, demonstrating the suitability of the developed SPR-based approach to detect β thalassemia mutations.

Another technique employed for post-natal diagnosis of the four β thalassemia point mutations was the qRT-PCR based on genotyping assays.

Exploiting genomic DNA of different genotypes, extracted from blood or buccal swabs, the designed genotyping assays, one for each mutation, were optimized and validated. Finally, the molecular diagnosis was performed for 25 unknown samples belonging to patients affected by β thalassemia. After sequencing, the diagnostic outcomes were confirmed for all the samples, demonstrating that qRT-PCR based on genotyping assays developed method may be efficiently employed for post-natal diagnosis of β thalassemia point mutations.

Established the suitability of genotyping assays for molecular post-natal diagnosis, we applied the same approach to non-invasive pre-natal screening, in order to detect, in ccffDNA, the four β thalassemia point mutations inherited from the father: a quite feasible case because the mutation is not present in maternal DNA, that is the main component of the circulating DNA extracted from plasma.

Twenty-six blood samples were collected from pregnant women whose partners were carrier of thalassemic mutations; the plasma was prepared and the ccffDNA pre-amplified and analyzed with the specific genotyping assay in order to determine if the fetus has inherited the paternal mutation.

The obtained data had shown that the developed genotyping assays could be efficiently employed for non-invasive pre-natal diagnosis of paternally inherited β thalassemia mutations, at least until the 9th gestational week.

In order to extend the non-invasive pre-natal diagnosis to paternally inherited thalassemic mutations at earlier pregnancy (prior to 9th), a new sensitive and precise approach had been proposed: ddPCR technology. In addition, this molecular approach was employed, also, for non-invasive pre-natal diagnosis of thalassemic mutations maternally or both maternally and paternally inherited.

$\beta^0$39 and β^+IVSI-110 genotyping assays were, at first, optimized in term of amplification efficiency and hybridization specificity, using genomic DNA of different genotype, not digested and enzymatically digested, with the aim to simulate the ccffDNA fragmentation. Subsequently, the two assays were validated using mixtures of two digested genomic DNA of different genotype, at different percentages, in order to simulate fetal and maternal circulating cfDNA at different gestational weeks. Finally, 36 blood samples were collected from pregnant women, the circulating cfDNA was extracted from plasma and the samples analyzed with the specific ddPCR assay in order to determine the fetal genotype.

In the samples in which the mutation was paternally inherited, the only presence of a mutated allele in a normal background indicated the heterozygous genotype of the fetus. For all the samples analyzed the diagnostic outcome was confirmed by DNA sequencing, also at 5th gestational week.

The case of maternally or both parents inherited mutation a precise quantification of normal and mutated alleles was required to detect the fetal genotype. Allelic balance is expected when the fetal genotype is identical to the mother's one, whereas allelic imbalance occurs if there is an under-representation (the fetus is homozygous for the normal allele) or over-representation (the fetus is homozygous for the mutated allele) of the mutant allele with respect to the normal one. Unfortunately, there were not samples with homozygous fetus for one of the two β thalassemia mutations considered.

The obtained results have been identify two diagnostic ranges of allelic ratio values statistically distinct and not overlapping, allowing the correctly determination of fetal genotype in all the samples analyzed until the 9th week, for lack of samples at earlier gestational weeks.

In conclusion, we have developed a simple and sensitive diagnostic approach, based on genotyping assays applied to ddPCR, for non-invasive pre-natal testing of β^+IVSI-110 and $\beta^0$39 mutations paternally and maternally inherited, and for the case in which both parents were carrier of the same mutation, at early gestational ages (until 9th week), confirming the sensitivity and accuracy of the technology.

However, in order to confirm the effective efficiency and applicability of the technology to β thalassemia mutations identification, will be necessary to increase the number of ccffDNA samples and recruit ccffDNA samples at early gestational weeks (prior to 9^{th}) to identify the gestational limit of the approach. Another future perspective could be extend the analysis to the other thalassemic point mutations.

In addition, both the post-natal and pre-natal developed approaches could be apply to point mutations causing other genetic diseases, with the aim to designed diagnostic protocols useful for patients and fetuses stratification in personalized therapy on the road of precision medicine.

1. INTRODUCTION

1.1. Genetic diseases

In wealthy countries, congenital malformations and genetic diseases have become the most common cause of infant mortality and morbidity [Galjaard, 1979].

In order to classify the genetic disorders is necessary to investigate the genetic origin of the pathologies. Among the different types of genetic disorders, there are chromosome alterations, such as aneuploidies (change in the number of individual chromosomes) or change in chromosome structure (*e.g.* translocations, insertions or deletions), or smaller mutations in DNA sequences such as microdeletions or microinsertions, causing a reading frameshift during mRNA translation, or point mutations caused by the replacement of a nucleotide with another.

Therefore, genetic diseases can be classified in the following categories: single gene disorders, X-linked disorders and aneuploidies.

1.1.1. Single gene disorders

Mutations just in a single gene cause these kind of disorders, which represent thousands of human diseases. Generally, the gene involved is not in a sexual chromosome but in an autosomal one, so they are also called “autosomal”. The frequency of these alterations has been estimated around 0.36% of live births [Baird et al., 1988]. The autosomal disorders can be divided in dominant, when the disease is clinically relevant even if the mutation is present in at least one of the two alleles, or recessive, when the mutation must be present in both alleles to give rise to the pathology. In such circumstances, if the genotype in heterozygous, where one allele is mutant while the other is wild type (wt), the subject is carrier of the disease and is called “healthy carrier”.

The **Table 1.1** reports some examples of monogenic diseases.

Disease	Involved gene	Clinical signs	Inheritance	References
Huntington's disease	Huntington disease gene (*HD*)	Neurodegeneration, involuntary movements, progressive dementia	Dominant	Barboza & Ghisi, 2018; Aziz et al., 2018
Myotonic dystrophy	Dystrophia myotonica protein kinase gene (*DMPK*)	Progressive skeletal muscle weakness, atrophy, myotonia	Dominant	Andrè et al., 2018; Wood et al., 2018
Cystic fibrosis	Cystic fibrosis transmembrane conductance regulator gene (CFTR)	Lung chronic bacterial infection and inflammation, multiorgan failure	Recessive	Dechecchi et al., 2018; Zolin et al., 2018
Thalassemia	Globin genes	Anemia, splenomegaly, iron overload in heart and liver	Recessive	Tatu & Sweatman, 2018; Bou-Fakhredin et al., 2017
Achondroplasia	Fibroblast growth factor receptor 3 gene (*FGFR3*)	Dwarfism, chondrodysplasia, multiple skeletal abnormalities	Dominant	Ornitz & Legeai-Mallet, 2018; Woodacre et al., 2018
Phenylketonuria	Phenylalanine hydroxylase gene (*PAH*)	Intellectual disability, microcephaly, motor deficits	Recessive	van Wegberg et al., 2017; Hofman et al., 2018

Table 1.1. Examples of single gene disorders. For each reported disease the involved gene, the clinical signs and the inheritance are indicated.

1.1.2. X-linked disorders

The expression of X-linked genes differ between males and females due to the imbalance of X chromosome between the two sexes. While females have both a maternal and a paternal X chromosome, males inherit only a maternal X, hence gene expression in females and males is not the same because the genes on the Y chromosome do not exactly pair up with those on the X chromosome. The X-linked disorders are genetic diseases caused by alterations in genes on X chromosome and they can be recessive or dominant. For further specification, see section **1.6**.

1.1.3. Aneuploidies

Aneuploidies are diseases in which there is a change in chromosomes number inside the cell, but they include, also, the gain and/or loss of chromosome arms, and variable sizes of chromosomal segments [Beach et al., 2017]. Generally, non-disjunction of chromosomes during the process of cell division, related to gamete production (meiosis), cause the abnormal number of chromosomes. Most aneuploidies lead to spontaneous miscarriage early pregnancy: it was estimated that aneuploid embryos account for at least 10% of human pregnancies, and for woman nearing the end of their reproductive lifespan, the incidence might exceed 50% [Nagaoka et al., 2012].

Usually, these abnormalities lead to clinical features including physical problems, sterility and learning impairment caused by an imbalance chromosomes, and then, gene dosage because of gaining or losing a particular genetic material [Hutaff-Lee et al., 2013]. On the contrary, milder symptoms are associated with mosaicism, which occurs when chromosomes incorrectly divide during the division of somatic cells (mitotis) after fertilization, resulting in a variable mixture of two types of cell populations in the body that are genotypically distinct [Gajecka, 2016].

The most common aneuploidies are summarized in **Table 1.2**.

Syndrome	Chromosome abnormality	Clinical features	Incidence	References
Trisomy 21 (Down syndrome)	Extra copy of the long arm of chromosome 21	Intellectual disability, motor dysfunctions	1:800	Gardiner, 2015; Watson-Scales et al., 2018
Trisomy 18 (Edwards syndrome)	Three copies of chromosome 18	Neurological impairment, growth disturbances, bone and skin malformations	1:8000	Cereda & Carey, 2012; Meyer et al., 2016
Trisomy 13 (Patau syndrome)	Three copies of chromosome 13	Multiple malformations of cardiac, central nervous, and urogenital systems	1:6500	Kroes et al., 2014; Tsukamoto et al., 2017

Table 1.2. Examples of common aneuploidies. For each reported syndrome the chromosome abnormality, the clinical features and the incidence are indicated.

An early identification of the genetic diseases is strongly necessary for allow a timely and accurate diagnosis, which has the final aim to set up a proper therapeutic regimen and, if possible, apply a personalized therapy, to minimize disease severity [Diagnosis of a genetic disease, 2008].

Genetic screening can be defined as any type of test performed for the early identification or exclusion of a hereditary disorder, to know the predisposition to some diseases or to determine a predisposition that may produce a hereditary disease in offspring.

Two different types of genetic screening can be distinguished: genetic screening after birth and pre-natal genetic screening. The first type includes pre-conception analysis of couples in age, neonatal, childhood and adulthood screenings, and has the purpose to confirm the genetic disease and determine the risk for children. Post-natal diagnosis is included in this issue. On the other hand, pre-natal genetic screening includes pre-implantation genetic diagnosis, maternal serum screening tests and pre-natal diagnosis after invasive or non-

invasive collection of fetal biological material. The ultimate purpose of pre-natal genetic screening is the possibility of identification of a genetic disorder early in pregnancy, in case of fetuses with increased risk of disease for family history and advanced maternal age.

1.2. Post-natal diagnosis of genetic diseases

The post-natal diagnosis of a genetic disease requires a clinical examination composed of three elements: a physical examination, a detailed family medical history and, important of all, clinical and laboratory tests. While the first two elements are not able to make a definitive diagnosis, testings are the only way for the confirmation of the disorder. The ultimate goal is to use information gained from analysis techniques to treat, cure or, if possible, prevent the development of disease [Diagnosis of a genetic disease, 2008].

1.2.1. Analysis techniques

The majority of molecular techniques employed to detect genetic diseases is based on the preventive amplification of the target DNA by polymerase chain reaction (PCR).

1.2.1.1. PCR

The PCR was introduced in 1985 [Saiki et al., 1988]. It is currently one of the most frequently used methods, as it ensures high simplification of the DNA sequence of interest, and therefore reduced amounts of samples for the following analyses are required.

The technique consists in the cyclic repetition of three processes (**Figure 1.1**): denaturation at high temperature of the double-stranded template DNA; hybridization with a pair of oligonucleotides (primers), each complementary to a strand of the denatured DNA, upstream and downstream the sequence to be amplified, respectively; elongation, by the action of a Taq DNA polymerase, a heat-resistant DNA polymerase that uses the oligonucleotides as primers and single-stranded DNA as a template for the synthesis of complementary nucleotide sequences, which extend up to the position of the primer in the opposite strand. At each reaction cycle the amplification products double, resulting in an exponential increase equal to 2^n, where n is the number of cycles carried out [Saiki et al., 1988].

Finally, the visualization of the PCR products can performed after agarose gel electrophoresis. In order to detect specific DNA alterations, PCR is usually employed in association with other specific methodologies [Silvestroni, 1998], as described below.

1.2.1.2. RFLP (restriction fragment length polymorphisms)-PCR

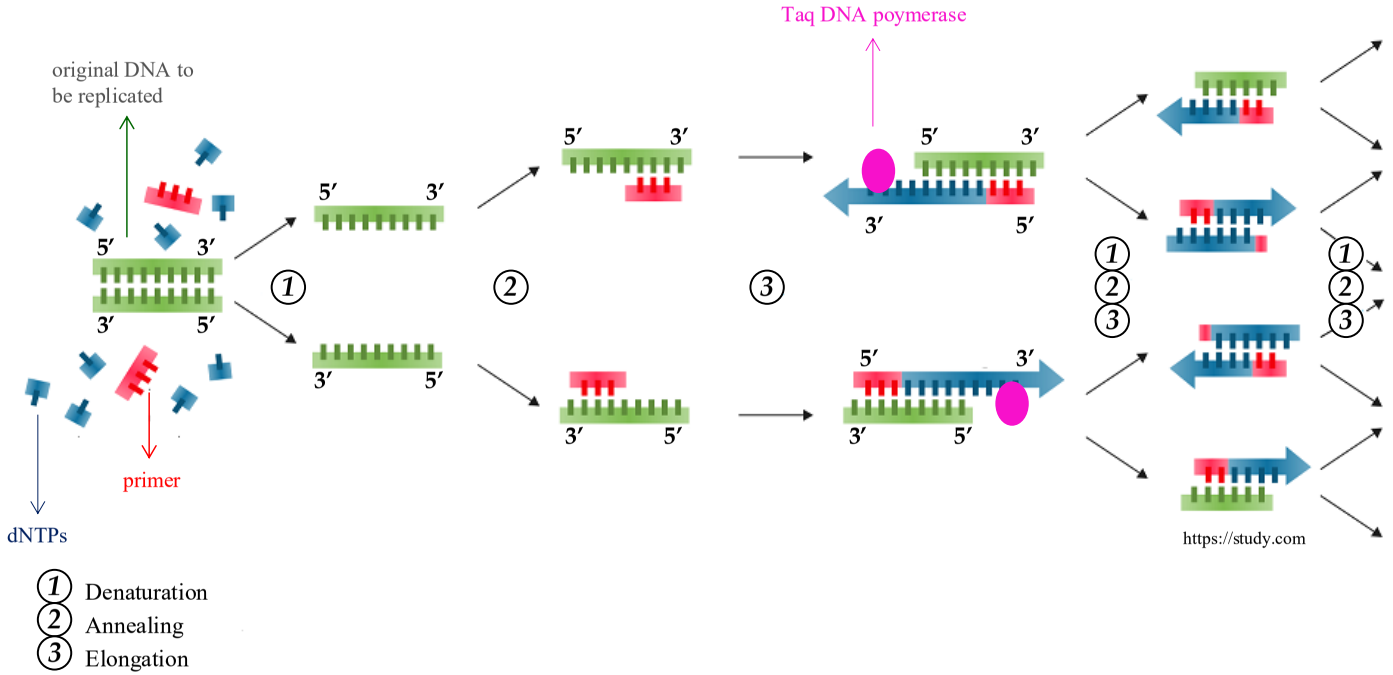


Figure 1.1. Polymerase chain reaction (PCR). The reaction requires a template DNA (in green), a pair of primers (in red), deoxyribonucleotide triphosphates (dNTPs, in blue) and a Taq DNA polymerase (in pink). Several thermal cycles are required, each consisting of three steps : 1) denaturation of the double-stranded template DNA; 2) annealing of primers to complementary sequences flanking the target; 3) elongation of primers by a Taq DNA polymerase, producing new DNA strands. At each cycle the amplification products double.

RFLP-PCR can be used to locate point mutations that abolish, create or move a restriction site. If a DNA or a PCR product is digested with an appropriate restriction enzyme [Pourzand & Cerutti, 1993] a series of fragments (restriction fragments) of specific and known length are obtained, separable and distinguishable by electrophoresis analysis. If there is a point mutation that alters a restriction site, the obtained restriction fragments will have length different from expected, allowing to detect the alteration [Pourzand & Cerutti, 1993].
This technique is inexpensive and does not required advantages instrumentation. In addition, the design of RFLP-PCR assay is easy and can be accomplished by using available public programs [Rasmussen, 2012]. However, it requires specific endonucleases and exact genotyping cannot be achieved if there is more than one nucleotide variation in a restriction enzyme recognition site. Moreover, the technique consists of many steps, and so long times are needed for diagnosis [Rasmussen, 2012].

1.2.1.3. ARMS (amplification refractory mutation system)-PCR

This technique, also called allele-specific PCR, consists in the establishment of two PCR reactions: one primer is the same for both the reactions, whereas the other is complementary to the sequence possibly carrying the mutation of interest. For example, in 3' it carries a nucleotide complementary either to the normal or to the mutated base: an amplification product will be obtained only when the primers recognize the complementary sequence on genomic DNA, allowing to identify the alleles present in the sample [Mamotte, 2006].
Although this technique is sensitive in mutation detection, ARMS-PCR can be time-consuming due to the high number of assays that need to be carried out for detection of different mutations [Kho et al., 2013].

1.2.1.4. DNA chip

The technique involves the use of a chip, consisting of a solid surface of nylon or glass, coated with a very large number of probes specific for different target DNA sequences, normal or carrying molecular defects to be characterized. The target DNA under investigation, usually, previously amplified by PCR, is fluorescently or radioactively labeled, or by exploiting the interaction of biotin and streptavidin, in order to detect the possible hybridization with the immobilized probes.
The technique, besides allowing the analysis of genotypic alterations, provides also quantitative information, since the amount of signal obtained is proportional to the amount of template that hybridizes with the probes [Bertrand et al., 1998].

It is a very fast and automated method for the study of genotype, for the search of DNA polymorphisms (SNPs, single nucleotide polymorphisms), nucleotide replacements and point mutations [Suzuki et al., 2012].
A cost-effective plastic fiber-based DNA chip was developed, resulting an advantageous platform for mass genotyping because of it low cost, rapid results, and reliability [Suzuki et al., 2012].

1.2.1.5. SPR (surface plasmon resonance)

DNA chips for the detection of point mutations are also used in techniques based on SPR, an optical phenomenon that allows to analyze the interaction between two biomolecules, as a consequence of a change in the refractive index on the active surface where the interaction occurs [Pattnaik, 2005]. In particular, as happens in Biacore^{TM} biosensors, a ligand is immobilized on the matrix of a chip called sensor chip, and then an analyte is injected in solution on the chip surface [Gambari, 2001]. The possible hybridization between ligand and analyte is monitored in real-time, and different interaction parameters can be evaluated and measured, as for example affinity, stability and kinetics [Gambari, 2001]. The advantage of SPR-based technology is high sensitivity; in addition, the times required for diagnosis are reduced, and the automation allows a great reproducibility [Feriotto et al., 2004].

1.2.1.6. SPR Imaging (SPR-I)

Techniques involving SPR-I are also based on the physical phenomenon of SPR, but they have the ability to detect the intensity variation of the reflected light on the entire chip by using a CCD (charge coupled device) camera, allowing a multiplexing approach in the investigation of biomolecular interactions. A further advantage for diagnosis is the very high sensitivity, as it is possible to work with femtomolar concentrations [D'Agata & Spoto, 2012]. The reduced amount of genomic DNA required for the analysis indicates the suitability of the technique for the analysis of non-amplified products [D'Agata & Spoto, 2012].

1.2.1.7. Reverse dot blot

Oligonucleotide probes for specific DNA mutations are bound to a nylon filter by an amino group at the 5' end. Then they are subjected to hybridization with the DNA under investigation: the sequence of interest is previously amplified by using as a precursor a biotinylated dUTP (2'-deoxyuridine 5'-triphosphate), in order to exploit a biotin-

streptavidin-alkaline phosphatase system to generate an easily detectable colorimetric reaction in case of successful hybridization [Lin et al., 2012].

1.2.1.8. Multiplex minisequencing

The technique of DNA minisequencing investigates up to ten SNP markers simultaneously by using PCR amplification, dideoxy single-base extension of an unlabeled primer, and capillary electrophoresis [Charfeddine et al., 2015]. In the single-base extension step, an unlabeled primer is designed to anneal the sequence adjacent to the SNP site. After primer annealing, the single-base extension occurs in the exact SNP position by addition of the complementary fluorescently labeled ddNTP (dideoxynucleoside triphosphate, missing the OH group in 3' with the aim to prevent the progress of the DNA synthesis). Each ddNTP is labeled with a different colour dye, so the resulting marker fragments for the different SNPs under analysis have all the same length, but vary by colour. After electrophoresis and fluorescence detection, the alleles of a single marker appear as different coloured peaks at roughly the same size in the electropherogram plot [Charfeddine et al., 2015]. This technique is very suitable for mutation analysis because it is sensitive and allows the simultaneous detection of several variations in a single reaction [Sagong et al., 2014].

1.2.1.9. Quantitative real-time PCR (qRT-PCR)

The qRT-PCR is a technique that combines microvolume PCR with rapid-cycle and fluorometry in this case detection and analysis of fluorescent signals produced by DNA-binding dyes or sequence-specific probes. In this way it is possible to monitor in real-time the accumulation of amplicons produced through the progress of a PCR reaction, whereby the increase in reporter fluorescent signal in proportional to the number of generated amplicons [Wilhelm et al., 2003]. This allows a quantitative analysis of the sample, as the increase of the amplicon is proportionally correlated to the initial amount of target template. **Figure 1.2** reports a representative example of a spectrogram that shows amplification curves obtained after performing a qRT-PCR run, where the fluorescence signal (in RFU, relative fluorescence unit) is plotted as a function of number of amplification cycles. After a first exponential phase, the amplification reaches a steady state (plateau) when the reagent amounts start to become limiting.

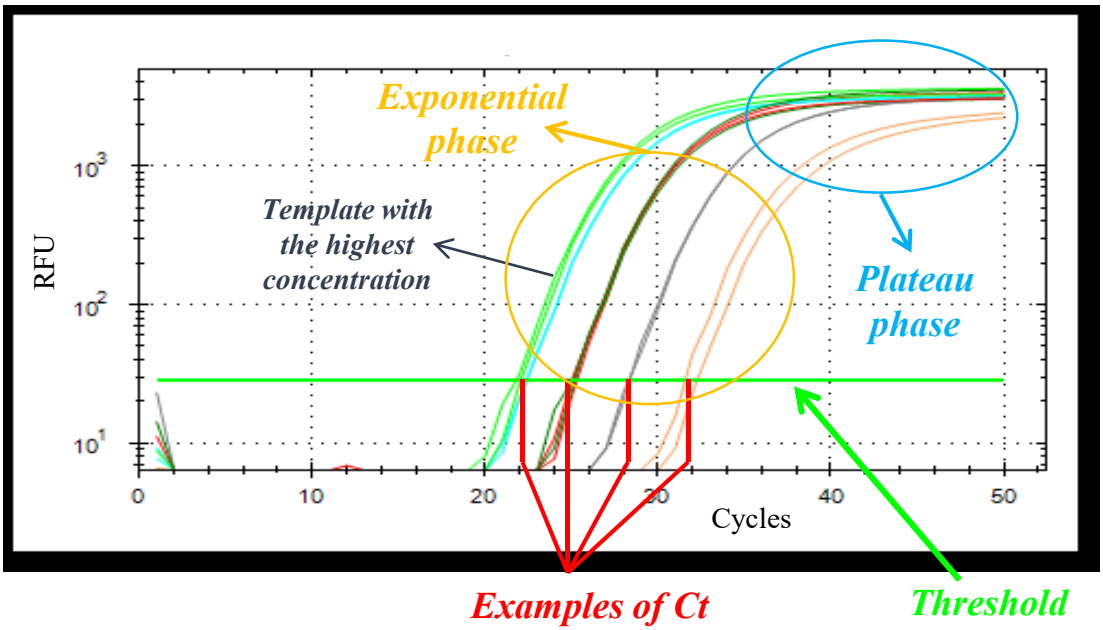


Figure 1.2. qRT-PCR spectrogram. Representative example of a spectrogram obtained after qRT-PCR amplification of different amounts of the same template, where fluorescence (in RFU, Relative Fluorescence Units) is plotted against the number of cycles (Ct). The threshold line, established by the operator, the Ct (threshold cycles) relative to the amplification of each sample (marked in red), the exponential (in orange) and the plateau phases (in light blue) are also indicated, as well as the amplification curve generated by the highest amount of template.

For quantification, it is first necessary to identify for the sample a threshold cycle value (Ct) (**Figure 1.2**, in red), at which the detected fluorescent signal is above the background (threshold value, **Figure 1.2**, in green, chosen by the operator), at the beginning of the exponential phase of amplification. More abundant the sample is, earlier this point is reached and more the amplification curve in shifted to the left (**Figure 1.2**).

Conversely of PCR, the qRT-PCR shows some advantages such as the lack of sample manipulation after the amplification, which allows to avoid any contamination, and a wide dynamic range of detection with a high degree of sensitivity [Hromadnikova et al., 2003].

Several types of detection chemistries are commercially available for sequence-specific probes suitable for qRT-PCR, among which very frequently used are TaqMan® probes or hydrolysis probes (**Figure 1.3**), consisting of allele-specific oligonucleotides complementary to a strand of the target DNA, and labeled with a fluorescent reporter at the 5' end and a quencher at the 3'end or internally. If the probe is intact, it does not generate fluorescence because reporter and quencher are close and so the fluorescence emitted by the reporter is completely quenched by the quencher. Instead, during the extension steps of PCR reaction, given that the probe is digested by the exonuclease activity of the DNA polymerase, reporter and quencher are found to be separated and, as a result, there is an emission of

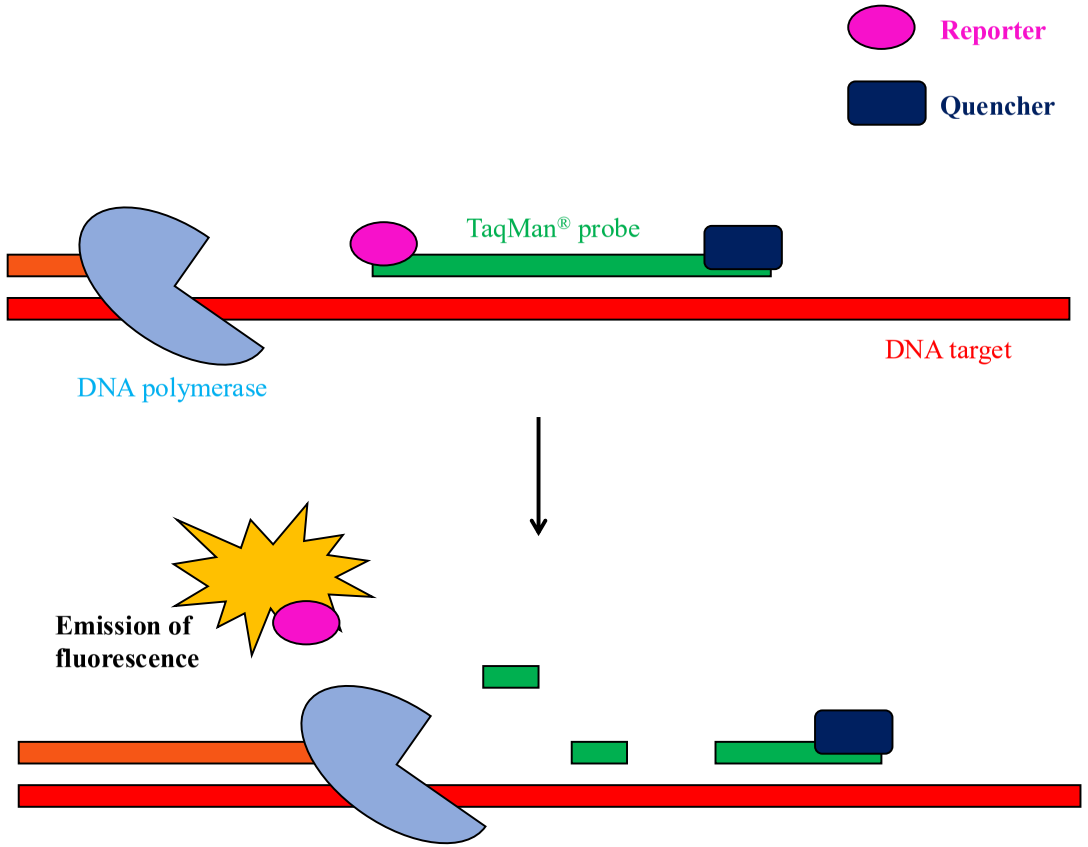


Figure 1.3. TaqMan® probe. TaqMan® probe is an allele-specific oligonucleotide complementary to a strand of the target DNA. It contains a fluorescent reporter at the 5'end, and a quencher molecule at the 3' end. During the extension step of the PCR reaction, the probe is digested by DNA polymerase, so reporter and quencher are separated. The result is an emission of fluorescence proportional to the amount of amplification product [Modified from: www.thermofisher.com].

fluorescence proportional to the amount of amplification product. Common types of reporter molecules are FAM™ (carboxyfluorescein) and VIC® (chlorophenyldichlorocarboxyfluorescein), whereas frequently found quencher molecule is TAMRA™ (carboxytetramethylrhodamine).

This method makes it possible to rapidly characterize the genotype of PCR products, and has already been widely applied for genotyping analysis in several genetic disorders, as described below which is useful for genetic diagnosis in monogenic diseases [Donohoe et al., 2000].

1.2.1.10. Genotyping analysis

Genotyping analysis is an end-point experiment used for determining the genotype of unknown samples, allowing the discrimination of SNPs [Kho et al., 2013]. A genotyping assay can distinguish two different alleles (for example mutated and non mutated allele) determining if the samples are homozygous for the non mutated allele, homozygous for the mutated allele or heterozygous.

It requires two subsequent steps: first amplification by PCR or qRT-PCR, in the presence of suitable probes, followed by the detection of the end-point result signals [Kho et al., 2013]. To distinguish the two different alleles, it is possible to use a pair of allele-specific oligonucleotide probes, one complementary to the mutated sequence and one to the non mutated one, marked with different fluorescent labels. In particular, two different TaqMan® probes are generally employed (**Figure 1.4.A**), each specific for each allele, each labeled with a different fluorescent dye. In particular, each probe contains a report dye at the 5' end, for example a VIC® dye and a FAM™ dye in the probes recognizing the non-mutated allele and the mutated one, respectively; and it contains also a quencher at the 3' end, for example a non-fluorescent quencher (NFQ).

During the amplification, each probe is able to anneal specifically to its complementary sequence on one of the two alleles (**Figure 1.4.B** and **Figure 1.4.C**). Therefore, the fluorescence signals generated during PCR indicate the alleles presence in the sample (**Figure 1.5**): only one fluorescence signal, produced by VIC® (**Figure 1.5.A**) or FAM™ (**Figure 1.5.C)** dyes, is detected if the sample genotype is homozygous, for the non mutated (**Figure 1.5.A**) or the mutated (**Figure 1.5.C**) allele respectively, whereas if the sample has both the alleles, both signal are expected to increase (**Figure 1.5.B**).

Indeed, the analysis is performed by measuring the end-point fluorescent signals at the end of the reaction. The final fluorescence intensity obtained from each probe, normalized with the reference dye ROX™ (rhodamine X) and defined as Rn, gives the final result (**Figure**

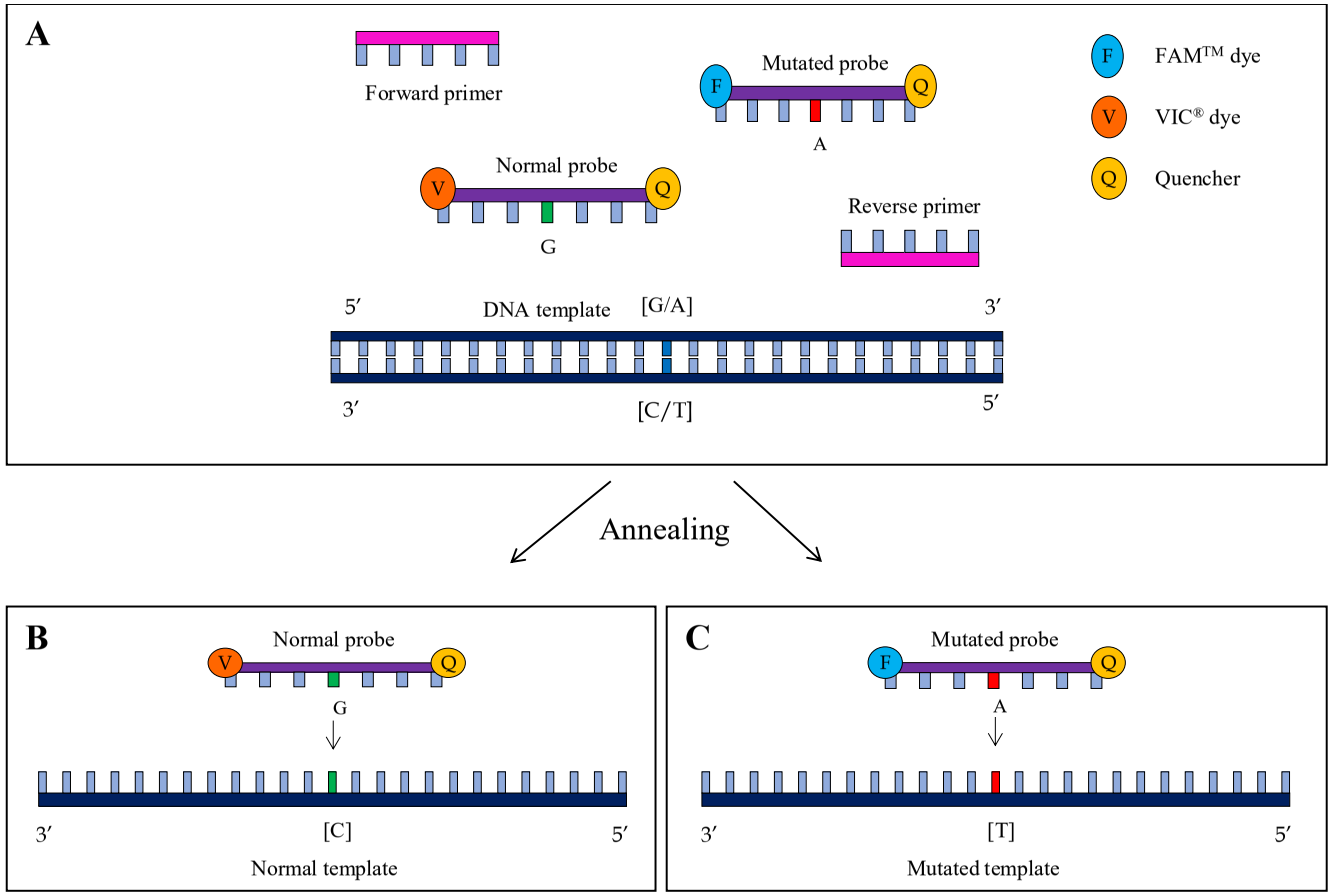


Figure 1.4. Schematic representation of the genotyping assay. (**A**) The reaction requires the template DNA (in blue) which may carry or not the mutation (in this example, G/A for 5'-3' strand), forward and reverse primers (in pink) and two probes (in violet): a mutated probe carrying the mismatched base (in red) and a FAM™ dye (in light blue), and a normal probe carrying the fullmatched base (C, in green) and a VIC® dye (in orange); both the probes carry a quencher (in yellow). The normal probe hybridizes with the normal template (**B**), wheres the mutated probe hybridizes with the mutated template (**C**) [Modified from: Cheng et al., 2004].

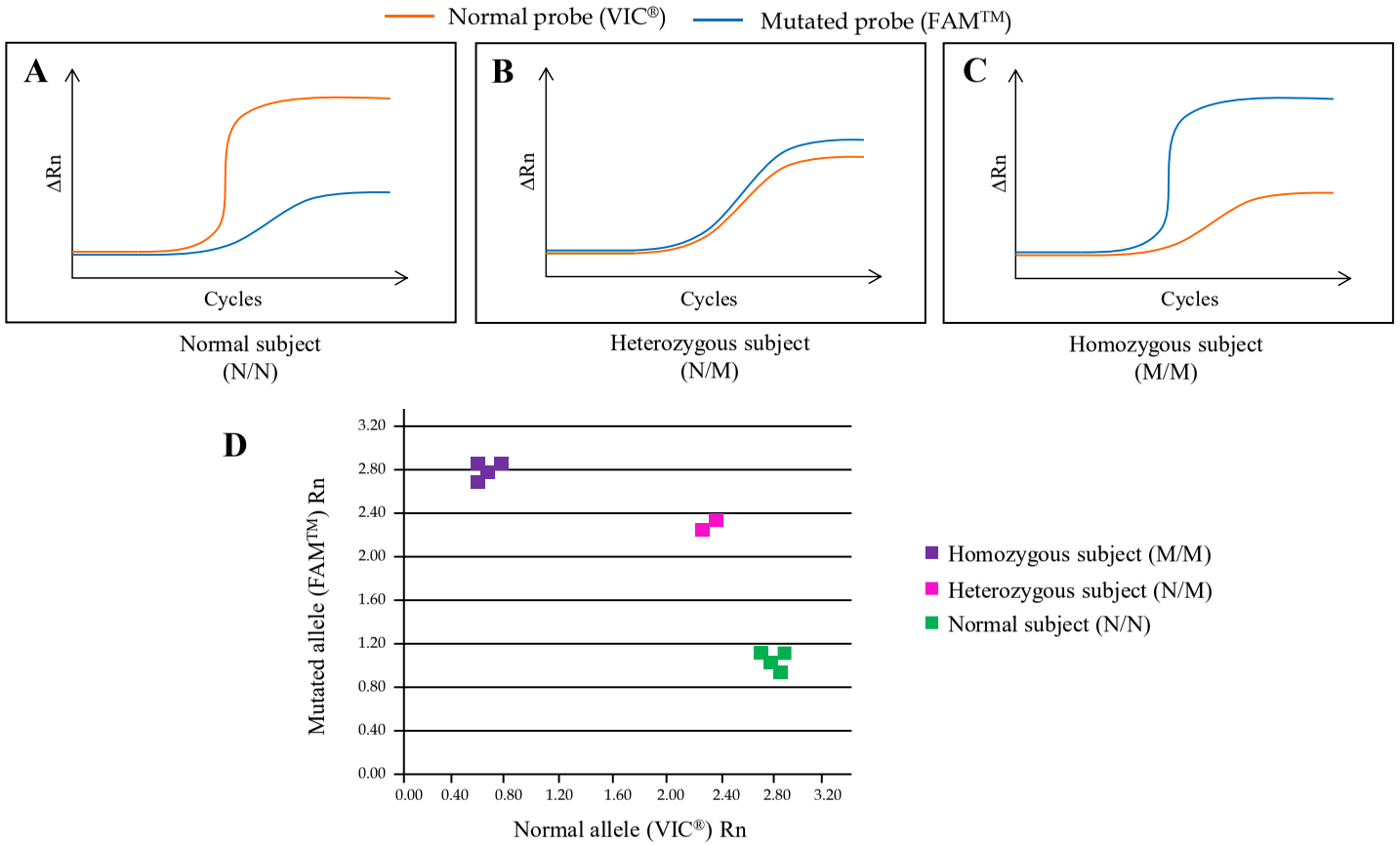


Figure 1.5. Representative examples of spectrograms obtained after genotyping assays. (**A-C**) Amplification plots from normal (**A**), heterozygous (**B**) and homozygous (**C**) subjects, after a genotyping assay that employs a VIC®-labeled normal probe (red curve) and a FAM™-labeled mutated probe (blue curve). ΔRn = Rn post-PCR – Rn pre-PCR. (**D**) Allelic discrimination plot obtained from normal (in green), heterozygous (in pink) and homozygous (in violet) subjects. VIC® Rn and FAM™ Rn indicate the normalized end-point VIC®/ROX™ and FAM™ /ROX™ signals, respectively, where ROX™ is the most commonly used reference dye [Modified from: Cheng et al., 2004].

1.5.D): a substantial increase of VIC® fluorescence (high VIC® Rn value), indicates a homozygous genotype for the non mutated allele; an increase of FAM™ fluorescence (high FAM™ Rn value), indicates a homozygous genotype for the mutated allele; an increase of both fluorescent signals indicates a heterozygous genotype.

TaqMan® genotyping probes can be designed to detect also multiple nucleotide polymorphisms (MNPs) and insertion/deletions [Zhou et al., 2013].

Thus, TaqMan® genotyping assay are suitable for large-scale screenings and high-throughtput analyses, as they are reliable, rapid and cost-effective. The simplicity and reproducibility of the assay permits its use in laboratories as a rapid (it allows detection of mutations in less than 40 minutes) and inexpensive diagnostic tool for mutations diagnosis [Kho et al., 2013].

1.2.1.11. FRET (fluorescence resonance energy transfer)

FRET occurs due to the interaction between the electron excited states of two dye molecules, where energy is transferred from one dye molecule (the donor) to the other (the acceptor) without emission of photons. This is distance-dependent: the donor and the acceptor dyes must be in close proximity. Therefore, the donor fluorophore excited by a light source, transfers its energy to a near acceptor fluorophore, that finally emits light at a longer wavelength, detected in specific channels [Gelsthorpe et al., 1999].

This phenomenon can be exploited in designing specific probes (FRET hybridization probes) for qRT-PCR, where the donor probe and the acceptor probe are labeled with fluorophores at the 3' end and the 5' end, respectively, and the interaction of the two dyes can only occur when both are bound to their targets. During qRT-PCR, the two different oligonucleotides hybridize to adjacent regions of the target DNA such that the fluorophores, which are coupled to the oligonucleotides, in close proximity in the hybrid structure. In order to identify mutations, FRET probes are designed to specifically hybridize with the mutated sequence on the target DNA, which is detected as the fluorescence emission of the acceptor fluorophore [Hung et al., 2010].

If the mutation to be discriminated is unknown, other techniques can be employed, allowing to identify the presence of a molecular defect in the template DNA. Only DNA sequencing is able to exactly detect which nucleotide alterations are present in the sample.

1.2.1.12. HRM (high resolution melt)-PCR

HRM-PCR analysis is a high-throughtput mutation scanning method based on profiles of DNA melting temperature (Tm), which is the temperature when half of the total quantity of double-stranded DNA has dissociated to become single-stranded DNA [Kho et al., 2015].

HRM-PCR analysis is performed on double-stranded DNA samples, typically after amplification of the DNA region of interest by PCR [Yue et al., 2014]. The process is simple: the PCR product is warmed from about 50°C up to about 95°C, for the separation of the two strands, by using intercalating dyes that have a unique property: when specifically bound to double-stranded DNA, they fluoresce brightly; when the sample is heated and the double-stranded DNA denatured, the fluorescence is reduced. As the temperature increases, the change in fluorescence is detected and allows determining the Tm of the template as dissociation of double-stranded DNA occurs [Kho et al., 2015].

The data are plotted in real-time in a graph known as melt curve, showing the level of measured fluorescence as a function of temperature. The Tm of a PCR product is predictable, and results changed if an alteration is present. So, in this case also the melt curve will appear different as expected.

However, different alterations may produce similar melting curves or very small differences in Tm that lead to ambiguous results, requiring probe-based assays, like qRT-PCR, to increase the detection accuracy [Kho et al., 2015].

1.2.1.13. DHPLC (denaturing high performance liquid chromatography)

This technique is based on the separation of DNA fragments by ion exchange liquid chromatography. DNA, previously denatured, binds to the stationary phase, constituted by beads of polystyrene, thanks to a TEAA (triethylammonium acetate) bridge molecule; then suitable elution buffers are used as mobile phase, to break the interaction DNA-TEAA. The DNA separation is monitored by spectrophotometric detection, while the result is shown in a chromatogram [Dastsooz et al., 2013]. In the presence of a mismatch, the interaction with the stationary phase becomes weaker, and therefore the mutated DNA is eluted previously, requiring a smaller volume of elution buffer.

DHPLC analysis is considered a higher validity, reliability and practicability method in detecting point mutations and polymorphisms [Dastsooz et al., 2013; Qin et al., 2013].

1.2.1.14. Sanger enzymatic DNA sequencing

The Sanger method for DNA sequencing (**Figure 1.6**) allows to determine the exact nucleotide sequence of a template DNA, and so also possible disease-causing alterations [Izmailov et al., 2002]. It is based on a sequence reaction that is an amplification of the genomic region of interest with a single specific primer (**Figure 1.6.A**) and a series of thermal cycles. The technique involves also the addition of fluorescently-labeled nucleotides, ddNTPs (**Figure 1.6.A**), missing the OH group in 3' with the aim to prevent the progress of the DNA synthesis, when incorporated during the elongation phase: the concentration of dNTPs and ddNTPs is such to obtain fragments staggered of one nucleotide in position 3', each terminating with a ddNTP (**Figure 1.6.A**). By electrophoresis separation of the fragments (**Figure 1.6.B**) and identification of the terminal nucleotide, it is possible to determine the sequence of the template DNA (**Figure 1.6.B**) [Sanger & Brownlee, 1970]. In automated DNA sequencing currently employed, a single reaction is performed by using the four different ddNTPs labeled with different fluorophores; electrophoresis occurs along capillaries, containing a network of gel or polymer. During the electrophoretic run, the fragments are read in order of increasing length from a laser beam that excites the fluorescent markers: the intensity of the emitted light is detected and measured, together with the identification of each fluorophore, and finally shown in a diagram, called electropherogram (**Figure 1.6.C**).

In the case of several nucleotides in the same position, that is typical example of a heterozygous genotype for a polymorphism or a point mutation, they will generate overlapping peaks of area proportional to the corresponding allelic ration [Sanger & Brownlee, 1970].

Currently, DNA sequencing is widely used in various fields, as it is the only technique that allows to determine with precision the exact nucleotide sequence of a portion of DNA [Zhang et al., 2013].

1.3. Pre-natal diagnosis of genetic diseases

Pre-natal diagnosis includes all the techniques that allow to obtain clinical information about a variety of fetal genetic, biochemical and physiological disorders. With pre-natal diagnosis, it is possible to detect the presence or the absence, of several diseases of different origin, such as genetic, infectious or iatrogenic (consequent to medical treatment during pregnancy). So, future parents could become aware of the fetus health and take appropriate decisions about pregnancy.

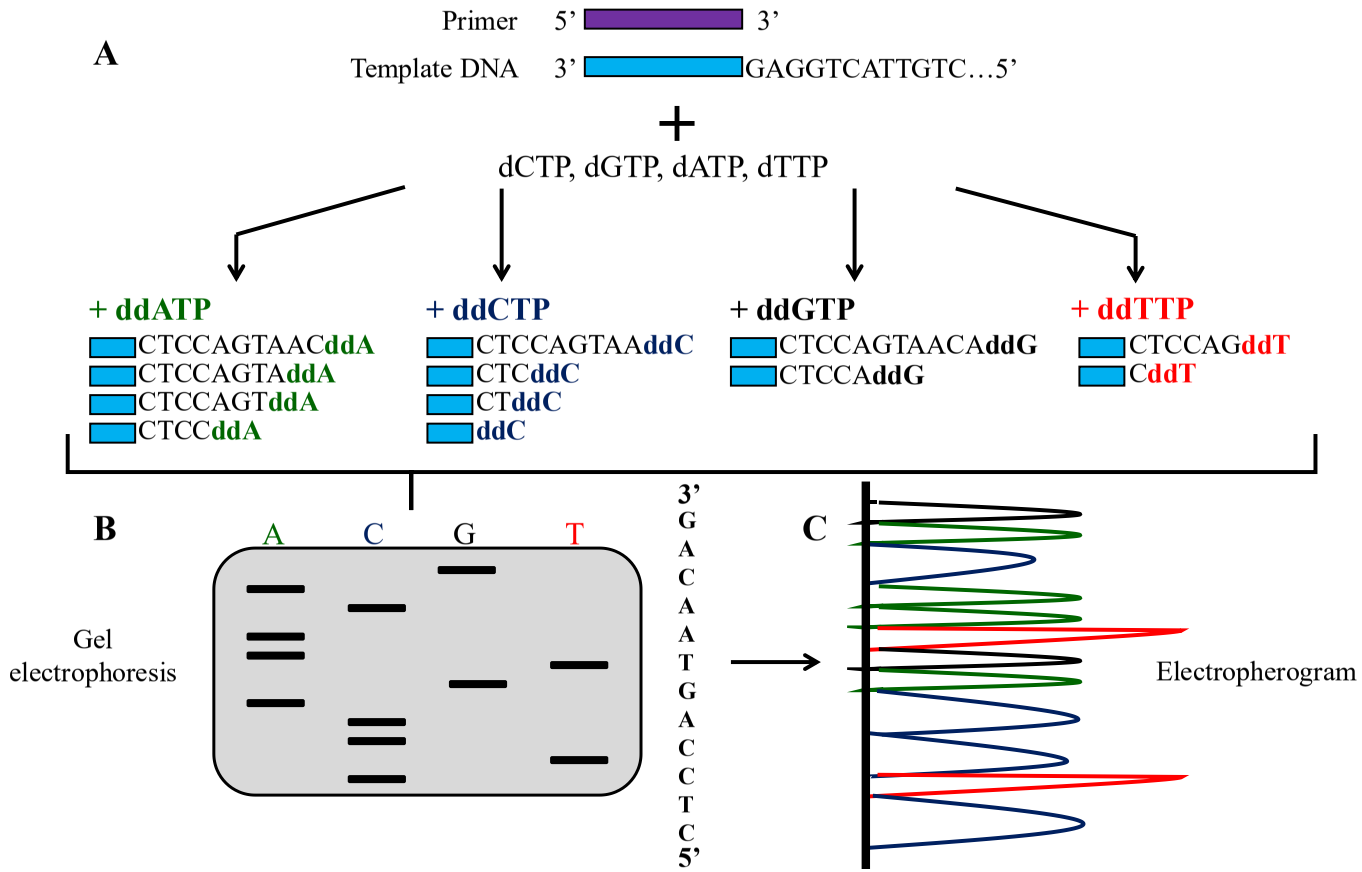


Figure 1.6. Schematic representation of Sanger's enzymatic DNA sequencing. (**A**) The reaction requires: a single stranded DNA template (in light blue), a DNA primer (in violet), normal deoxynucleoside triphosphates (dNTPs), and modified dideoxynucleoside triphosphates (ddNTPs) each labeled with a different fluorophore and able to terminate DNA strand elongation due to the lack of hydroxyl group in 3'. The fragments of different lenght obtained after the reaction are then separated by gel electrophoresis, allowing the identification of the nucleotide sequence (**B**), that is finally shown in a diagram, called electropherogram (**C**) [Modified from: Anasagasti et al., 2012].

Different fetal material sampling techniques are available. Invasive pre-natal techniques, the first to be introduced in obstetrics about 40 years ago, are still currently used but carry a quite high risk of fetal loss. On the contrary, in the last years the new approach of non-invasive pre-natal diagnosis has been developed, with the desire to know the fetus health without risk for himself and for the mother.

1.3.1. Screening tests

Pre-natal screening tests play an important role in the identification, among apparently normal pregnancies, of fetuses at sufficient risk to justify subsequent invasive procedures or costly pre-natal diagnostic tests [Cuckle et al., 1984].

Bi-test is typically done at 11^{th} -14^{th} gestational week [Wald et al., 2003; Malone et al., 2005; Driscoll & Gross, 2009] and is based on the evaluation of two elements: the sonographic nuchal translucency and maternal serum biochemical analyses including β-human chorionic gonadotropin (β-hCG) and pregnancy-associated plasma protein-A (PAPP-A).

Tri-test is performed from the 15^{th} to the 17^{th} gestational week and consists of three biochemical markers: free β-hCG, estriol and α-feto protein (AFP).

Quad is a maternal serum analyte screening which can be offered at 15-20 gestational weeks [Wald et al., 2003; Malone et al., 2005; Driscoll & Gross, 2009]. It is based on the quantification of four biomarkers in the maternal blood: serum AFP, β-hCG, unconjugated estriol and inhibin-A.

Based on the obtained results and few other variables, the parents can have the information about the risk of aneuploid fetus.

In addition, the evaluation of specific markers can be directly predictive of some pathologic conditions: for example, the measurement of acetylcholinesterase and AFP levels is useful for considering neural tube defects, generally related to high values; instead, the measurement of 17α-hydroprogesterone is helpful in case of risk of congenital adrenal hyperplasia disease.

1.3.2. Invasive pre-natal diagnosis

Invasive pre-natal techniques are fetal material sampling procedures which hide a risk of miscarriage, approximately from 0.5 to 1% [Mujezinovic & Alfirevic, 2007; Kozlowski et al., 2008].

The collected fetal material (tissues, cells, DNA), through these procedures, is generally analysed with biochemical and molecular techniques aimed to identify possible alterations in order to perform a diagnosis of fetal diseases.

1.3.2.1. Current invasive sampling techniques

As previously underlined, in order to perform pre-natal diagnosis of genetic diseases, fetal biological material is highly needed. The currently used sampling techniques allow to collect fetal material for genetic analysis with the improvement of medical tools. They are defined invasive because involve diagnostic tools, like needles, inside the uterine environment, so all of them carry a risk of fetal loss that cannot be ignored [Daffos et al., 1983].

For this reason and because of the coast of each single exam, the utilization of invasive procedures is generally limited to pregnancies with high risk, such as advantaged maternal age (>35 years old), positive serum screening tests, a history of a previous affected child, a parent carrying a balanced chromosomal rearrangement of an autosomal recessive disorder, a mother carrying an X-linked disorder and a fetus with structural abnormalities identify with ultrasounds.

The following invasive sampling techniques are currently employed:

- amniocentesis (**Figure 1.7.A**);
- chorionic villus sampling (**Figure 1.7.B**);
- cordocentesis or fetal blood sampling (**Figure 1.7.C**);
- embriofetoscopy;

The first to be used was amniocentesis followed by embriofetoscopy, fetal blood sampling and chorionic villus sampling. Their development was facilitated by improvements in instrumentation and technology, and has gone with the advancement of cytogenetics and molecular genetics techniques [Monni et al., 2010].

The more suitable technique depends on several factors, such as gestational.

1.3.2.1.1. Amniocentesis

Amniocentesis (**Figure 1.7.A**) is routinely offered as a standard practise to women who will be at least 35 years of age. This choice represents a reasonable balance between the hazards of an invasive diagnostic procedure and the probability of an undiagnosed fetal disorder.

Amniocentesis consists in a transabdominal sampling of the amniotic fluid with a spinal needle. Thanks to a continuous ultrasound monitoring during the insertion of the needle, the incidence of bloody and dry taps is lowered, and decreases the need of multiple insertions.

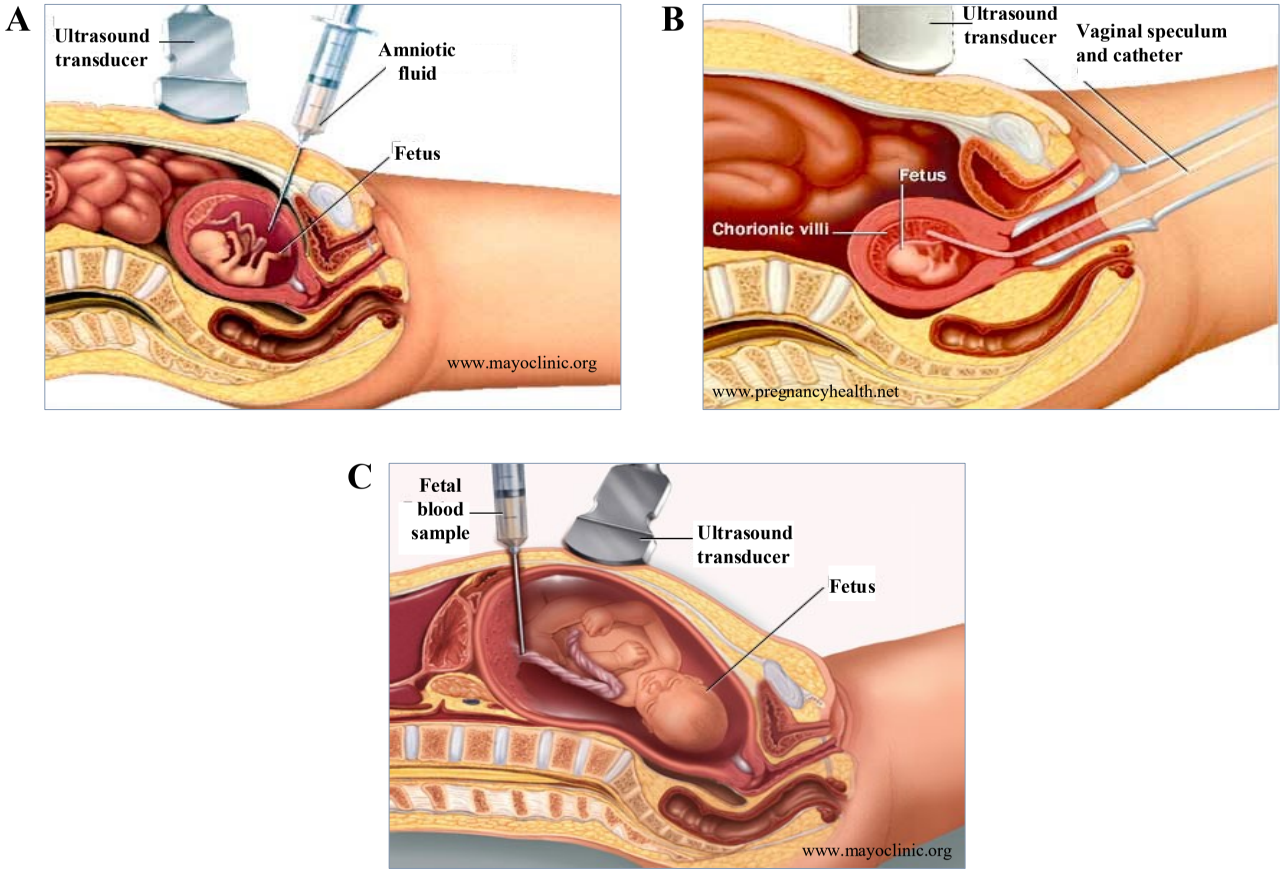


Figure 1.7. Pre-natal invasive sampling techniques. Amniocentesis (**A**), consisting in amniotic fluid sampling by the use of a needle and under a continuous ultrasound monitoring, transcervical or transabdominal chorionic villus sampling (**B**), and cordocentesis or percutaneous umbilical blood sampling (**C**), are represented.

It is usually performed after 15 weeks of gestation, when the uterus can be sampled with minimal risk of injury maternal and fetal health. Between 15 and 18 weeks of gestation, the uterus contains about 150-250 mL of amniotic fluid [Alfirevic et al., 2003].

The technique presents, unfortunately, some complications like pregnancy loss, rupture of membranes, vaginal bleeding or intrauterine infection [Alfirevic et al., 2003]. The risk of a pregnancy loss is between 0.5 and 1% but is very difficult to estimate it, because it is impossible to know the real risk of a miscarriage caused by amniocentesis [Scott et al., 1978]. Sampling earlier in gestation is not suggested.

After sampling the amniotic fluid, the retrieved fetal cells are concentrated and cultured for 5 or 7 days before being analysed. The final laboratory results usually become available within 10-14 days.

1.3.2.1.2. Chorionic villus sampling

Chorionic villus sampling (**Figure 1.7.B**) is a technique that can be performed during the first trimester, between 11 and 24 weeks of gestation, with a risk of miscarriage between 1 and 2.5%. Sampling earlier in gestation may be associated with increased risk of fetal abnormalities and should not be routinely carried out.

The indications for this procedure are the same as those for amniocentesis, except that some fetal abnormalities, such as neural tube defects and anterior abdominal wall defects, are amenable to pre-natal diagnosis only by analysis of amniotic fluid. Therefore, patients with a high risk for fetal abnormalities are not candidates for chorionic villus sampling.

There are two types of chorionic villus sampling: transcervical and transabdominal, both aimed to obtain optimal specimens constituted by 10 to 20 mg of chorionic villi, consisting of the embryonic part of placenta [Shulman et al., 1992]. Both are equally efficacious; the transcervical modality is preferred when the placenta is located on the posterior uterine wall, whereas transabdominal sampling is particularly indicated when the placenta is implanted in a fundal or high anterior location. Both approaches usually require two operators, the first performing the sampling and the second guiding with ultrasounds. The operator experience is fundamental for the success of the exam and for minimizing the risk of a possible miscarriage [Saura et al., 1994].

The collected chorionic villi consist of two cell types: cytotrophoblastic cells, which are rapidly dividing cells used for direct metaphase analysis, and mesenchymal core cells that are used to initiate cell cultures. So, the direct analysis of cytothrophoblasts can provide rapid results, usually within 24 to 72 hours, whereas, for the mesenchymal cells, the results are available after three weeks.

1.3.2.1.3. Cordocentesis or fetal blood sampling

Cordocentesis (**Figure 1.7.C**) is a technique based on percutaneous umbilical blood sampling, usually close to the insertion into the placenta. It is performed between 18 and 20 weeks of gestation, with an estimated risk between 3% in the low risk population and 7.2% in all cases, not only for the miscarriage but also for infections, bleeding and leakage of amniotic fluid [Dugoff & Hobbins, 2002].

Therefore, although this approach remains controversial, now it is only performed for potentially lifesaving therapeutic in utero transfusion procedures in severe cases of fetal anemia or alloimmune thrombocytopenia.

1.3.2.1.4. Embryofetoscopy

This technique can be performed from the 5th week of gestation. It is the only approach that allows a direct visualization of the human embryo *in vivo*. So, it is useful for the confirmation of anomalies diagnosed by ultrasounds in early pregnancies or for the determination of anatomic anomalies. In addition, it is possible to sample small aliquots of fetal blood from the umbilical vessels or the chorionic plate blood vessels. Another use of this technique is a direct intervention for a possible fetal surgery [Reece, 1999].

1.3.2.2. Analysis techniques

The laboratory techniques employed after sampling, with the aim of a pre-natal diagnosis, include biochemical studies, performed on maternal blood, and molecular analysis, performed on fetal genetic material.

Biochemical analyses are aimed to identify and quantify same markers in the maternal blood that may be predictive of possible fetal anomalies.

On the contrary, molecular analyses are performed to detect possible alterations in fetal DNA. They include cytogenetic and DNA analyses.

Molecular cytogenetic analyses such as karyotyping or fluorescence *in situ* hybridization (FISH) with chromosome-specific probes allow the identification of macroscopic anomalies, both in the number and in the structure of chromosomes. So they mainly employed for the diagnosis of common autosomal trisomies and sex chromosome aneuploidies.

Instead, in order to identify specific DNA sequences and to find pathogenic point mutations, DNA analyses are required, such as comparative genomic hybridization (CGH), qRT-PCR, quantitative fluorescent (QF)-PCR, multiplex ligation-dependent probe amplification

(MLPA), Sanger DNA sequencing and next generation sequencing (NGS). Most of them are based on PCR and are useful for the diagnosis of monogenic diseases or X-linked disorders.

1.3.2.2.1. Karyotyping

Karyotyping is the mainly employed technique in cytogenetics (**Figure 1.8**).

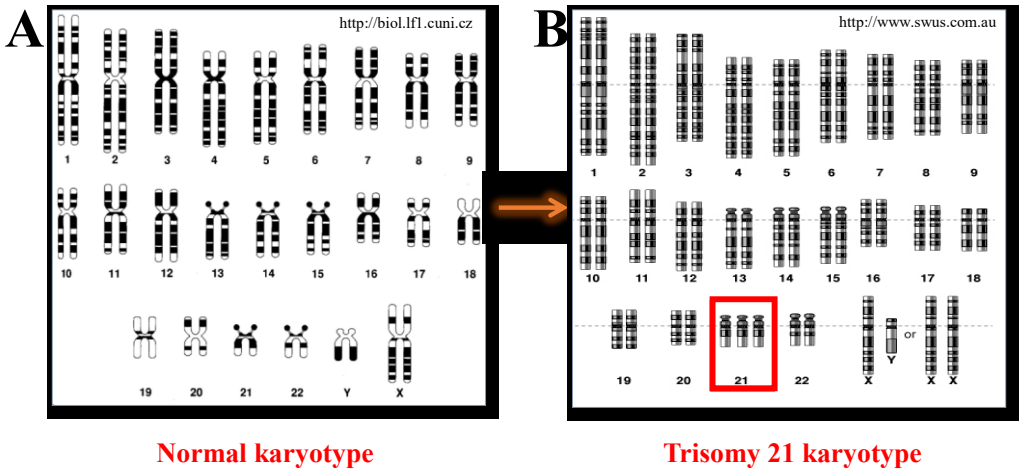


Figura 1.8. Human karyotype. (**A**) Normal karyotype, consisting of 46 couples of chromosomes, and (**B**) karyotype presenting the trisomy 21 or Down syndrome, characterized by three chromosomes 21.

In pre-natal diagnosis, this molecular analysis has become an indispensable diagnostic tool for the identification of chromosome abnormalities such as aneuploidies or unbalanced structural rearrangements. It consists in the representation of the ordered set of the individual chromosomes. In order to obtain a karyotype, chromosomes in a particular phase of the cell cycle, the metaphase, are needed because in this stage the chromatin is condensed. The use of colchicine, a substance that blocks the metaphase chromosomes, is required [Levan, 1938] and permits to increase the number of metaphase spreads available for analysis in a preparation because it inhibits spindle formation during mitosis.

In addition, the treatment of cells with a hypotonic solution facilitates chromosome spreading, leading to a better definition useful for counting the chromosomes [Tjio & Levan, 1956].

Karyotyping has been the first technique used for the identification of the aneuploidy 21 (**Figure 1.8.A**) [Lejeune et al., 1959]. Subsequently, the chromosomal abnormalities causing Klinefelter and Turner syndromes were identified [Ford et al., 1959; Jacobs & Strong, 1959].

This method is usually carried out through the biological material collected during invasive techniques.

1.3.2.2.2. FISH

The first application of molecular techniques to chromosome slide preparations, called *in situ* hybridization (ISH), was an attempt to identify and locate specific nucleic acid sequences inside cells or on chromosomes [John et al., 1969]. The technique is based on the hybridization between a target DNA in cells, nuclei or metaphase chromosomes with a suitable complementary probe. The hybridization can be visualized by autoradiography, which has been applied to human chromosomes since 1960s [German & Bearn, 1961]. Subsequently, an approach with the use of non-radioactive probe was developed, where the hybridization can be visualized through avidin or streptavidin fluorescent labeling [Langer et al., 1981].

FISH employs fluorescently labeled probes, resulting in an increase of analysis resolution that allows to identify chromosome rearrangement at submicroscopic levels. This technique is based on the fixation and denaturation of target DNA in cells, nuclei or metaphase chromosomes on the surface of a slide. The probe DNA must be labeled with a nucleotide that is either conjugated to fluorescein (direct labeling) and/or a non-fluorescent hapten (indirect labeling). It is first denatured and pre-hybridized with unlabeled repetitive DNA, then, it is hybridized with target DNA for 16-48 hours at 37°C.

After hybridization, the unbound single-strand DNA is removed by a wash. Then, an anti-fade solution containing DAPI (4'-6-diamidino-2-phenylindole), a fluorescent stain, is applied to the slide. At this point, it is possible to observe the specimen at the epifluorescence microscope with suitable filters [Reichman, 2000]: a camera captures the image and the fluorescent signals are subsequently quantified [Hiraoka et al., 1987].

Many different probes can be used for FISH: currently, a range of commercial probes is available for the detection of certain constitutional and acquired chromosomal abnormalities. In addition, depending on abnormality to look for, several approaches have been developed, such as reverse-FISH [Carter et al., 1992], fiber-FISH [Heiskanen et al., 1995], M (multicolour)-FISH [Speicher et al., 1996], SKY (spectral karyotyping)-FISH [Schröck et al., 1996], flow-FISH [Rufer et al., 1998], Q (quantitative)-FISH [Martens et al., 1998], COBRA (combined binary ratio labelling)-FISH [Tanke et al., 1999], cenM (centromere-specific multicolour)-FISH [Nietzel et al., 2001], pod (parental origin determination)-FISH [Weise et al., 2008] and other modified FISH approaches.

While with the standard FISH it is not possible to simultaneously detect all of the chromosomes in the entire genome, the most advanced FISH-based approaches permit trough colour karyotyping to distinguish different chromosomes because differentially labeled.

In pre-natal diagnosis through the use of fluorescent probes specific for whole chromosomes or portions of them, it is possible to identify submicroscopic rearrangements and detect very small partial trisomies or microdeletions, not visible with other cytogenetic techniques [Bishop, 2010].

1.3.2.2.3. CGH and microarray CGH

CGH was developed as an efficient genome wide screening strategy for detecting DNA copy number imbalances [Kallioniemi et al., 1992] within a single experiment. This technique, unlike FISH, not only provides high-resolution detection of genomic alterations, but also allows refinement of breakpoints on chromosome rearrangements.

It is based on a comparison between the DNA content of a test and a reference genome by differentially labeling the two genomic DNA with distinct fluorochromes: the green fluorescein isothiocyanate (FITC) and the red (Texas red) fluorescent dyes, denatured and subsequently co-hybridized to normal metaphase chromosomes. Due to the simultaneous hybridization to the normal denatured metaphase chromosome spreads, there is a competition from DNA hybridization to homologues sites. After washing to remove the unbound DNA, a fluorescence microscope observation of the metaphase spreads is performed, and the resulting fluorescence intensities of test and reference hybridization are digitally quantified along the length of each chromosome. If chromosomal regions are equally represented in both samples, the chromosomes appear yellow, because there is an identical amount of green and red dyes.

In some studies, it has been demonstrated that, beyond the limits detectable by microscopy, CGH is a useful tool for detecting deletions and duplications in patients with mental retardation or learning difficulties [Vissers et al., 2003; Shaw-Smith et al., 2004]. It is also suitable for the analysis of individuals with known chromosome abnormalities by using a custom designed array [Bejjani et al., 2005]. The clinical utility of CGH in the pre-natal setting has been demonstrated in systematic reviews [Hillman et al., 2011; Hillman et al., 2013].

This approach offers several potential advantages over conventional parental testing: in fact, in addition of being sensitive and comprehensive, it may also be amenable to automation, thus leading to a reduction of coasts, labour and reporting time of results. The major

challenge for the large scale implementation of this technique appears to lie in the interpretation of results [Vetro et al., 2012]. Nevertheless, it requires metaphase chromosomes as targets for hybridization, limiting the high resolution of the method around 3 Mb [Kirchhoff et al., 2004].

A new method has been designed: the array-based CGH. It has been developed to replace the metaphase chromosomes with DNA sequences, in order to increase the resolution for detecting copy number changes in the human genome, leading to more detailed information on genomic gains and losses. The fundamental principle of array-CGH is the same as that in CGH, but the first involves comparative genomic hybridization using an array rather than a metaphase spread as the substrate [Pinkel et al., 1998]. The current microarrays are constituted by thousands of spots of reference DNA sequences applied in a precisely gridded order on the slide. At the beginning, the arrayed DNA segments were large human DNA segments (about 150 Kb) inserted into a bacterial artificial chromosome (BAC) or bacterial/P1-derived artificial chromosome (PAC) [Ishkanian et al., 2004]. Then, as the resolution of the array yields improved, shorter sequences have been used as targets, including smaller cDNA fragments [Pollack et al., 1999; Ishkanian et al., 2004], PCR products [Mantripragada et al., 2004] and oligonucleotides [Rouillard et al., 2002]. The coverage and properties of array-CGH are dependent on the design and density of the array used, but this technique provides resolution at the nucleotide level. So, the arrays developed to detect SNPs have the highest resolution of all the available array-based platforms [Le Scouarnec & Gribble, 2012]. The genotype information obtained from these SNP array enables the identification of recessive disease genes or mosaic aneuploidies [de Leeuw et al., 2012].

Given the recent advantages in techniques for the study of chromosomes, it has been suggested that this technique could gradually replace classical cytogenetics in clinical diagnosis.

1.3.2.2.4. QF-PCR

QF-PCR is a technique that allows visualizing and quantifying specific DNA sequences using fluorescent primers [Guzel et al., 2012; Kamyab et al., 2012].

The principle of this approach is based on the amplification of selected short tandem repeats (STRs), witch are genomic repetitive sequences comprised between 2 and 5 nucleotides. Each STR has a polymorphic nature related to the different number of repeats at a given locus, generating several alleles of different lengths. So it is quite easy to analyze STRs by PCR amplification with primers flanking the polymorphic sequences, because fluorescent

products of different lengths are generated, whose amount is directly proportional to the amount of target sequence in the initial template.

QF-PCR is employed to assess fetal sex by using the nonpolymorphic Amelogenin gene: with this marker it is possible to generate X- and Y-specific products of different lengths, besides detecting sex chromosomal disorders [Crigliano et al., 2004; Crigliano et al., 2005]. This method is rapid and also enables pre-natal diagnosis of aneuploidies involving chromosomes 21, 18, 13, X and Y within short time, such as 24 or 48 hours from the sampling [Guzel et al., 2012; Papoulidis et al., 2012].

1.3.2.2.5. MLPA

MLPA has been developed in recent years as a method based on PCR for detection of aneuploidies. It is a multiplex PCR-based assay that allows to determine the copy number of up to 50 different genomic DNA sequences in only a single reaction tube [Schouten et al., 2002] (**Figure 1.9**).

This technique employs several probes of unique length composed of two parts that, when hybridized to adjacent target sequences on genomic DNA, can be joined together by the enzyme DNA ligase: this permits the amplification of all target sites, by using a single primer pair complementary to the two free ends common to all the probes. Then products are run on a capillary electrophoresis system and separated by size: each peak corresponds to the amplification product of a specific probe.

This technique is widely used in pre-clinical settings, where it permits, for example, the detection of abnormalities with uncharted clinical relevance. Compared to karyotyping or FISH, it is less labour-intensive and cheaper [Yan et al., 2011].

In pre-natal diagnosis, MPLA is considered a clear and inexpensive genetic screening method for pregnancies at high risk [Sellner & Taylor, 2004].

1.3.3. Non-invasive pre-natal diagnosis

Considering the risks associated to conventional invasive techniques, in recent years, non-invasive pre-natal diagnosis has been becoming increasingly important, allowing the investigation of the fetal health status without risk for the fetus or the mother, although with only a predictive/probabilistic value.

Non-invasive pre-natal diagnosis is aimed to identify genetic abnormalities from the analysis of maternal blood during the pregnancy, and is based on the discovery of circulating cell-free fetal DNA (ccffDNA) within maternal plasma in 1997 [Lo et al., 1997]. The discovery of the ccffDNA has permitted the advancement of safer and earlier tests based to a simple

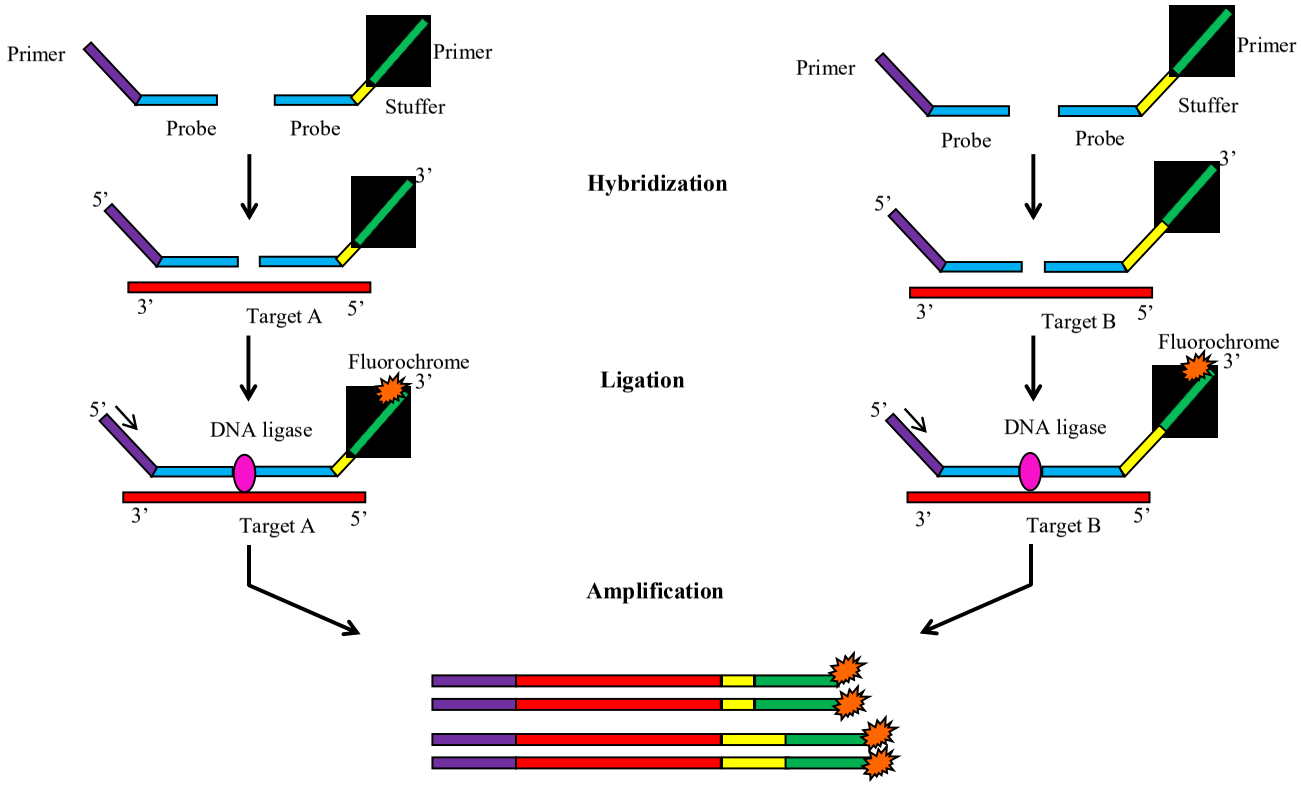


Figure 1.9. MLPA (multiplex ligation-dependent probe amplification). Two probes are required for the amplification of each target DNA (for example, Target A on the left, and Target B on the right), containing a sequence complementary to the target (in light blue) and a portion useful for the annealing of universal primers; one of them contains also a "stuffer" sequence (in yellow), whose length is different for different targets. After the hybridization with the target sequences, the two probes can be joined together by the enzyme DNA ligase (in pink): this allows the amplification of all target sites, by using a single primer pair complementary to the two free ends common to all the probes. One of the primers is usually conjugated with a fluorochrome. At the end of the reaction, the amplification products of different lengths can be easily separated and analyzed [Modified from: Höming-Hölzel et al., 2012].

maternal peripheral blood sample [Drury et al., 2016]. Non-invasive pre-natal testings provide the possibility to determine fetal sex [Wright et al., 2012; Perlado-Marina et al., 2013], fetal rhesus D (RhD) genotyping [Clausen et al., 2014; Fasano, 2016], some pregnancy-associated conditions, including preeclampsia [Contro et al., 2017; van Boeckel et al., 2017; Rolnik et al., 2018], aneuploidies [Mennuti et al., 2015; Skrzypek & Hui, 2017], and the identification of paternally inherited monogenic disorders [Wright & Burton, 2009; Bustamente-Aragonés et al., 2012; Skrzypek & Hui, 2017]. Lately, significant advancements have been investigated extending the potential applications to fetal whole-genome sequencing and maternally inherited mutations [Perlado et al., 2016; Hui et al., 2017; Beulen et al., 2017; Kagan et al., 2017; Hudecova & Chiu, 2017; Hayward & Chitty, 2018; Chiu et al., 2018; Lee et al., 2018].

1.3.3.1. Fetal cells

In maternal circulation, different fetal cell types have been found, including trophoblasts, leukocytes and erythrocytes/nucleated red blood cells [Sekizawa et al., 2007].
However, it has been demonstrated that significantly more ccffDNA is present in maternal plasma or serum compared to fetal DNA extracted from the cellular component of maternal blood [Lo et al., 2000]. Therefore, ccffDNA is generally preferred for diagnostic purposes.

1.3.3.2. ccffDNA

As previously mentioned, the discovery of ccffDNA in maternal circulation was made by Lo and his colleagues in 1997, applying a simple and sensitive Y chromosome-specific PCR assay to detect ccffDNA from women bearing male fetuses [Lo et al., 1997]. One year later, the same group described the presence of ccffDNA in maternal serum as early as the 7th gestational week, with increasing concentration as pregnancy progresses, and rapid disappearance after delivery [Lo et al., 1998]. Furthermore, using qRT-PCR, they determined the concentration of ccffDNA in maternal plasma as 3.4-6.2% of total circulating cfDNA, at early and late gestation, respectively [Lo et al., 1998; Rossa et al., 2001].

1.3.3.2.1. Amount

The ccffDNA reaches a mean of 25.4 genome equivalence (GEq)/mL, in early pregnancy and 292.2 GEq/mL in late pregnancy, where one GEq is defined as the amount of a sequence of DNA present in one diploid cell [Lo et al., 1998; Rossa et al., 2001].
The ccffDNA comprises only a small portion of total circulating cfDNA: the earliest studies suggested that the fetal fraction was only 3-6% [Lo et al., 1998], but more recent studies

have found that, in last gestational weeks, it may be closer to 10-20% [Lun et al., 2008]. It is possible to detect ccffDNA from 4 weeks of gestation until delivery [Illanes et al., 2007]. The proportion of ccffDNA grows by 0.1% every week between the 10th and 21st gestational week, then, increases faster with almost 1% increment/week after the 21st week [Drury et al., 2016; Zhou et al., 2016].

In addition, the amount of circulating cfDNA depends, besides the gestation period, on other factors, such as maternal diseases and body weight [Vora et al., 2012; Zhou et al., 2015; Kim et al., 2016], aneuploidies [Zhou et al., 2015] and twin pregnancies [Attilakos et al., 2011], but in general, the increasing concentration of ccffDNA, correlated to pregnancy progression, has been observed [Bischoff et al., 2005].

Anyway, the very low amount of ccffDNA in maternal plasma is a critical point, requiring both specific and optimized techniques for its purification and very sensitive detection approaches.

1.3.3.2.2. Size

Circulating cfDNA in maternal plasma is highly fragmented. It was found that the 85.5% of ccffDNA is shorter than 0.3 Kb in early pregnancy. It constitutes the 28.4% of the < 0.3 Kb fraction in maternal plasma, increasing to 68.7% in the third trimester [Bischoff et al., 2005]. The 99% of fetus-derived DNA is shorter than 312 bp, whereas maternal cfDNA has a medium length of about 400-500 bp. In conclusion, the length of ccffDNA is comprised between 150-300 bp, but the entire fetal genome is represented [Chan et al., 2004; Li et al., 2004].

1.3.3.2.3. Source

Although the ccffDNA origin is still uncertain, the most accredited sources are the following:

- Fetal hematopoietic cells apoptosis in maternal blood [Sekizawa et al., 2000; Sekizawa et al., 2007];
- Transfer of cffDNA through placenta [Bianchi et al., 2004; Sekizawa et al., 2007];
- Trophoblasts disruption [Jackson, 2003; Sekizawa et al., 2007];

It seems that the principal origin of the fetal nucleic acids in maternal plasma is the trophoblasts breakdown as apoptotic fragments included in microvesicles due to the continuous trophoblasts turnover, but quickly absent after birth when the placenta is removed [Jackson, 2003; Alberry et al., 2007]. Moreover, the increasing ccffDNA concentration,

during pregnancy progression, seems to be caused by placental expansion [Sekizawa et al., 2003; Sekizawa et al., 2007].

1.3.3.2.4. Stability

The ccffDNA stability is a fundamental issue to obtain an optimal DNA yield after extraction and to perform the subsequent diagnostic analyses. ccffDNA seems quite stable in maternal plasma [Angert et al., 2003], but it is promptly cleared after birth: it is not possible to detect fetal fragments after birth, so there is not the risk of persistence from one pregnancy into the next one [Lo et al., 1999; Hui et al., 2008].

After isolation of ccffDNA from maternal plasma, its stability was found very low [Ordonez et al., 2013] recommending storage on ice and particular care during purification and handling, as well as a storage temperature of -80°C.

Moreover, it has been found that the extraction efficiency is also affected by the storage temperature of blood prior to plasma preparation, suggesting 4°C as the optimum temperature after sample collection [Hidestrand et al., 2012]. In addition, in maternal plasma, the fetal fraction can be reduced due to a maternal blood cells lysis in the time-lapse between blood sampling and plasma preparation [Ordonez et al., 2013]. To overcome this problem, Dhallan et al. proposed to treat blood samples with formaldehyde [Dhallan et al., 2004]. Later, Zhang et al. demonstrated that the formaldehyde prevents cell lysis and plasma DNase activity, permitting to increase the fetal DNA recovery [Zhang et al., 2008].

1.3.3.2.5. Purification and enrichment strategies

The plasma results the source of election for the best recovery of ccffDNA [Hui et al., 2017]. About the purification techniques suitable for the ccffDNA extraction, several studies have been performed where some commercially available DNA extraction kits were compared [Schmidt et al., 2005; Clausen et al., 2007; Repiska et al., 2013]. Some works in literature report also that phenol-chlorophorm procedure can allow extracting ccffDNA from maternal plasma with very high yields, greater than any commercial purification kit [Yuan et al., 2012; Chen et al., 2012]. However, the two routinely employed purification kits are QIAamp® DNA Blood Mini Kit (Qiagen) and QIAamp® DSP Virus Spin Kit (Qiagen) [Schmidt et al., 2005; Clausen et al., 2007; Repiska et al., 2013].

In non-invasive pre-natal testing, the background due to circulating maternal DNA interferes with the sensitivity and detection of fetal genetic features, and, therefore, the ccffDNA concentration in maternal plasma becomes a critical limit, requiring enrichment strategies.

Therefore, the developed techniques are aimed to enrich fetal cells into maternal circulation. For example, the fluorescence-activated cell sorting (FACS) technique has been successfully reported for the enrichment of fetal leukocytes from maternal plasma [Herzenberg et al., 1979]. Bianchi et al. discovered a technique to enrich erythrocytes/nucleated red blood cells (NRBCs) containing ccffDNA through the use of FACS technique and the employment of a monoclonal antibody against the transferrin receptor, highly expressed on fetal erythroblasts despite maternal ones [Bianchi et al., 1990]. Other investigators using different monoclonal antibodies and cell enrichment techniques successfully confirmed this kind of approach [Price et al., 1991; Zhao et al., 2002; D'Souza et al., 2007; Sekizawa et al., 2007].

Another method was performed with the use of magnetic cell sorting following the NRBCs enrichment [Genshirt-Ahler et al., 1993]. Fetal haemoglobin has been used in order to discriminate maternal from fetal NRBCs. Nonetheless, they still obtained a maternal origin for a 20% of fetal haemoglobin positive NRBCs [de Graaf et al., 1999].

As for enrichment of ccffDNA in maternal plasma, one of the most studied approach exploits the difference in length between maternal and fetal DNA fragments. The purification of circulating DNA fractions of lower molecular weight (100-300 bp), after agarose gel electrophoresis, might increase the fraction of ccffDNA and, also, to improve the detection of point mutations of other genotypic features [Li et al., 2004; Li et al., 2006; Li et al., 2009]. Alternatively, it is possible to measure only short DNA molecules, amplifying short and long amplicons by PCR [Lun et al., 2008; Drury et al., 2016]. For example Lun et al. described a relevant discrimination between maternal and fetal molecules using 179 and 64 bp amplicons obtained with primers for *ZFX* (zinc finger X-linked gene)/*ZFY* (zinc finger Y-chromosome gene) [Lun et al., 2008]. Routinely, enrichment based on size is not applied to clinical protocol.

On the same bases, COLD-PCR (co-amplification at lower denaturation temperature-PCR) was proposed as enrichment method, which selectively amplifies minority alleles from background of wt alleles [Li et al., 2008; Li & Makrigiorgos, 2009]. This technique could be used for the detection of fetal alleles in maternal blood because the shorter fragments of ccffDNA could be denatured using a temperature at which maternal DNA fragments would remain double-stranded, allowing only ccffDNA to be amplified by COLD-PCR [Li et al., 2008].

Finally, the new frontier for ccffDNA enrichment could be based on DNA methylation, the most studied mechanism of epigenetic regulation. Indeed, in the case of hypermethylation of ccffDNA, MeDIP (methylation DNA immunoprecipitation) can achieve an enrichment, a

technique that employs immunoprecipitation to enrich the DNA sample of hypermethylated sequences [Ioannides et al., 2014].

1.3.3.3. Non-invasive pre-natal testing clinical applications

Since the discovery of ccffDNA in maternal plasma [Lo et al., 1997], non-invasive pre-natal testing has been applied on different fields such as fetal gender determination, X-linked disorders, fetal RhD genotyping, aneuploidies and monogenic disorders.

1.3.3.3.1. Fetal gender determination

The first application of ccffDNA in maternal plasma or serum was aimed to the determination of fetal sex [Rossa et al., 2001; Sekizawa et al., 2002]. This is extremely important in those cases where the mother is carrier of an X-linked disorder, such as DMD or hemophilia because pregnancies with male fetuses are primarily at risk, or for those at risk of conditions associated with ambiguous development of external genitalia, for example congenital adrenal hyperplasia, where early maternal treatment with dexamethasone can reduce the degree of virilisation of female fetuses [Forest et al., 1998; Hyett et al., 2005]. Furthermore, the value of ccffDNA in maternal plasma by copy number of Y specific sequence has been reported as an indicator for preeclampsia [Swinkels et al., 2002; Zhong et al., 2002].

The most commonly used technology for detecting male fetus specific DNA in maternal plasma is represented by qRT-PCR amplifying the single copy *SRY* (sex-determining region Y) gene located on chromosome Y as target gene [Rossa et al., 2001], the single copy sequence *DYS14* (testis specific protein Y-linked) [Lo et al., 1990] and the multicopy *DAZ* (deleted in azoospermia) gene [Stanghellini et al., 2016]. However, during early gestation, it is quite difficult to detect very low amount of ccffDNA [Lo et al., 1990; Rossa et al., 2001; Brich et al., 2005; Stanghellini et al., 2016].

In a systematic review, including a PubMed based meta-analysis, 146 publications have been identified used to determine the clinical validity of non-invasive pre-natal sex determination using ccffDNA in maternal blood and urine, based on PCR and qRT-PCR. Despite the expected variability among the considered studies, the overall sensitivity (95.4%) and specificity (98.6%) of the employed technologies were high but when the analytical tests were performed prior to 7 weeks of gestation using blood, they were found to be unreliable [Devaney et al., 2011]. This conclusion has been reported in other recent studies [Wright et al., 2012; Perlado-Marina et al., 2013].

In addition, non-invasive pre-natal diagnosis identification of female fetuses, by employing Y chromosome specific sequences, is based on a null result, but this may be the source of false negative results if the amount of fetal male DNA is so little not to be detected by qRT-PCR. Otherwise, this method could lead to false positive results if male DNA contaminates the sample, during the extraction of ccffDNA from maternal plasma.

So, other approaches would be required for detecting also fetal female DNA, for example based on epigenetic markers. Some genes have been identified with the differential methylation pattern in maternal blood cells and in fetal placenta [Chim et al., 2005; Chan et al., 2006], allowing to distinguish maternal and fetal DNA. For example, the *mapsin* gene promoter is unmethylated in the placenta but hypermethylated in maternal blood cells [Chim et al., 2005; Bellido et al., 2010]. After bisulphite treatment, that permits the conversion of non-methylated cytosines in uraciles, a methylation specific PCR with suitable primers can distinguish the T and C alleles of U-*mapsin* based on size [Tong et al., 2006].

Although the bisulphite conversion causes DNA degradation up to 96%, some strategies have been developed to overcome DNA degradation and to perform paternal diagnosis of fetal sex [Weber et al., 2005].

On the contrary, if the target sequence is unmethylated in maternal cells and hypermethylated in the placenta, as for *RASSF1* (Ras association domain family member 1) gene, a tumor suppressor gene, a digestion of maternal DNA with a restriction enzyme sensitive to methylated sequences can enrich fetal sequences [Tong et al., 2010; Go et al., 2011]. In both cases, DNA sequences belonging to both male and female fetuses can be identified.

Another approach to detect fetal gender has been suggested by Tang, who successfully identified female fetuses from maternal plasma using paternally inherited STRs located on the X chromosome [Tang et al., 1999].

A further aspect in non-invasive pre-natal diagnosis of X-linked disorders is first the investigation of the maternal defect in maternal plasma and then the detection of disease status in the male fetuses.

In a recent study, Tsui et al., analysed maternal mutations in 7 pregnancies carrying haemophilia risk, in order to quantify a difference between mutant/wt alleles in maternal plasma to identify the fetal genotype [Tsui et al., 2011].

1.3.3.3.2. Fetal RhD genotyping

The development of a non-invasive method for the determination of RhD status in Rh- pregnant women has proved of fundamental importance both to provide a prophylaxis, administrating anti-D immunoglobulins, in pregnancies at high risk for this condition, and

to avoid risks, such as miscarriage, haemorrhage and sensitization, caused by conventional sampling methods [Lo et al., 1998].

Non-invasive pre-natal diagnosis of fetal RhD genotype is already being performed in the UK, France, and the Netherlands [Bianchi et al., 2005], and several studies confirmed that pre-natal diagnosis with non-invasive techniques based on PCR can be accurately performed [Brojer et al., 2005; Van der Schoot et al., 2006; Clausen et al., 2014]. However, small percentages of cases can be false positive or negative results, due to: lack of ccffDNA in maternal plasma at early gestation, or low sensitivity of technology to detect ccffDNA [Sekizawa et al., 2007; Bianchi et al., 2005; Brojer et al., 2005; Van der Schoot et al., 2006].

1.3.3.3.3. Pregnancy-associated conditions

An increase of ccffDNA levels has been found in some pathologies correlated with pregnancy, such as preeclampsia [Lo et al., 1999; Zhong et al., 2001], preterm labour [Leung et al., 1998], invasive placentation [Sekizawa et al., 2002], hyperemesis gravidarum [Sugito et al., 2003], intrauterine growth restriction [Caramelli et al., 2003], feto-maternal haemorrhage [Lau et al., 2000] and polyhydramnios [Zhong et al., 2000]. In these cases, the quantification of ccffDNA sequences may have an important diagnostic value.

For example, preeclampsia is a multisystem pathology characterized by hypertension and proteinuria that occurs late in pregnancy [Redman et al., 1999]. In preeclampsia, placenta shows failure of trophoblast cells to invade and remodel mother spiral arteries, causing a restriction in the blood flow to the fetus and thus contributing to onset of the disease that leads to maternal mortality. The quantification of cffDNA in maternal plasma as an indicator for preeclampsia has first ben reported by Lo in a small scale study where in plasma of preecamptic women the cffDNA was increased approximately 5-fold than in plasma deriving from unaffected pregnant women. Other studies have recorded a significant increase in the copy number of Y specific sequences of ccffDNA in plasma of preeclamptic women [Swinkels et al., 2002; Zhong et al., 2002].

1.3.3.3.4. Aneuploidies

Non-invasive pre-natal diagnosis has been successfully applied to the detection of the most common aneuploidies, in order to identify pregnancies at sufficient risk for trisomy 21, 18 and 13 [Mennuti et al., 2015; Skrzypek & Hui, 2017]. Currently, commercially available non-invasive pre-natal testings permit to disclose common chromosomal aneuploidies, sex chromosome abnormalities, triplody and are based on sequencing approach for gathering the genetic information of ccffDNA. Now these approaches are available from the 9^{th} -10^{th} week

of gestation and extended to the detection of microdeletions, microduplications and paternally inherited mutations causing genetic diseases or/and carrier status [Sekizawa & Saito, 2001; Bianchi, 2012; Ferrari et al., 2015]. Unfortunately, these types of investigations are considered as screening tests, so positive results should be confirmed using amniocentesis or chorionic villus sampling.

Another advanced technology applied to aneuploidies is the digital PCR (dPCR). Several articles reported the use of the dPCR for accurate measurement of chromosomal aneuploidies using ccffDNA obtained from maternal plasma [Evans et al., 2012; Lee et al., 2018].

1.3.3.3.5. Monogenic diseases

The development of a simple, quick and cheap non-invasive pre-natal technology for monogenic disorders is fundamental for a timely diagnosis with the aim to start, as soon as possible, the specific conventional or the possible personalized therapies. However, the detection of fetal single point mutations from ccffDNA is extremely difficult. The maternal DNA sequences are very similar to those fetal and so the diagnosis of monogenic disorders remains limited to the detection of paternally inherited alleles [Wright & Burton, 2009; Liao et al., 2014].

When the disease is autosomal dominant and the father carries the mutation or this spontaneously occurs *de novo* during oocyte or sperm formation, it can be easly detected by using the same approach employed in fetal sex or RhD determination based on qRT-PCR [Ferrari et al., 2015]. But, in case of an autosomal recessive disorder, it is significantly harder to diagnose, because the maternally inherited portion of fetal genome and maternal DNA are identical. So it is necessary to develop strategies able to compare the mutant and the wt alleles at a quantitative level, in order to disclose if the fetus has inherited the mutant or the normal one [Liao et al., 2014].

Moreover these non-invasive testing should be highly sensitive because ccffDNA in maternal plasma is about 20% of total DNA in the third trimester of pregnancy but only 10% or less in the first trimester useful for non-invasive pre-natal testing [Ferrari et al., 2015]. The possible difficulties, encountered in applying the current non-invasive pre-natal testing approaches into clinical practice, are: low sensitivity and reproducibility, expensive coasts, possible contaminations, laborious techniques; adequate and sophisticated instrumentation, qualified and specialised staff [Ferrari et al., 2015].

Recently, the advanced technologies such as dPCR, have permitted the clinical application and the implementation of non-invasive pre-natal testing because of the high sensitivity and capability to reveal the entire fetal genome from maternal plasma DNA and in the future

they could permit also the detection of maternally inherited mutations [Liao et al., 2014; Wong & Lo, 2016; Perlado et al., 2016; Camunas-Soler et al., 2017].

1.3.3.4. Pre-natal non-invasive molecular techniques

In case of non-invasive pre-natal diagnosis, molecular analysis technique are required in order to detect fetal genetic alterations from ccffDNA. Some of the used method are described also for post-natal diagnosis such as qRT-PCR, other are described in the section of molecular techniques for pre-natal diagnosis after collecting fetal material using invasive techniques.

Other approaches suitable for non-invasive pre-natal diagnosis are listed below, also able to discriminate very low amounts of fetal DNA, the crucial issue of non-invasive sampling.

1.3.3.4.1. MALDI-TOF (matrix-assisted laser desorption/ionization-time of flight)

An alternative strategy, developed by the laboratory of Dennis Lo in Hong Kong, was the use of MALDI-TOF mass spectrometry for the non-invasive detection of fetal point mutations [Ding et al., 2004].

Circulating cfDNA purified from maternal plasma is first amplified by PCR for the region of interest. PCR products are then subjected to standard base extension and Single Allele Base Extension Reaction (SABER), the latter being restricted to the fetal-specific allele by addition of a single species of dideoxynucleoside triphosphate without any dNTP [Ding et al., 2004]. The fragments obtained are then analyzed with MALDI-TOF, a soft ionization technique, and finally identified from the mass spectrum [Ding et al., 2004], where mutations or polymorphisms in the fetal DNA inherited from the father are shown as peaks of different molecular weight with respect to maternal ones, and so easily distinguishable and identifiable [Ding et al., 2004].

In the field of non-invasive pre-natal diagnosis, it has been demonstrated that the enrichment for size fractionation of ccffDNA may lead to the improved detection of fetal point mutations causing β thalassemia by MALDI-TOF [Li et al., 2009].

The advantages of this method derive from the analytical specificity conferred by the base extension reaction, and from the sensitivity of mass analysis, even though the technique is very laborious [Ding et al., 2004].

1.3.3.4.2. dPCR

The dPCR is a very sensitive method for DNA quantification by using PCR. The basic concept is to greatly dilute the sample and distribute very small aliquots (1-2 DNA molecules) for each reaction. Then a PCR reaction is performed with fluorescent probes (such as in qRT-PCR) and the end-point fluorescence is measured at the end of the reaction, in order to discriminate positive and negative samples [Whale et al., 2012]. In addition, the results are subjected to a calculation of the statistical distribution (Poisson distribution) [Whale et al., 2012]. The precision and the reproducibility of this method depend on the number of aliquots of diluted sample: much more the aliquots are, much more the technique is sensitive and accurate [Pinheiro et al., 2012].

The dPCR is a very useful technique, because it allows to detect mutations present only in a small portion of template DNA, such as the genetic alterations carried by the fetus [Pinheiro et al., 2012]. For further specification, see section **1.5**.

1.3.3.4.3. Nested PCR

Nested PCR involves two sets of primers, used in two successive runs of PCR, the second set intended to amplify a secondary target within the first run product [Fucharoen et al., 2003]. So, the target DNA undergoes the first amplification with the first set of primers, then the obtained product undergoes a second amplification with the second set of primers. It is very unlikely that any of the unwanted PCR products contain binding sites for both the new primers, ensuring the product from the second PCR has little contamination from unwanted products of primer dimers, hairpins, and alternative primer target sequences [Fucharoen et al., 2003].

By using a combined nested PCR and subsequent restriction analysis, the successful application of this strategy to non-invasive pre-natal detection of the paternally inherited HbE (caused by the GAG to AAG conversion at codon 26 of the β globin gene), has been demonstrated [Fucharoen et al., 2003].

Nested PCR increases both the sensitivity and the specificity of amplification but requires the subsequent restriction analysis [Fucharoen et al., 2003].

1.3.3.4.4. PAP (pyrophosphorolysis-activated polymerization)

Pyrophosphorolysis is the reverse reaction of DNA polymerization: in the presence of pyrophosphate, the 3' nucleoside is removed from the DNA duplex to generate nucleoside triphosphate and a shortened 3’ end duplex DNA [Liu & Sommer, 2000].

PAP is a method for nucleic acid amplification, where pyrophosphorolysis and polymerization are serially coupled by utilizing a pyrophosphorolysis-activable oligonucleotide acting as a primer. A very high specificity results from both reactions [Liu & Sommer, 2000], and one mutated allele can be detected in 10^9 wt alleles [Phylipsen et al., 2012].

With this technique, it has been possible to specifically amplify ccffDNA mutations inherited from the father or polymorphisms that are absent in the mother DNA, allowing in all cases the detection of the paternal allele in maternal plasma at 10^{th} to 18^{th} week of gestation [Phylipsen et al., 2012].

1.3.3.4.5. NGS

NGS, which was introduced in 2005, indicates a high-throughput DNA sequencing technology capable to analyze large amounts of different DNA samples in a single reaction [Voelkerding et al., 2009; Jones et al., 2014].

Different commercially platforms are currently available for this technology: Genome Analyzer and HiSeq 2000 (Illumina), HeliScope (Helicos BioSciences), SOLiD and Ion Torrent (Life Technologies), Roche/454 (Roche). In all of them, either templates, primers or polymerase enzymes are immobilized on a solid support or on beads before the sequencing reactions [Metzker, 2010], allowing the instruments to process millions of microreactions carried out in parallel on each spatially distinct template [Natrajan & Reis-Filho, 2011].

All of NGS approaches need a double-stranded DNA template, such as genomic DNA, circulating DNA or immunoprecipitatd DNA. This DNA sample is then converted in a library of sequencing reaction templates (sequencing library) through the following common steps:

- fragmentation, to obtain small DNA fragments;
- size selection, where the size depends on each sequencing platform specifications;
- adapter ligation, which adds a platform-specific synthetic DNA (which works as a primer) to the end of each fragment [Linnarsson, 2010].

In this way, an ideally sequencing library that accurately represents the sample DNA population is created.

Depending of the type of NGS platform employed, the library can require a previous amplification to produce targets to be sequenced, or be directly sequenced from the single-molecule template [Loman et al., 2012].

The two technologies are known as second generation sequencing (Illumina, SOLiD and Roche/454 platforms) or third generation sequencing (Helicos BioSciences platform), respectively. In the first approach, to better detect the last nucleotide added by the instrument, the amplification step can introduce errors (such as artificial mutations) into experiments [Metzker, 2010]. On the other hand, single molecule sequencing bypasses the need for amplification step or manipulations and requires far less starting material for sequence detection [Hart et al., 2010]. For these reasons, this approach is considered to have a greater dynamic range of sequence detection, including the possibility of sequencing DNA from only a single cell [Navin & Hicks, 2011].

Depending on the platform used, sequencing and recording steps are different:

- Illumina technology is based on a cyclic reversible termination method with four fluorescent colours. At the first step, there is the incorporation of one fluorescently modified nucleotide, then after washing and imaging for detection of the incorporated nucleotide, a cleavage step removes the fluorescent dye and novel incorporation step is performed. These steps are done in a cyclic manner, 72 or 100 times or more [Bentley et al., 2008].

- The HeliScope platform employs only one fluorescent colour and uses a cyclic reversible termination approach as well [Braslavsky et al., 2003].

- SOLiD is a technique based on sequencing by ligation. It employs a DNA ligase and a cleavable two-base-encoded probe consisting of two nucleotides combined with a particular dye [Valouev et al., 2008].

- Roche/454 is an instrument based on the synthesis principle of pyrosequencing: after the incorporation of a dNTP, a pyrophosphate (PPi) is released and converted into a detectable light through a series of enzymatic reactions [Ronaghi et al., 1996].

- The Ion Torrent technology is based on voltage: every time a new nucleotide is incorporated, a change in pH occurs (because a proton is released) that is converted in a detectable voltage change [Rothberg et al., 2011].

Anyway the final step of NGS is the genome alignment, performed through several strategies for filtering and scoring the different variants and for detecting disease-causing mutations [Glissen et al., 2012].

Tanks to the advent of NGS, resolving the whole genome of a fetus from maternal blood sources has become possible. In 2010, Lo and colleagues sequenced DNA extracted from maternal plasma, and used the parental SNP genotypes to distinguish fetal from maternal reads [Lo et al., 2010]. This experiment demonstrated that the entire fetal genome is represented in maternal circulation and that these technologies can be applied in pre-natal diagnosis for detection of severe monogenic disorders and fetal chromosomal aneuploidies [Lo & Chiu, 2012].

NGS still presents some limits for its application in pre-natal diagnosis, as sequencing to sufficient depth to detect fetal DNA genotype is still quite expensive and time consuming. Nevertheless, it is the basis of pre-natal non-invasive screening tests commercially available, currently offered to detect fetal sex, common chromosomal aneuploidies and small insertions and deletions.

Although an invasive approach to obtain fetal DNA currently still provides the gold standard test for pre-natal diagnosis, many women decide not to undergo invasive testing, either because it is unpleasant and carries a small but significant risk of miscarriage [Wright & Burton, 2009]. So, non-invasive pre-natal diagnostic techniques would be highly preferred. The development of a simple, quick and cheap non-invasive pre-natal technology is fundamental, but one limit of this approach is that an invasive diagnostic procedure is anyway required to have a definitive diagnosis and be authorized to properly decide about pregnancy.

1.4. Biosensors based on SPR: an innovative technology

1.4.1. SPR

SPR (**Figure 1.10**) is an optical phenomenon that occurs in thin conducting films between media of different refractive indexes [Jason-Moller et al., 2006; Ahn et al., 2018]. Specifically, at the interface between two transparent media with different refractive indexes, the light coming from the medium with higher refractive index is partly reflected and partly refracted: this occurs up to a certain angle of incidence, said limit angle, beyond which no light is refracted through the interface and total reflection takes place. Despite this, in particular conditions in the less refractive medium the propagation for a short distance of a component of the electromagnetic field of the incident radiation, said evanescent wave, occurs [Pattnaik, 2005; Ahn et al., 2018]. At a certain combination of angle of incidence and energy, the incident light excites the very mobile electrons (also called plasmons) in the metal conducting film, generating surface oscillations or plasma waves, a phenomenon

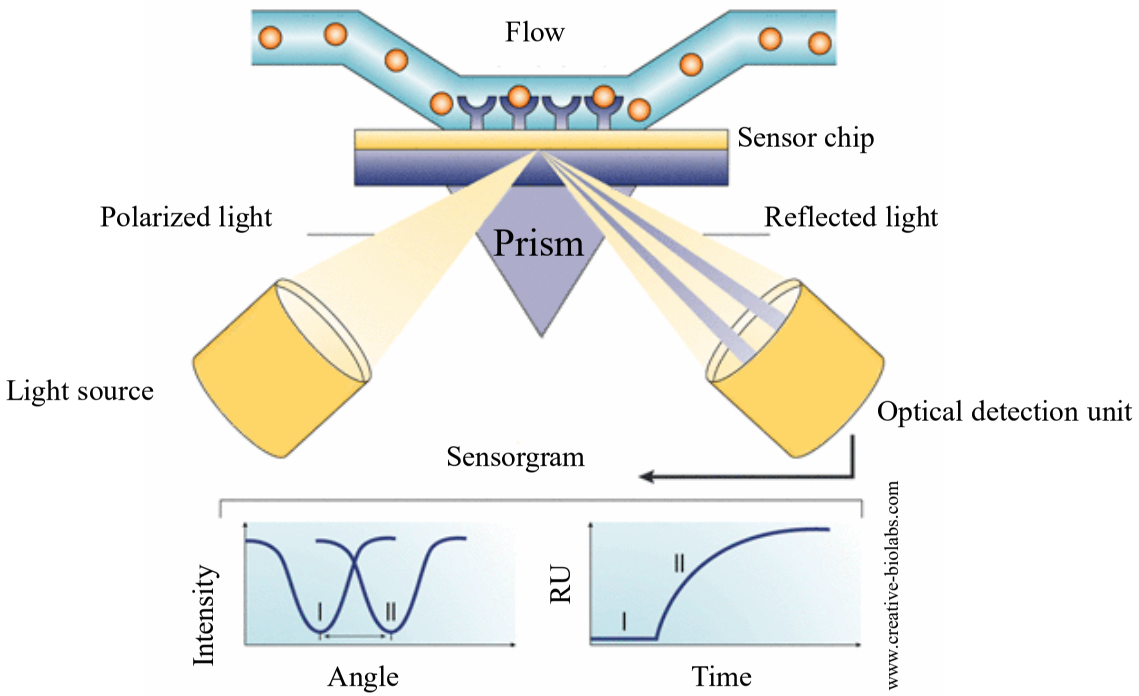


Figure 1.10. Surface plasmon resonance (SPR). The phenomenon allows to detect changes in refractive index at the sensor chip surface. After the interaction, the increase in mass results in a change of the SPR angle (from I to II, lower left panel), which is monitored in real-time as resonance unit (RU) signal as a function of time, in a graph called sensorgram (lower right panel).

known as SPR. As a result, a characteristic absorption of energy via the evanescent wave field occurs and SPR is seen as a drop in the intensity of the reflected light [Pattnaik, 2005] (**Figure 1.10**, lower left panel).

This phenomenon can be exploited for the detection of biomolecular interactions on a suitable metallic surface (called sensor chip), where one of the two investigated molecules (called ligand) is anchored, while the other (called analyte), in solution, flows on the surface. Indeed, the characteristics of the metal film, the wavelength of the incident light and the refractive indexes of the two different media are the parameters influencing the SPR phenomenon, where the two media are the device where the interaction takes place, with a high refractive index, and the sample solution, with a low refractive index [Torreri et al., 2005]. While metal film, wavelength of incident light and device material are constant and depend on the instrument or the experimental assembly, the refractive index of the solution on the device surface depends on mass, according to a relationship of direct proportionality, *e.g.* the concentration and the molecular weight of the analyte in contact with the ligand on the chip surface [Torreri et al., 2005].

So, both when the detecting molecule is attached to the device, or when the analyte binds to the ligand, the solute concentration at the sensor chip surface increases and, consequently, an increase of the refractive index on the surface occurs, leading to a change in the SPR signal. In fact, a detector measures the intensity of the reflected light and the corresponding angle of reflection, and it is able to detect the fall of the signal occurring in case of SPR (**Figure 1.10**, lower left panel). The angle of reflection at which this phenomenon is observed is called SPR angle.

The value of SPR angle is measured very precisely by the optical reader of the instrument, thanks to a series of light sensitive diodes capable of detecting the reflected beam. Its measure is expressed in resonance units (RU) (**Figure 1.10**, lower right panel), where 1000 RU correspond to a change of 0.1° in the SPR angle or to 10^{-3} units of refractive index, or also to about 1 ng/mm^2 biomolecule density [Biacore^{TM} handbook].

The measured response is related to the mass of bound analyte and is largely independent of the nature of the analyte. Anyway, this strategy allows to obtain not only quantitative information about the biomolecular interaction, but also to characterize specificity, thermodynamics and kinetics for a wide range of molecular weights, including small molecules, without the use of markers [Jason-Moller et al., 2006]. The unknown

concentration of an analyte placed in solution can be determined as well [Jason-Moller et al., 2006].

1.4.2. Affinity biosensors based on SPR

Biosensors are compact analytical devices for the analysis of biological activities, characterized by the incorporation of a sensitive biological or biological-derived element, integrated within or intimately associated with a physicochemical transducer. They are therefore physical or biochemical devices containing biological mediators, immobilized according to particular operational schemes and coupled to suitable signal transducers, capable of reacting or interacting with the molecule of interest present in the sample, whose activity or concentration is so recorded [Newman & Turner, 1992]. The biological mediator of recognition is typically a biomolecule (antibody, enzyme, DNA, RNA), but this can also be a whole cell, a fragment of biological tissue or a bacterium [Newman & Turner, 1992]. In addition to the mediator, the majority of biosensors is constituted by other two main components: a substrate linked to the biomolecule, which can be silicon, gold, glass, a polymeric material; a physical transducer of the signal of various nature (electrochemical, electro-optical, piezoelectric, mechanical), able to convert the biological activity in an easily measurable signal [O'Connor & Glynn, 2010; Briones & Moreno, 2012].

SPR-based biosensors are affinity biosensors for the analysis of interactions among biological molecules. They are able to ensure real-time analysis of the interaction between different biomolecules, such as antigens and antibodies, ligands and receptors, as well as between double-stranded DNA and transcription factors or between single-stranded DNA molecules and complementary sequences [Turner, 2000], the latter very useful for molecular diagnostics.
The Biacore™ (GE Healthcare) instrument, is an example of biosensor based on SPR.

1.4.2.1. Biacore™ instruments

Biacore™ instruments, amongst which Biacore™ X100 employed in the current thesis (**Figure 1.11.A**), can monitor interactions between molecules in real-time using a detection system based on SPR; a computerized system, instrument integral part, performs analysis and data processing.

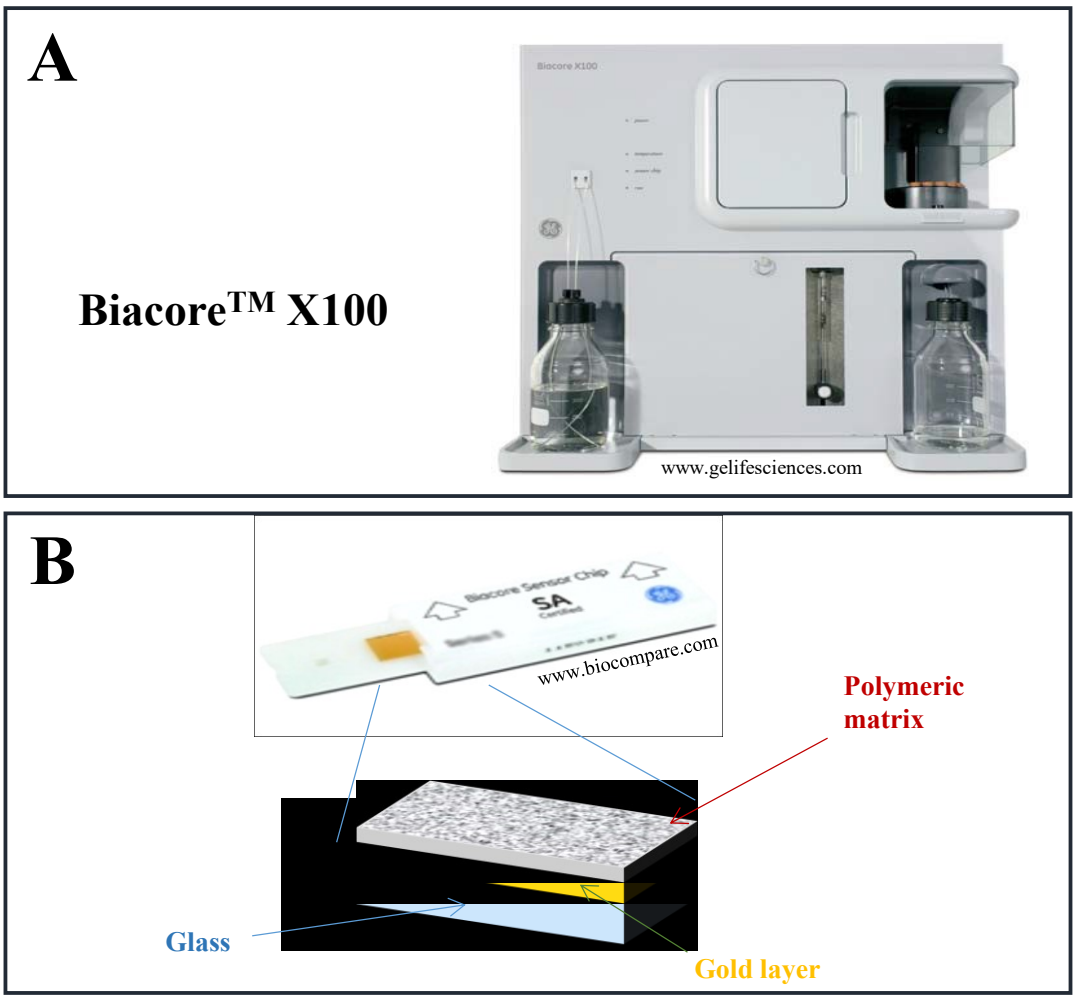


Figure 1.11. Biacore™ X100 instrument. (**A**) Affinity biosensor Biacore™ X100. (**B**) The sensor chip is constituted by a glass lamina, coated on one side with a thin gold layer about 50 nm thick, that binds a polymeric matrix through linker molecules of inert material.

The experimental strategy provides for a first ligand immobilization on a suitable surface, and the subsequent injection of the analyte: the potential interaction is detected in real-time [Pögel Neè Steinicke et al., 2018].
The main components of the instrument are: sensor chip, integrated microfluidic system and optical unit. Each element is described in the sections below.

1.4.2.1.1. Sensor chip

The interactions take place on a removable device known as sensor chip (**Figure 1.11.B**). The active surface, on which the reactions occur, consists of a glass lamina coated on one side with a thin layer of gold, with a 50 nm thickness, which binds a polymeric matrix through linker molecules of inert material. Depending on the chemical properties of the polymeric matrix, different molecules can be immobilized through suitable ligation reactions [Biacore^{TM} X100 Handbook]. In this connection, numerous different types of sensor chips are commercially available.
CM5 is an example of sensor chip composes of a carboxymethyl dextran surface matrix that can immobilize a wide range of ligands, from small organic molecules to proteins, nucleic acids and carbohydrates [Biacore^{TM} X 100 Handbook].
Another, widely employed, kind of sensor chip presents a dextran matrix surface on which streptavidin (SA) molecules are attached. SA molecule is a tetrameric protein with a strong binding affinity for the biotin, so the chip surface has a high affinity with biotinylated ligands. The interaction between the two molecules is not a covalent bond but so strong to be compared to it. Considering that, the biotinylated ligand cannot easily be removed to regenerate the SA surface. This sensor chip is particular appropriate for working with nucleic acid ligands [Homola et al., 2002].
The ligand immobilization on the sensor chip surface can occur with three different mechanisms:

- Covalent immobilization: covalent stable interactions link the molecule according to the functional groups that the ligand exhibits, resulting from amine, thiol or aldehyde coupling;

- Bonding affinity: a second molecule called capturing molecule, immobilized on the sensor chip surface with a covalent bond, links the ligand by weak interaction;

– Hydrophobic adsorption: the ligands, represented by hydrophobic molecules, link the sensor chip surface forming hydrophobic interaction through single or double lipid layer;

In Biacore™ instrument, the sensor chip is inserted manually inside an accommodation, where it is in contact with both the microfluidic system and the optical unit. The device has useful properties such as biocompatibility, low aspecific binding and high stability, in fact once immobilized a ligand it can be re-used different times for several interaction analysis with various analytes.

1.4.2.1.2. Microfluidic system

The interactions between molecules, to be studied, take place on the surface of the sensor chip, where the molecules are available thanks of a microfluidic system also known as Integrated Microfluidic Cartridge (IFC). The system includes a series of microchannels and pneumatic valves enclosed in a plastic housing, that enable the carrying of the injected solutions and reagents at the sensor chip surface.

When the sensor chip is docked against the IFC, separate flow cells are formed; Biacore™ X100 instrument has two flow cells, but the number can change according to the instrument model. The IFC forms two grooves in the region in contact to the gold layer. The cells do not need to be used in the same time: they are independent one from each other for the analysis; in fact, different ligands can be immobilized and may be used in series or individually. Generally, performing the analysis, it is suggested to keep on of the two flow cells empty to be used as a negative control of the interaction.

A pump system and the IFC valves finely control flow and delivery of sample and buffer to the flow cells. An autosampler, through a needle, allows the reagents transferring from the sample rack to the IFC. Thanks of this system, a continuous flow of liquid is maintained over the sensor surface throughout an analysis, switching between buffer and sample with minimum disturbance or dispersion of the sample boundary. This high degree of precision in sample delivery is important for assay procedures reproducibility, and provides the controlled conditions necessary for interpreting kinetic data obtained from the interaction studies [Biacore™ X100 handbook].

1.4.2.1.3. Optical unit

As mentioned above, the SPR phenomenon enables the interaction detection between the ligand and the analyte. This optical phenomenon occurs in thin conducting films at an

interface between media of different refractive indexes. In Biacore™ systems, the glass of the sensor chip, with a high refractive index, and the sample solution, with a low refractive index are the media; instead, the thin gold layer on the sensor chip surface represents the conducting film [Torreri et al., 2005].

In Biacore™ instruments, the optical unit is constitutes by two different elements: a glass prism, which contacts the glass surface of the sensor chip once it is placed into the instrument; and a light-emitting diode (LED), that generates a monochromatic radiation that passes through a polarizer and is focused on the sensor chip, whose wavelength is near the infrared spectrum. With the aim to reach a total internal reflection condition, at the interface in which the sensor chip meets the solution, the glass is pressed against the prism by using a silicon interface to have a good optical contact. Under the condition just described, the SPR phenomenon can occurs in any thin film conductor, but the wavelength at which it occurs and the adsorption profile energy are unique characteristics. The gold layer use, in Biacore™ system, has the aim to promote SPR phenomenon and allows a high stability of interactions between molecules.

Therefore, the ligand-analyte interaction is detected as a change in SPR signal, where the shift in the angle of reflection at which SPR occurs is measured in resonance units (RU) [Biacore™ X100].

1.4.2.1.4. Data processing and sensorgram

During the analysis, the changing of SPR signal is continuously monitored and registered; this results in a plot called sensorgram, displayed in real-time on the computer screen, as RU as a function of time, in which it is possible to detect the investigated molecules interactions represented by real-time association and dissociation [Gambari, 2001; Wilson, 2002].

The typical sensorgram is shown in **Figure 1.12**, where the different phases of interaction analysis are distinguished:

- The continuous flow of a buffer solution on the sensor chip, on which the ligand is immobilized, results in a constant baseline signal at a specific RU value (**Figure 1.12.a**).

- The subsequent phase is the analyte injection on the sensor chip surface; if the analyte interacts with the ligand an increase of RU value occurs (**Figure 1.12.b)** and, if the binding kinetics is sufficiently quick, the RU measured can reach a maximum and constant value. This steady status is defined plateau and remain constant until the end

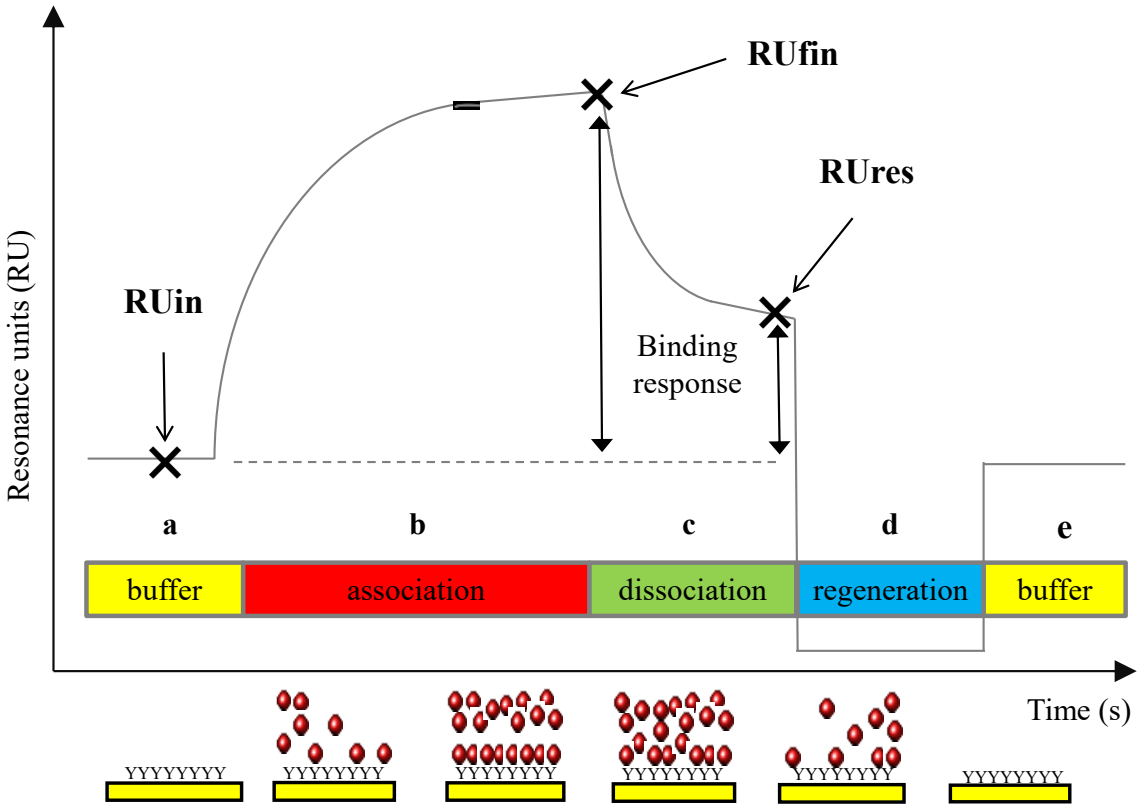


Figure 1.12. **Representation of a typical sensorgram, where resonance units (RU) values are recorded in function of time.** The initial RU (RUin), measured before the injection of the sample, are only due to the presence of the ligand on the sensor chip surface, where the buffer solution (buffer) flows (**a**). During the association phase following the analyte injection, a corresponding signal increase occurs, where the curve slope is related to the kinetics and extent of analyte-ligand interaction (**b**); the curve may reach a steady state where all the ligand sites are saturated by the analyte. At the end of the association phase, the final RU (RUfin) are measured. During the washing step (**c**), a partial dissociation of the analyte from the ligand can occur, measured as residual RU (RUres) that reflect therefore the stability of the complex. The final regeneration phase (**d**), carried out with a suitable denaturing solution, allows the complete detachment of the analyte from the ligand: the RU stabilize at the initial value again (**e**) and the sensor chip is ready for a new use.

of injection corresponding to a condition in which all the ligand sites have been saturated. After the analyte injection phase, it is possible to get information about the interaction between the two molecules in terms of affinity, because the signal increase is directly related to the mass increase on the sensor surface, depending on the entity of analyte-ligand binding. The shape and the slope, of the association curve, give information from a qualitative point of view, from which it is also possible to derive the relative rate constant. Instead, the difference between the RU values measured at the end (RUfin, final RU) and before (RUin, initial RU) the injection allows a quantitative analysis.

- The following step is, usually, a washing (**Figure 1.12.c)** with a proper buffer, resulting in an almost significant decrease of the signal due to the partial dissociation of the ligand-analyte complex, that depends on the stability of the association.

- between the two molecules. In fact, during washing, the weak interactions are removed, so the signal measured, at the end of washing, depending on just stable interaction. Here, the shape and the slope of the generated dissociation curve provide information about the interaction stability, after subtracting RUin values to those measured after the washing step (RUres, residual RU). In this case, too, it is possible to calculate the kinetics dissociation constants of the complex.

- The last step, known as regeneration phase (**Figure 1.12.d)**, provides for the completely removal of the analyte from the sensor chip surface by the employment of suitable denaturing solutions which have the aim to make RU come back to the initial values (**Figure 1.12.e)** and prepare the support for a new analysis [Gambari, 2001; Wilson, 2002; Torreri et al., 2005].

The bulk effect is another phenomenon that characterized the commonly obtained diagrams: it depends on the simple flow of solution through the microfluidic cell and takes place in absence of interaction. The liquid flow determines an alteration of the refractive index, which produces an abrupt change in resonance units remaining constant during the whole injection and at the end return to the initial values. Therefore, finally, a common ligand-analyte interaction results in a combination of the bulk effect with the specific signal obtaining from the molecules association.

The report points are specific time intervals where RU values are measured throughout the analysis, which the operator can set: if the response refers to one or another report point, set as reference by the operator, is relative, otherwise it can be absolute. In addition, the instrument software allows operating the difference between the obtained analytical curves: in case of an empty flow cell, as control of interaction, the signal obtained after the analyte injection is subtracted from the measured into the ligand-containing flow cell (reference subtraction).

The percentage of saturation of the ligand sites from the analyte can be calculate on the relationship between the value [RUfin-RUin] obtained from the analysis, and the theorical value corresponding to a 100% saturation, calculate by the relationship:

[RUfin-RUin] analyte = [RUfin-RUin] ligand x MW analyte / MW ligand

where *MW* is the molecular weight [Feriotto et al., 2002].

1.4.2.2. Advantages

SPR biosensors have a broad range of applications and current uses, thanks to the numerous advantages of the technique, which are summarized in the following points:

- interactions monitoring in real-time;
- label-free reagents;
- quickness of responses;
- cheap experimental procedures;
- simple analytical method;
- user-friendliness;
- high specificity, sensibility and selectivity;
- medium-high processivity;
- possibility of analysis of small molecules;
- reduced samples pre-treatment;
- reusing of sensor chip several times;

1.4.2.3. Applications

Thanks to their numerous advantages previously summarized, SPR sensors have been used for detect and analyse a wide range of biological molecules interactions, deriving qualitative and quantitative information, through kinetic, affinity and concentration analysis [Karlsson, 2004; Lee et al., 2005; Benounis et al., 2015].

The interactions usually investigated are:

- peptide-receptor;
- antigen-antibody;
- protein-DNA;
- nucleic acid-nucleic acid;
- protein-small molecules (< 100 dalton (Da));
- membrane receptor-ligand [Benounis et al., 2015].

This innovative and advantageous technology can be applied in several different fields, among these: the diagnosis of point mutations responsible of certain disorders, such as β thalassemia and cystic fibrosis [Feriotto et al., 2002; Feriotto et al., 2004; Breveglieri et al., 2018]; the searching of genetic modified organisms (GMOs) in food [Feriotto et al., 2002; Huang et al., 2015]; the glucose sensing in biological fluids [Hartono et al., 2018]; the detection of pathogens, toxins and chemical additives in foodstuffs, like the identification of *Salmonella* [Savas et al., 2018] or *Listeria monocytogenes* bacterium [Radhakrishnan & Poltronieri, 2017], or the detection of the B enterotoxin of the *Staphylococcus* [Homola et al., 2002], or the presence of toxic metalloid arsenic derived from herbicides [Chen et al., 2015]. This method can also allowed the identification of viruses causing infections [Benounis et al., 2015], like HIV-1 [Lifson et al., 2017] and has contributed to the searching and non-invasive diagnosis in the field of Alzheimer disease [Li et al., 2016]. In addition, SPR sensors can be applied to analyse variations or interactions in real-time, also, in live cells [Ahn et al., 2018].

When applied to the field of molecular diagnosis, including genetic diseases, DNA-DNA interactions are generally employed.

1.5. Digital droplet PCR (ddPCR): an innovative technology

The ddPCR, employed in the current thesis, is a new generation technology based on a system able to produce small water-in oil droplets, inside each presumably occurs a single amplification reaction [Corbisier et al., 2015].

In a common PCR, a single sample corresponds to a single measurement, instead, with this new technology, the target molecule, in order to be investigate and amplify, is divided into almost 20000 droplets of around microliter sizes. In this way, thousands independent amplification events can occur, one inside each droplet: this is the key of the instrument sensibility [www.bio-rad.com].

The ddPCR performs a random distribution thanks to the microfluidic and the chemical proprieties of surfactants [Hindson et al., 2011]. After the amplification reaction, the droplets are analysed thanks to a reader able to distinguish the positive from the negative droplets. The droplets are defined positive if contain at least a copy of the template, and, for this reason, are associated to an high fluorescence; on the contrary, the negative droplets do not contain the target sequence, so no fluorescence increase takes place. The possibility to distinguish the positive from the negative droplets and the application of Poisson statistic allow to obtain the absolute quantification of the samples, after the data analysis. The quantification is precise and accurate and lacks in potential underestimations.

An underestimation can occur only if each droplet contains more than one molecule of template consequence of its high amount [Hudecova, 2015].

The **Figure 1.13** schematically shows the ddPCR workflow. The main steps are:

- Mix reaction preparation;
- Droplets generation;
- Amplification reaction;
- Detection;
- Analysis;

1.5.1. Droplets generation

After the preparation of the reaction mixture (**Figure 1.13.A**), which contains the sample, a supermix (which contains all the reagents required for the amplification reaction) and the oligonucleotide primers and probes, the droplets generation takes place. An automated instrument called Automated Droplet Generator makes this procedure [www.bio-rad.com]. For each sample, the instrument gives rise to thousands droplets in a completely automated way, abolishing, in this way, the related variability due to the manual and individual droplets preparation.

A specific amount of an appropriate oil is added to the sample in a cartridge; the result is the generation of the emulsion water in oil (**Figure 1.13.B**), thanks to a microfluidic system combined with a vacuum system [Hindson et al., 2011].

In this way, each sample is well partitioned and, consequently, diluted allows a sensible and precise analysis. The next stage is the amplification of the obtained emulsion.

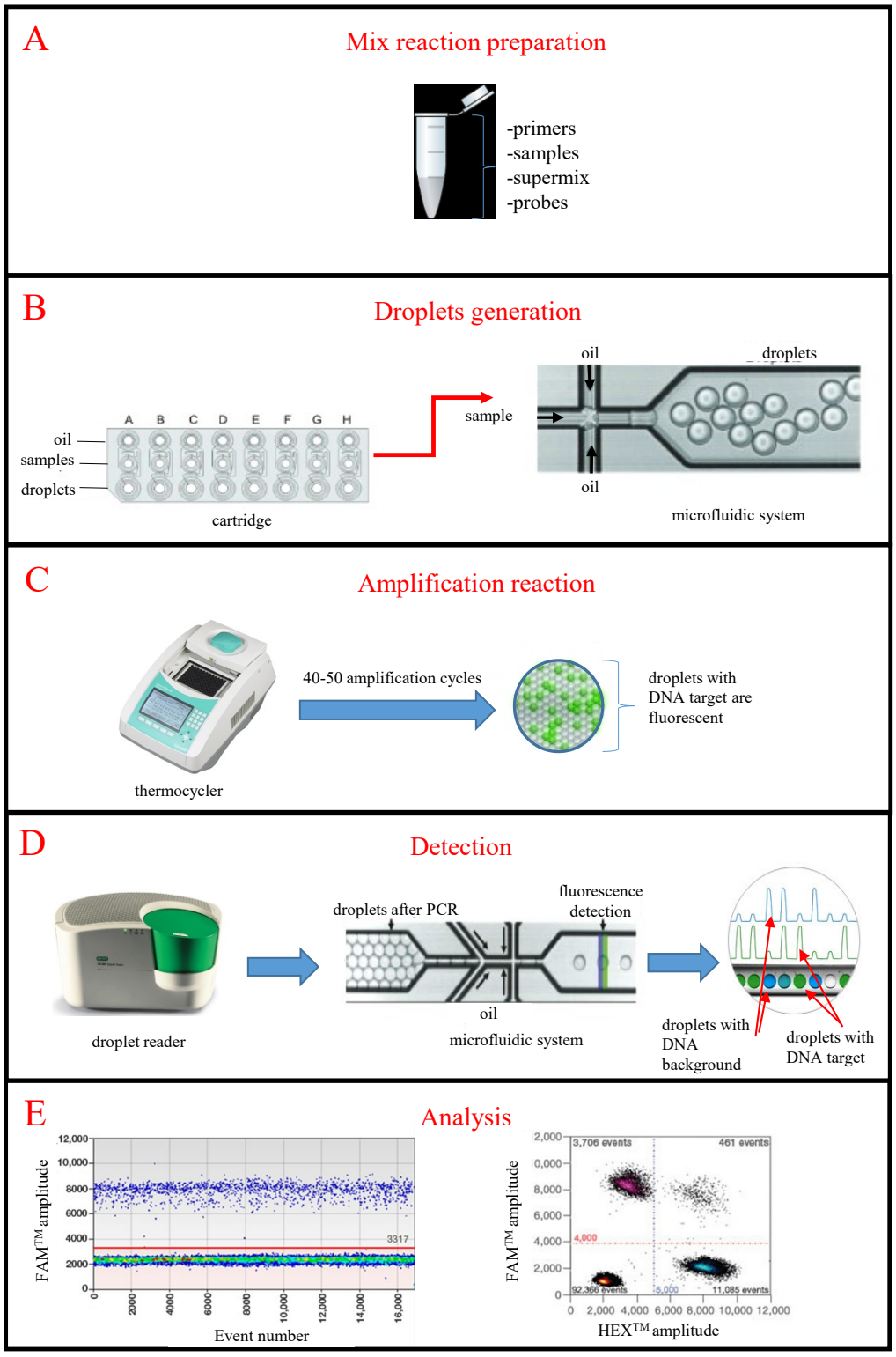


Figure 1.13. ddPCR workflow. (**A**) Reaction mix preparation containing primers, probes, the samples and the supermix, in which the reaction enzyme is present. (**B**) An automated system blends oil samples into a cartridge and generates droplets of nanolitre size by a microfluidic system and vacuum generation. (**C**) The emulsion is transferred to the thermocycler for the amplification. The presence of the target template in the droplets will be detected by the fluorescence development. (**D**) Fluorescence detection using the Droplet Reader. The system, after the injection of a reader oil, separates and aligns each droplet, which is analyzed by two lasers to detect fluorescence. (**E**) Analysis of the data represented in graphs as the fluorescence (FAM™ or HEX™) relative to each droplet (Event number) [Modified from: D'Aversa et al., 2018].

1.5.2. Amplification reaction

If the target is present inside the droplet, the amplification takes place because the primers and the probes hybridize with the target sequence and a specific enzyme performs the extension of the complementary strand [Pohl & Shih, 2004].

As previously pointed out, the fluorescence emission, using fluorophore labeled probes, allows the detection of the target amplification. Such as qRT-PCR, the ddPCR can operate with several kinds of probes. Usually, TaqMan® probes are empoyed, associated with two fluorophores: a quencher and a reporter. If both the fluorophores are linked to the probe, they are closer, so the quencher absorbs the reporter energy so no fluorescence is emitted; instead, when the polymerase, proceeding the synthesis of the new strand, degrades the probe the two fluorophore are moving away one from the other and the reporter can emit fluorescence (**Figure 1.13.C**), demonstrating the target presence. The fluorescence is directly proportional to the thermal cycle increase because the amplified and labeled template increases with each cycle.

Maximum two probes, labeled with different fluorophore, can be employed, generally FAM™ and VIC® or HEX™ (carboxyhexachlorofluorescein), which have different emission spectrums.

Commonly, two probes are designed and employed: one for the sequence of interest and one that hybridizes with a reference sequence, in order to detect a background DNA, to be sure that the reaction took place (**Figure 1.13.D**).

At the end of the amplification reaction, positive droplets, in which the sequence of interest and/or the background DNA were amplified, and negative droplets, are obtained: strictly necessary for the statistic of Poisson application [www.bio-rad.com].

1.5.3. Detection

At the end of the amplification reaction, the plate, in which the samples are aplified, is placed inside an instrument called Droplet Reader that is able to detect the different fluorescences [www.bio-rad.com]. The instrument, using a needle, aspires the emulsion from the plate wells, flows it in the microfluidic system, where, thanks to a splitter fluid, the droplets are separated one from the others and are aligned and stacked to be strike from two lasers. The instrument detector registers, at the same time, the spectrums of fluorophores emission (**Figure 1.13.D**) [Hindson et al., 2011]. During this step, the instrument distinguishes the positive from the negative droplets and displays them, using a specific software, in graphs (**Figure 1.13.E** and **Figure 1.14**). Finally, the positive droplets are counted to determine an absolute quantification [www.bio-rad.com].

1.5.4. Analysis

A specific software, connected to the Droplet Reader instrument, processes the information, analyses the results and displays the relating graphs. The obtained fluorescence intensity, from each droplet, is recorded in two 1D graphs: one that displays the FAM™ fluorescence intensity, and the other the HEX™ or VIC® one, as a function of the events number. The events are displayed as colourful spots: one for the single droplet. The graph in Figure **1.14.A** shows the positive events, for which the fluorescence intensity is high, in the upper part; instead, the negative events display, as a cordon, in the lower part of the graph because have a baseline fluorescence due to the intrinsic fluorescence of the fluorophore [www.bio-rad.com].

The same results can be visualized in a 2D graph (**Figure 1.14.B**), in which the FAM™ fluorescence intensity is a function of the VIC® or HEX™ one, for each event. The 2D graph allows to discriminate between four different events populations: the one located in the lower left represents the negative droplets; the one located in the upper left represents the positive droplets for FAM™; the population in the lower right represents the positive droplets for HEX™ or VIC®; and the droplets placed in the upper right are positive for both the fluorophores, for which the amplification of both sequences has taken place.

The ddPCR allows an absolute quantification of the samples by applying the statistic of Poisson. The final quantification, expressed in copies/µL (**Figure 1.15**), is based on the following equation:

$$\lambda = ln\ (1-p)$$

where λ is the number of the target molecules for each amplification and p is the portion of the positive droplets [Hindson et al., 2004].

1.5.5. Advantages

The ddPCR has a broad range of applications, thanks to the numerous advantages of the technique, which are summarized in the following points:

- Absolute quantification: the ddPCR technique does not required a calibration curve for the analysis, as for qRT-PCR, but applies the Poisson statistic and allows an absolute quantification. This is an advantage because, as well as reducing the analysis cost, lets to eliminate the mistakes related to the relative quantification by using the calibration curve [www.bio-rad.com].

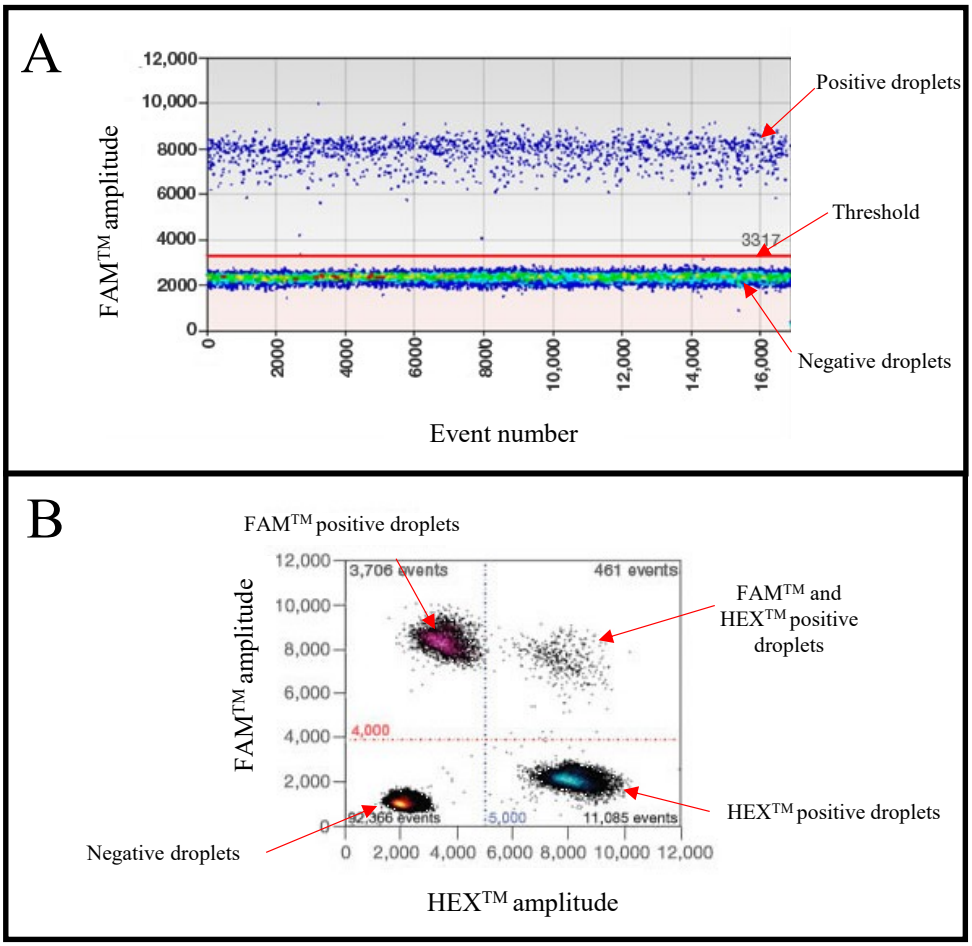


Figure 1.14. Examples of ddPCR graphs. (**A**) ddPCR 1D graph, in which the FAM™ amplitude is a function of the events number. The positive droplets, in which the DNA target was amplifyed, are in the upper part of the graph, instead, the empty negative droplets are in the lower part of the graph. The positive droplets are separated from the negative ones by a threshold, set by the operator. (**B**) ddPCR 2D graph, in which the FAM™ amplitude is a function of HEX™ amplitude. Four population groups are identified: the one located in the lower left represents the negative droplets; the one located in the upper left represents the positive droplets for FAM™; the population in the lower right represents the positive droplets for HEX™ or VIC®; and the droplets placed in the upper right are positive for both the fluorophores, for which the amplification of both sequences has taken place [Modified from: www.bio-rad.com].

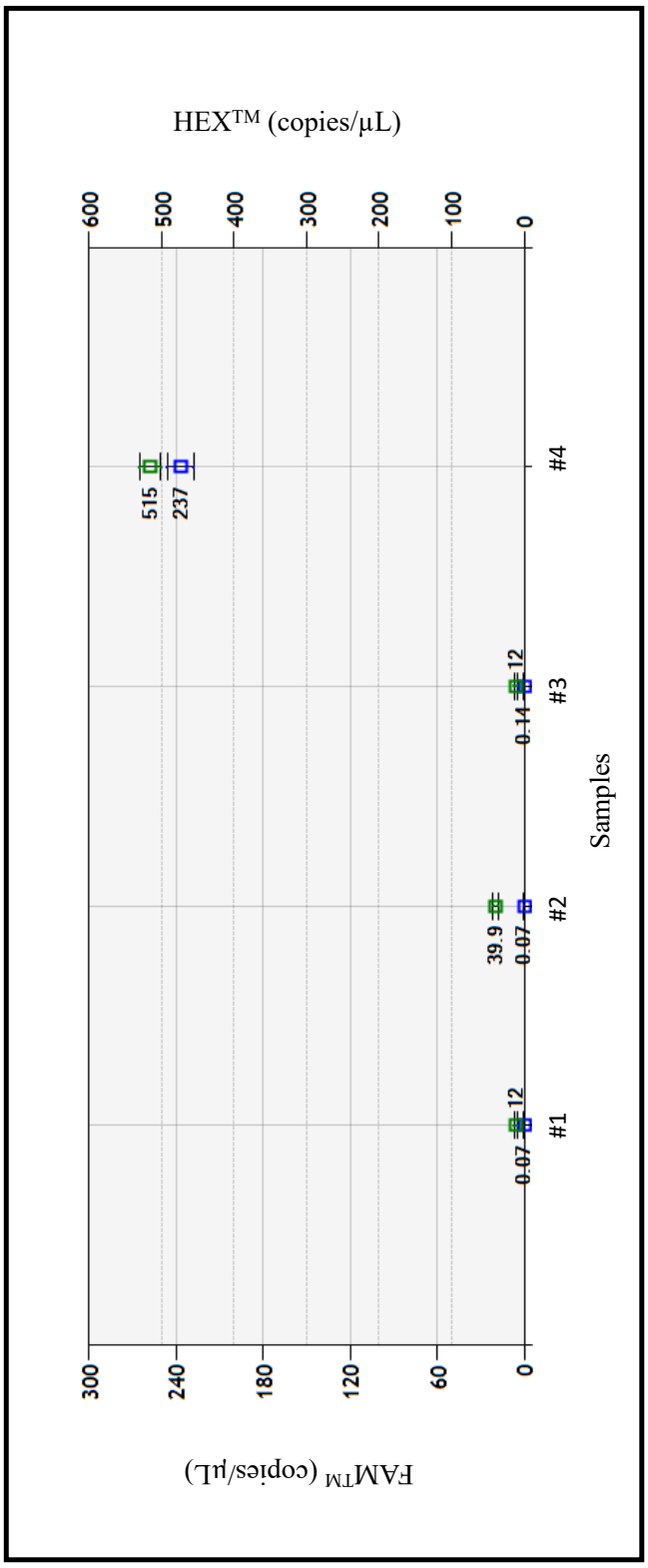


Figure 1.15. Examples of absolute quantification by ddPCR software. For each sample, the absolute quantification (copies/µL) of FAM™ labeled target (in blue) and of HEX™ labeled background DNA (in green) is indicated [Modified from: www.bio-rad.com].

- Precision and accuracy: the ddPCR ability to have only one template in each droplet, ensures the detection of very low quantity of the template, resulting in a precise and accurate quantification. In addition, the extreme dilution allows the correct separation of the positive from the negative droplets, which constituted the background noise strictly required for the analysis based on Poisson relationship [www.bio-rad.com].

- High tolerance to inhibitors: the technology has a high tolerance for substances that inhibit the enzymes taking part to the reaction [Hudecova et al., 2015].

- Reduction in cost: although the costs concerning the instrument and the required reagents are high, the reaction volumes are in the range of nano- or picoliters and, additionally, the numbers of wells employed is reduced, compared to qRT-PCR, because is not necessary to load the sample in duplicate and perform a calibration curve [www.bio-rad.com].

1.5.6. Applications

Considering the relevant advantages, just described, the ddPCR technology can be applyed in numerous fields, among these:

- Rare mutations identification: the detection of rare mutations finds broader application in cancer research. In fact, the acquisition of mutations in sensitive genes, which occurs in a few number of cells, in enough to determine the beginning and development of the cancer. Considering that the mutations are found in a restricted number of cells, only a very sensitive technique can detect them. The ddPCR helps the researchers to achieve this aim because is able to measure extremely low levels of mutated alleles. For example, in a recent study directed by Ono, the ddPCR technology was employed for detecting low-copy *KRAS* mutations, responsible for the colorectal and pancreatic cancer. The cut-off limit of reference intervals for mutant *KRAS* was determined to be 0.09% based on samples from healthy individuals [Ono et al., 2017]. Another example is represented by a study carried out from Zorofchian research group; they, with the help of the ddPCR, detect the *MYD88 p.L265P* mutation: a rare alteration responsible of secondary central nervous system lymphomas: a rare malignant cancer that exhibits aggressive clinical behaviour [Zorofchian et al., 2018].

- Copy number variations (CNV) detection: the CNV are genome alterations in which the copy number of a target DNA sequence differs from the standard sequence. The alterations are insertions, deletions or amplifications of gene segments usually found in tumorigenesis processes [Hindson et al., 2011; Hudecova et al., 2015]. For instance, Lodrini and his group, using the ddPCR technique, analyzed *MYCN* and *ALK* increased copy number from patients with neuroblastoma [Lodrini et al., 2017]. The technology can also be used to predict treatments response and survival outcome, in fact, in a recent work, Li and his group have exploited the ability of ddPCR, in determine CNV, to predict outcome after osimertinib treatment in lung cancer, quantifying *T790M* mutant copy number. Patients who achieved partial response had a higher mutant copy levels than those with progressive disease [Li et al., 2018].

- Gene expression analysis: the microRNAs (miRNAs) are small molecules of RNA able to regulate the gene expression and involved in some processes such as cell cycle, apoptosis and cell differentiation; several recent studies have shown that these molecules can be used as blood biomarkers to detect the cancer onset, development and replace of the malignancy [Hudecova et al., 2015]. In a recent study directed by Zhao, based on ddPCR, four miRNAs (miR-21, miR-93, miR-106a, and miR-106b) were identified related to the presence of gastric cancer. All the miRNAs in gastric cancer patients have shown very high levels compared to the ones in healthy patients [Zhao et al., 2018].

- Absolute quantification of ccffDNA: in several studies the ddPCR technique has shown to have great potential for the absolute quantification of ccffDNA [Hindson et al., 2011; Manokhina et al., 2014; D'Aversa et al., 2018]. Hindson and his group, quantifying the ccffDNA and comparing it with the maternal one, obtained the same results achieved by NGS technique [Hindson et al., 2011].

- Viral load quantification: measuring the viral load, in biological samples derived from patients, is important and necessary in clinical. It is hard to obtain a viral load quantification using the qRT-PCR because the technique needs the instrument calibration and the availability and exploitation of standard samples as positive controls [www.thermofisher.com]. Actually, the ddPCR is the optimal approach for the viral load absolute quantification as demonstrated by a study of Rutsaert. In this research, the ddPCR was employed to measure the HIV (human immunodeficiency

virus) persistence [Rutsaert et al., 2018]. In a 2017 study, the ddPCR was used to detect and quantify the influenza virus A defective interfering particles [Schwartz & Lowen, 2017].

- NGS library quantification: a further application of the ddPCR technique is the precise and accurate quantification of the libraries produced by NGS method, without the employment of a calibration curve [www.thermofisher.com; Day et al., 2013; Aigrain et al., 2016].

- Detection and quantification of GMOs: considering the increasing of the number of GMOs, the ddPCR found a great applicability in this field. In a 2018 study, the ddPCR was employed in order to detect and quantify the GMOs in food with accurate and reliable results [Demeke & Dobnik, 2018].

1.6. X-linked diseases

As previously underlined, the pre-natal diagnosis includes all the techniques, invasive and non-invasive, that allow to monitor the fetus health during the pregnancy with the aim to identify fetal diseases. In particularly, the pre-natal sex determination is useful to evaluate, at an early stage, the risk of X-linked diseases. An early diagnosis allows a timely therapeutic plan application and affects the potential decision of the parents to pregnancy interruption [D'Aversa et al., 2018].

Beyond the 44 autosomal chromosome, the human has two sex chromosomes that are XX for females and XY for males. The X chromosome presents several genes involved in human growth; instead, the Y chromosome is smaller and has only genes for male development and sperm production.

The X-linked diseases are monogenic disorders due to singe gene mutations. Y chromosome defects do not led to this kind of diseases, but causes only male fertility impairment. Therefore, the X-linked diseases are due to gene mutations located in X chromosome. These disorders can be divided into two categories: X-linked recessive and dominant diseases [Mandieh et al., 2013].

1.6.1. X-linked recessive diseases

As previously mentioned, a female has two sex chromosomes X: if a female has a recessive mutated allele located in one of the two X chromosomes, she will be healthy carrier for the genetic disorder. Usually, a carrier subject does not present phenotypic characteristics of the

disease, but sometime mild symptoms can occurs. Therefore, in case of X-linked recessive diseases, a female will have the disorder symptoms if inherits the mutated alleles from both the parents. The child with a male gender, having a XY chromosome set, will present the pathological phenotype if inherits a mutation on X chromosome, because there is not another copy of the gene to atone the mistake.
Several X-linked disorders are well known, among them the main are Duchenne muscular dystrophy, hemophilia and fragile X syndrom.

1.6.1.1. Duchenne muscular dystrophy

The Duchenne muscular dystrophy is a lethal X-linked recessive neuromuscular disorder caused by mutations such as frame-shifts, duplications and non-sense mutations, in the dystrophin gene, that result in absent or insufficient functional dystrophin: a protein of the cytoskeletal that allows the strength, stability and functionally of myofibres [Birnkrant et al., 2018]. Symptoms include atrophy, muscular weakness and degeneration. The rapid onset of the disease is in childhood, between 2 and 6 years, and the disease development is slow but severe and leds to death, around 25-30 years, due to cardiomyopathy and respiratory impairments [Gintjee et al., 2014; Birnkrant et al., 2018].
The Duchenne muscular dystrophy prevalence has been reported as 1/6250 live male births [Birnkrant et al., 2018], and predominantly affects the male individuals. Being an X-linked recessive disorder, the females are usually asymptomatic even if a small proportion of carries have mild symptoms of the pathology. There is not a real treatment for the disease, the common therapy is the administration of corticosteroids, as soon as a motor delay occurs with the aim to strengthen the muscles [Basil et al., 2014].

1.6.1.2. Hemophilia

Hemophilia is an inherited bleeding recessive X-liked disorder caused by plasma deficiency of coagulation factors, characterized by bleeding episodes that occur spontaneously or at the time of trauma [Pevvandi & Garagiola, 2018]. Two different type of haemophilia are distinguished:

- A haemophilia: mutations in the gene coding for the VIII coagulation (*F8*) factor cause this kind of hemophilia. Its incidence is 1/5000 male births. Also in this case, the disease affects predominantly male individuals, and the females are usually asymptomatic except a small portion of carriers that have mild symptoms of the pathology [Franchini et al., 2016].

– B hemophilia: mutations in *F9* gene coding for the IX coagulation factor are responsible for the onset of this kind of hemophilia. The incidence observed is higher than the hemophilia A one, and corresponds to 1/40000 male births. Because it is a recessive X-linked disorder, the same considerations of the A type can be applied [Franchini et al., 2016].

The diagnosis of both the types provides a simple and rapid test: a little skin cut is performed and the blood coagulation time is measured. If the measured coagulation time exceeds respect to the standard one, the dosages of VIII and IX coagulation factors are measured in the bloodstream, in order to determine the type and the severity of the genetic disorder.

In both cases, the bleedings appear when the newborn starts to walk, and are localized mainly in joints and muscles, even if each body site can be involved if affects by trauma or lesion. The disease severity depends on defect size of coagulation factor: the hemophilia is considered severe if the factor biological activity is less than 1%, and comes with spontaneous and frequent bleedings. If the factor biological activity ranges between 1 and 5% the clinical picture is mildly severe and, obviously, associated to milder symptoms; instead if it ranges between 5 and 40% the disease is classify as minor [Franchini et al., 2016].

The treatment provides for a substitutive therapy of the missing coagulation factors by administration of plasma derivates and recombinant proteins, just after a bleeding episode, or to prevent it. The mayor complication of this approach is the antibody production against the coagulation factor administrated, however, with the treatment, the disease prognosis is positive.

1.6.1.3. Fragile X syndrome (FXS)

FXS, also known as Martin-Bell syndrome, is an inherited X-linked disorder that causes intellectual disability [Ciaccio et al., 2017], that manifests itself in mental retardation, of variable severity, and impairments of memory and speech. These disorders are associated to behavioural problems, like anxiety, mood lability until autism, and characteristic physical signs, such as close and stretched face and laxity of joints [Ligsay et al., 2016].
The severity of the clinical picture is higher in males than females, for which the cognitive disorders are mild. The actual worldwide prevalence is estimated to range between 1/6000 men and 1/5000 women [Ciaccio et al., 2017].

FXS is caused by an expanded trinucleotide repeat (CGG) on the 5' UTR of the *FMR1* gene (fragile X mental retardation 1) in the first exon [Ciaccio et al., 2017; Raspa et al., 2017]. A normal range is between 6 and 44 repeats; individuals with 45 to 54 repeats are considered to have intermediate expansion; those with 55 to 200 repeats have the per-mutation that will become unstable in future generations; the affected individuals have more than 200 trinucleotide repeats. In this last case, cytosines methylation occurs during gestation silencing *FMR1* transcription, leading to a reduction or absent FMRP (fragile X mental retardation protein), which regulates the protein synthesis and some signalling pathways in neurons, necessary for normal brain development [Saul et al., 2012; Raspa et al., 2017]. The conventional therapy is a maintenance strategy that provides the administration of selective serotonin reuptake inhibitors or stimulants and antipsychotic agents to ameliorate the mental symptoms [Saul et al., 2012]. In addition, new approaches are developed, such as mGluR5 receptors antagonists, GABA-A and GABA-B receptors agonists and minocycline, which can modify the course of the disease, determining an amelioration of the prognosis [Ligsay et al., 2016].

1.6.2. X-linked dominant diseases

Some X-linked rare diseases are dominant inherited: one single allele is sufficient to determine the pathological phenotype. If the mutation is maternally inherited, the sons will have 50% probability to be affected, regardless of gender. If the mutation is paternally inherited, the mutated allele will inherit only from the daughters, which will have the pathological phenotype; on the contrary, all the males will be healthy.

There are only few examples of X-linked dominant disorders, among there: vitamin D resistant rickets and *incontinentia pigmenti*.

1.6.2.1. Vitamin D resistant rickets

The vitamin D resistant rickets, a rare disease, is the most common form of genetic rickets resulting in an inadequate mineralization of growing bone due to an impairment of vitamin D biosynthesis and action, whose leads to bone metabolism failure, growth retardation and bone pain and deformity, but also findings related to hypocalcemia. Mutations in some genes coding for either enzymes involved in vitamin D biosynthesis or vitamin D receptor, are responsible for the disease [Hirao et al., 2016; Acar et al., 2017].

1.6.2.2. *Incontinentia pigmenti*

The *incontinentia pigmenti*, also known as Bloch-Sulzberger syndrome, is a rare dominant X-linked disorder. It has an incidence of 1/40000 newborns. This pathology is generally lethal of male, instead arises after birth for the females, leading to warty plaques, alopecia, teeth alterations, and just in some cases retina and central nervous system problems. The disease is the result of inherited or *de novo* mutations on *IKBKG* (inhibitor of nuclear factor kappa B kinase gene), a modulator gene of NF-kB (nuclear factor kappa B), a critical transcription factor that upregulates the immune system and prevents the cell death, being involved in apoptosis process [Rafatjoo & Kashani, 2016; Mangalesh et al., 2018].

1.7. β thalassemia

The term thalassemia derives from the Greek: *thalassa* and *haima* that mean sea and blood, respectively. In fact, β thalassemia is the most common autosomal recessive hereditary blood disorder worldwide, characterized by reduced (β^+) or absent (β^0) hemoglobin (Hb) β chains synthesis, resulting in reduced Hb in red blood cells (RBC) leading to variable phenotypes from severe anemia to clinically asymptomatic individuals [Galanello & Origa, 2010].

1.7.1. Hemoglobin

Hb is the oxygen carrying protein of the RBC. The human Hb molecules are a set of very closely related proteins formed by symmetric pairing of dimer of polypeptide chains into a tetrameric structural and functional unit, held together by ionic bonds, hydrogen bonds, hydrophobic interactions and van der Waals forces. Their main function in mammals is to efficiently transport oxygen from the lungs to tissues of the body.

The functional properties of Hb molecules are primarily determined by the characteristic folds of the amino acids chains on the globin proteins, including 7 stretches of peptide α helix in the α chains and 8 in the β chains. These helices are in turn folded into a compact globule that heterodimerizes and then forms the tetramer structure. These four polypeptides of Hb tetramer have a large central space into which a heme prosthetic group, a protoporphyrin molecule that can be reversibly bind to oxygen [Weatherall, 1980], in which the iron (Fe2+) is positively charged [Schechter, 2008]. The combination of two α (HBA) and two β globins (HBB), $\alpha_2\beta_2$, forms the major adult Hb [Silvestroni, 1998; [Sankaran & Orkin, 2013] (**Figure 1.16**).

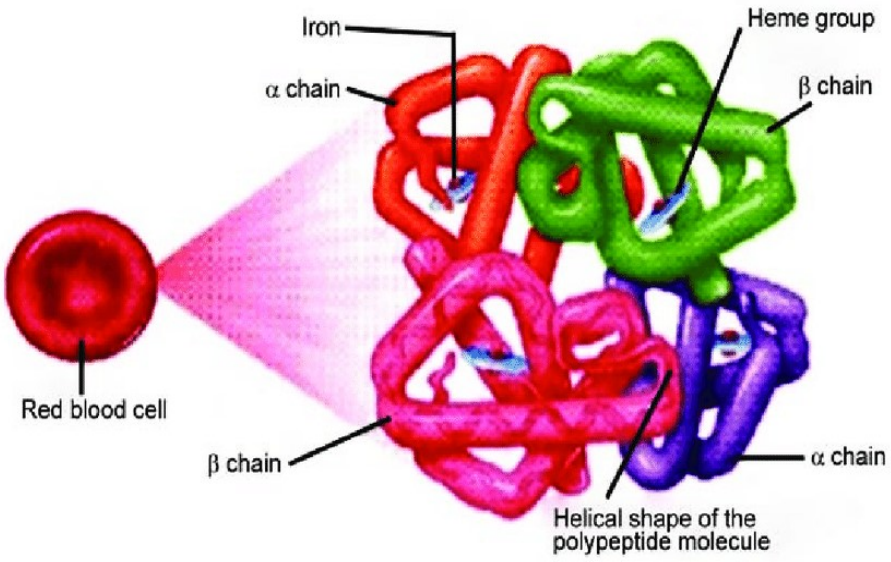


Figure 1.16. Structure of adult hemoglobin (HbA). It is composed of two α and β globin chains, each with an heme group binding an iron ion [From: Saxena et al., 2017].

1.7.2. Structure of globin genes

During the life span, in healthy individual, three types of Hb are produced differing for the types of globin chains: adult Hb, expressed during post-natal period, consisting of 98% HbA, made of two α and two β globin chains ($\alpha_2\beta_2$), and 1-2% HbA_2, consisting of two α and two δ globin chains ($\alpha_2\delta_2$); fetal haemoglobin (HbF), characteristic of the intrauterine period, made of two α and two γ globin chains ($\alpha_2\gamma_2$); and the embryonic hemoglobin comprising Hb Grower1, Portland and Hb Grower2, which have different types of globin chains, $\zeta_2\varepsilon_2$ $\zeta_2\gamma_2$ $\alpha_2\varepsilon_2$ respectively [Silvestroni, 1998; Farashi & Harteveld, 2017] (**Figure 1.17**).

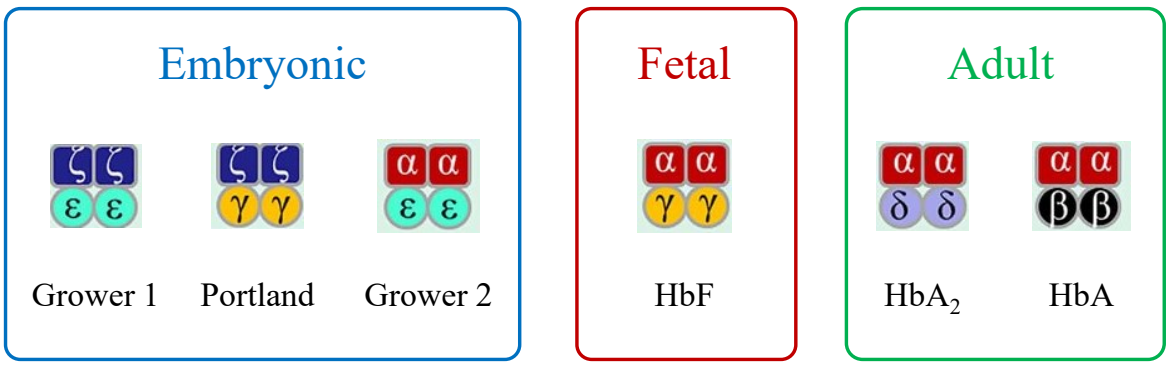


Figure 1.17. The six kinds of normal human hemoglobin [Modified from: Farashi & Harteveld, 2017].

The synthesis of globin chains varies according to the development period (**Figure 1.18**).

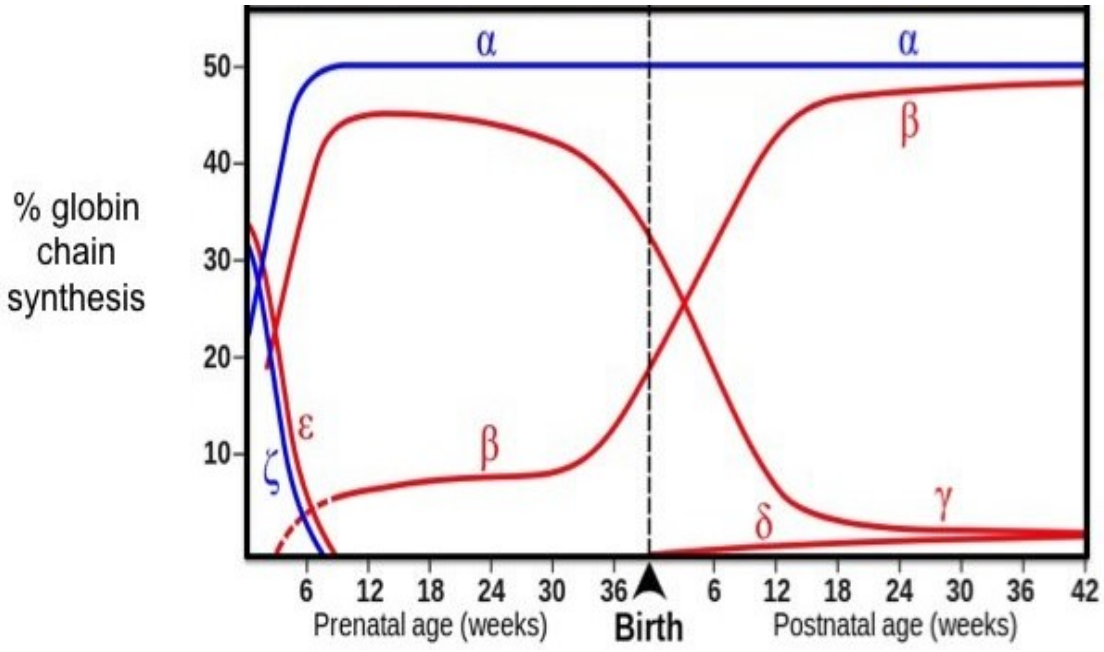


Figure 1.18. Developmental globin chains synthesis profile. The timeline of human globin genes expression is shown on the X-axis [Modified from: Di Giovanni, 2014].

Up to the 10th week of gestation, only chains ζ and ε are present in the embryo. When the pregnant woman achieves the 6th week the production of α and γ chains (^{G}γ and ^{A}γ, differing for the aminoacid residue 136, in a 3:1 ratio) starts to generate HbF, then, the globin switch from fetus to adult begins, with the gradual transition from the synthesis of γ chains to the synthesis of β chains. In the first weeks after birth, the synthesis of β chains rapidly increases, whereas γ chains are sharply reduced, until at the 6th month of life HbF is totally missing [Silvestroni, 1998; Sankaran & Orkin, 2013; Rivella, 2015].

The genes encoding the different human globin chains are organized in two clusters: α like and β like, also named non α (**Figure 1.19**). The α like globin genes, represented by ζ, α_2 and α_1, are located on chromosome 16 (**Figure 1.19**, on the top), instead the β like globin genes are located in the short arm of chromosome 11 containing the embryonic ε, the fetal ^{A}γ and ^{G}γ, δ, and β genes (**Figure 1.19**, on the bottom) [Cao & Galanello, 2010; Debarchana et al., 2014; Rivella, 2015].

In both cases, the position of genes on the chromosomes is arranged in order of their developmental expression.

In particular, *HBB* gene spans 1.6 Kb and contains three exons, two introns and both 5' and 3' untranslated regions (UTRs). TATA, CAAT and duplicated CACCC boxes are located in a promoter, in 5' position, which has a regulating function on the gene. The region that has the major regulatory action, on the expression of non α cluster genes, is the locus control

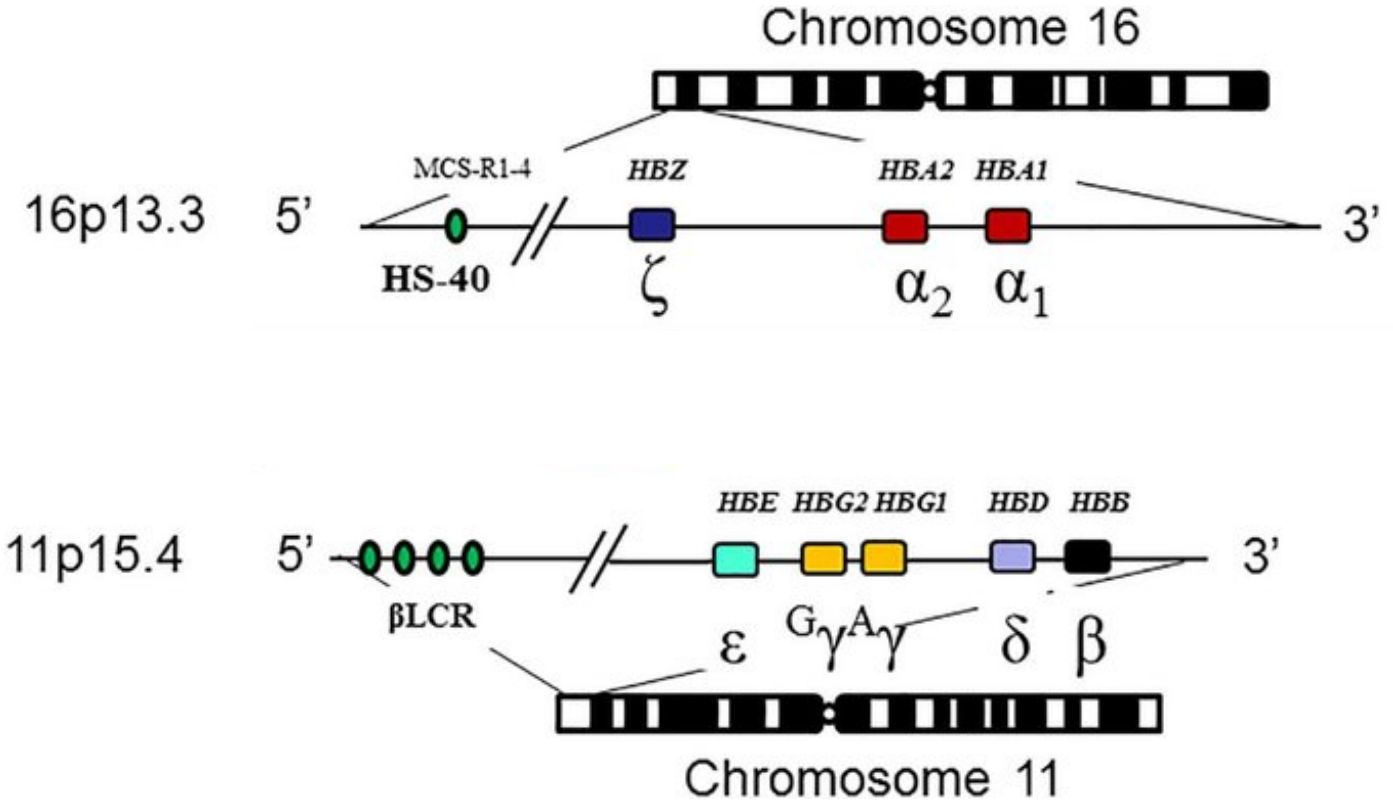


Figure 1.19. The α and non α clusters. Schematic representation of the cluster α, on chromosome 16, and non α, on chromosome 11, containing the genes encoding for globin chains. The β LCR, the HS-40 and the chromosome locations are also indicated [Modified from: Farashi & Harteveld, 2017].

region (LCR), located on chromosome 11 (**Figure 1.19**) [Manning, 2010]. This region, containing a strong enhancer, maps 50 Kb from the *HBB* gene and contains four erythroid specific DNAse hypersensitive sites (HSs), HS1 to HS4, which are hallmarks of DNA protein interaction. A combination of several DNA motifs, interacting with transcription factors, constitutes each HS site [Cao & Galanello, 2010].

HS40, located on chromosome 16 (**Figure 1.19**), is the human α globin upstream regulatory element, a region sensitive to digestion by nuclease enabling the transcription of the individual globin genes [Manning, 2010].

1.7.3. Pathophysiology and clinical features

Three main clinical and hematological conditions of increasing severity are been described, depending on the reduction in β chains: thalassemia major also known as "Cooley's anemia" or "Mediterranean anemia", thalassemia intermedia and β thalassemia carrier state [Galanello & Origa, 2010].

The absence or the reduced presence of the β globin chains result in a relative increase in the unattached α globin chains (α/β chains imbalance) that precipitate and form insoluble hemi-chromes in the erythrocyte progenitors. The occurrence of hemi-chromes leads to rigidity and mechanical fragility of cells and oxidative damages of the erythrocyte membranes that results in an ineffective erythropoiesis (IE) [Rivella & Rachmilewitz, 2009], a severe intramedullary erythrocyte apoptosis, and a severely shortened RBC lifespan due to extra-medullary hemolysis, leading to severe anemia [Silvestroni, 1998]. IE triggers a cascade of compensatory mechanisms resulting in erythroid marrow expansion, extramedullary hematopoiesis, hepato-splenomegaly, and increased gastrointestinal iron absorption [Silvestroni, 1998; Rivella, 2009]. Consequently, children with abnormality in β globin chains become symptomatic around 6 months of age [Soni, 2017]. The clinical severity of β thalassemia is related to the extent imbalance between the α/non α globin chains [Cao & Galanello, 2010].

1.7.3.1. Thalassemia major

Being homozygous for the β thalassemia is a necessary condition for thalassemia major. This condition is a severe transfusion dependent anemia, in fact individual with thalassemia major require start early regular blood transfusions to survive. A transfusion program is necessary to maintain a minimum Hb concentration of 95-105 g/L. If the major thalassemic patients do not receive the appropriate treatments die within 6 months after diagnosis [Mohamed, 2017].

Major problems are progressive hepato-splenomegaly from extramedullary hematopoiesis, recurrent infections, and spontaneous fractures [Dong & Rivella, 2017].

Even if the transfusions are strictly necessary to patients survival, they hide a relevant consequence: the iron overload, also due to increase absorption of metal. The main complications of siderosis are growth retardation, failure of sexual maturation and iron precipitation in specific organs like heart, liver and endocrine glands causing myocardiopathy, hepatitis and hormone secretion relative problems [Cao & Galanello, 2010; Manglani & Kini, 2017]. Other complications are hypersplenism, usually related to late transfusions, osteoporosis, associated mainly with bone marrow expansion, and hypoparathyroidism [Manglani & Kini, 2017].

1.7.3.2. Thalassemia intermedia

The clinical picture of thalassemia intermedia is characterized by clinically and genotypically very heterogeneous disorders, ranging in severity from the asymptomatic carrier state to the severe transfusion dependent type [Cao & Galanello, 2010]. Transfusions are mostly occasionally or not required. Iron overload occurs mainly from increased intestinal absorption of iron caused by IE. Patients have blood Hb concentration of 7-10 g/dL. They display a broad spectrum of clinical signs, depending on the degree of α/non α globin chains imbalance and several genetic and environmental factors. They suffer from different complications, including pulmonary hypertension, thrombotic events, infections, endocrine dysfunctions and leg ulcers [de Dreuzy et al., 2016].

1.7.3.3. β thalassemia carrier state

Carriers of β thalassemia, which result from heterozygosity for the disease, are usually asymptomatic but, in rare cases, a mild anemia occurs. Examination of the blood smear reveals microcytosis (reduced RBC volume) and hypochromia (reduced RBC Hb content). There is a 25% risk of having sons with homozygous thalassemia, at each pregnancy, when both parents are carriers [Galanello & Origa, 2010].

For therapeutic purposes, based on their transfusion needs, β thalassemia patients are classified as transfusion dependent (TDT) of non-transfusion dependent thalassemia (NTDT).

1.7.4. Epidemiology

It is estimated that more than 60000 babies are born annually with thalassemia major and more than 80 million peoples are carriers of β thalassemia [Mohamed, 2017].

β thalassemia has a broad distribution throughout Mediterranean countries, the Middle East, Cental Asia, India, Southern China and the Far East as well as countries along the north coast of Africa and South America. The highest carrier frequency is reported in Cyprus (14%) and Sardinia (12%) islands [Galanello & Origa, 2010; www.osservatoriomalattierare.it]. The frequency of the disease in these areas is most likely related to the selective pressure from *Plasmodium falciparum* malaria: the RBC reduced half-life makes difficult the natural reproductive cycle of malarial *Plasmodium*, even though the mechanism is not known yet [Weatherall, 1997]. The disease, therefore, is mostly spread in *Plasmodium* endemic areas, although global migration has now turned thalassemias into a concern for many non-endemic countries as well [Lederer et al., 2009].

1.7.5. Molecular bases

The continuous assessment of methods for DNA study has allowed the detection of a high number of defects in globin genes or regulatory elements facilitating the identification of genotype-phenotype correlation for β thalassemia [Silvestroni, 1998; Thein, 2005].

More than 300 mutations are now describe responsible for the disease [Soni, 2017], the majority of mutations are single nucleotide substitutions (point mutations), deletions, or insertions of oligonucleotides leading frameshift, causing a change in mRNA reading frame. A complete update list of β thalassemia mutations is available through the Globin Gene Server Web Site (www.globin.cse.psu.edu).

Gene deletions are rare, have variable extension and can affect the entire *HBB* gene or its promoter, and usually result in β^0 thalassemia [Silvestroni, 1998; Cao & Galanello, 2010]. In fact, the partial or total LCR deletion, even if leaves intact the *HBB* gene, is responsible of its inactivation [Cao & Galanello, 2010].

Insertions are usually of a single o few nucleotides, and in these cases lead to a frameshift that can generate a premature stop codon. Insertions of entire codons allow the reading frame to remain in phase, and the remaining amino acids are normal [Thein, 2013].

Point mutations, affecting *HBB* expression, belong to four different categories according to the molecular mechanism:

- Mutations leading to defective *HBB* gene transcription

  In many cases this category results from point mutations in *HHB* gene promoter [Lacerra et al., 2013] and 5' UTR mutations [Cao & Galanello, 2010], causing

transcription reduction from 75% to 80%, generally giving rise to β^+ thalassemia [Lacerra et al., 2013].

- Alterations affecting mRNA processing

  This group includes some 3' UTR mutations and defects impairing polyadenylations and splicing processes. The half part of β thalassemia mutations are splicing defects [Silvestroni, 1998]. If the mutation affects introns (IVS, intervening sequences) different effects can occur: (1) destruction of one of the two invariant dinucleotides, GT or AG, necessary for the normal splicing process, thereby degradation of mRNA resulting in β^0 thalassemia; (2) efficiency reduction of the splice site, by altering its consensus sequence, generally resulting in β^+ thalassemia; (3) new splice site creation, by generating a new GT or AG in a cryptic site, generally resulting, also in this case, in β^+ thalassemia; Mutations that affect exons activate a cryptic site located in a coding sequence, which will be used together with the normal site. Resulting phenotype is a mild β^+ thalassemia because the new site is rarely recognized [Cao & Galanello, 2010; Lacerra et al., 2013].

- Defects resulting in abnormal mRNA translation

  Mutations causing defects of translation are nonsense, introducing a termination codon. They are numerous and usually give rise to β^0 thalassemia [Silvestroni, 1998; Cao & Galanello, 2010].

- Production of unstable globin chains

  These defects include frameshift, nonsense mutations or small deletions giving origin to unstable globin chains without physiological function, which rapidly precipitate in RBC [Silvestroni, 1998].

In the Mediterranean area, the most frequent mutations for β thalassemia are four: $\beta^0$39, β^+IVSI-110, β^+IVSI-6, β^0IVSI-1 [Rigoli et al., 2001; Cao, 2010; Cao & Galanello, 2010].

- $\beta^0$39 C→T

  The prevalent β thalassemia mutation in Italy, with an incidence of 67%, and the second most common in the Mediterranean area, is the $\beta^0$39 one, caused by a single nucleotide substitution, from CAG to TAG at codon 39 of the *HBB* gene (**Figure 1.20.A**) [Orkin & Goff, 1981; Silvestroni, 1998; Huang, 2001; Thein, 2015].

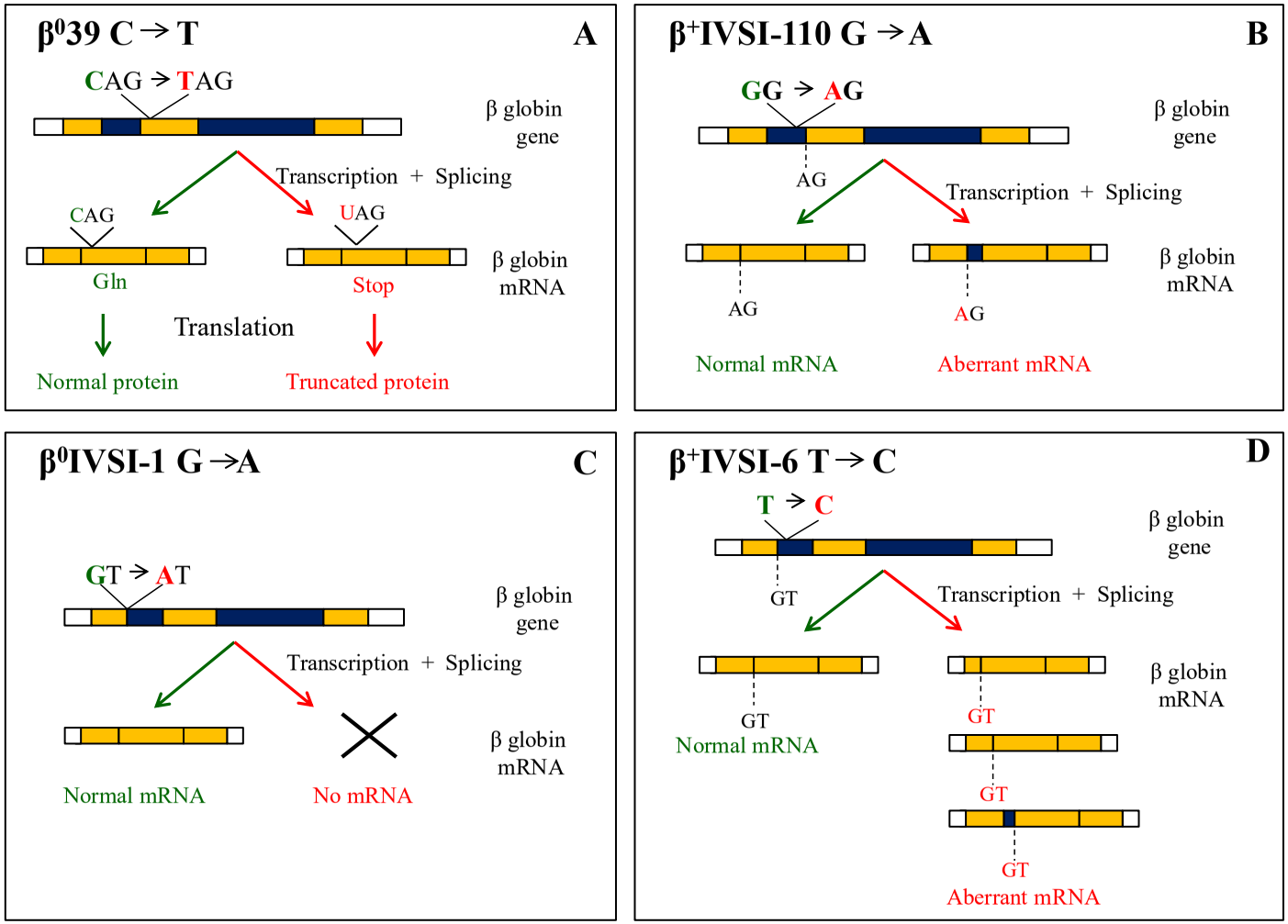


Figure 1.20. Representation of the pathogenic mechanism of the four most frequent β thalassemia mutations in Italy. $\beta^0$39 C→T (**A**), β^+IVSI-110 G→A (**B**), β^0IVSI-1 G→A (**C**), β^+IVSI-6 T→C (**D**).

The codon 39 is located in the second exon of the *HBB* gene. The nucleotide substitution turns a cytosine in a thymine, and, in mRNA, as a result, a CAG codon, coding for a glutamine, converts in the stop codon UAG, determining a nonsense mutation. The premature termination codon (PTC) stops the translation, resulting in a truncated protein that loses its function and leads to β^0 thalassemic phenotype [Silvestroni, 1998].

If patients are homozygous, the nonsense mutation results in β thalassemia major, while in case of heterozygous patients the clinical picture is different depending on the presence of other pathogenic mutations and can range from thalassemia major (for example in association with β^+IVSI-110) to thalassemia intermedia (for example in association with β^+IVSI-6) [Rigoli et al., 2001].

– β^+IVSI-110 G→A

The mutation β^+IVSI-110, in which G is substituted with A, is the second prevalent β thalassemic mutation in Italy, with 11% of incidence (**Figure 1.20.B**), and the first most common in Mediterranean countries, as well as the first base substitution identified in the *HBB* gene [Spritz et al., 1981; Thein, 2013].

The point mutation gives rise to an alternative intronic AG acceptor splice site, 19 nucleotides far from the normal AG acceptor site of the first intron (IVSI), causing a defect in the recognition of the exon/intron junction region. The 80-90% of pre-mRNAs exposes in abnormal processing resulting in abnormal and unstable RNA molecules responsible of β^+ thalassemic phenotype [Huisman et al., 1997].

Both in homozygous and heterozygous patients, the resulting thalassemic clinical condition, in most cases, is thalassemia major; but in some cases, according to the mutation present in the other allele, thalassemia intermedia can occur (for example in association with β^+IVSI-6) [Rigoli et al., 2001].

– β^+IVSI-6 T→C

In Italy the mutation β^+IVSI-6 has a frequency of 7% [Huisman et al., 1997]. This splicing mutation is caused by the substitution of the nucleotide thymine, near the GT dinucleotide in 5', with the cytosine in the consensus sequence of the splice donor site in the first intron of the *HBB* gene (**Figure 1.20.C**) [Thein, 2013]. The conserved pre-mRNA splicing processing is reduced and altered, resulting in β^+ phenotype [Silvestroni, 1998]. Generally, this mutation is related to a mild thalassemia, but the

clinical picture can extend to several phenotype depending on chromosomal background [Rund et al., 1997].

- β^0IVSI-1 G→A

  The splicing mutation β^0IVSI-1, which has an incidence of 4% in Italy [Huisman et al., 1997], is caused by the substitution of a guanine with an adenine in the invariant GT dinucleotide at position 5' of the first intron of the *HBB* gene (**Figure 1.20.D**). This nucleotide replacement alters the pre-mRNA splicing process; in fact, the normal splice site is destroyed, impairing the functional mRNA production [Silvestroni, 1998]. The result is β^0 thalassemic phenotype in homozygous patients as well as in heterozygous ones associated with other mutations [Huisman et al., 1997].

1.7.6. Therapy

In contrast with the subjects carrier of β thalassemia, which do not require specific treatments, and with β thalassemia intermedia patients, whose therapeutic approach changes depending on the diverse clinical picture, patients with thalassemia major require a specific treatment necessary for survival, as noted above [Borgna-Pignatti et al., 2004].

The therapeutic strategies can be divided in two main categories: maintenance and definitive therapy. Blood transfusions and pharmacological chelation therapy are considered together the conventional therapy and belong to maintenance strategies, while bone marrow transplantation is considered the current definitive ttreatment [Manglani & Kini, 2017]. In addition, the innovative gene therapy is considered a definitive therapy but, even though the promising results obtained, is still under clinical trials. At last, considering the many different types of mutations leading to β thalassemia, personalized therapeutic approaches are widely being studied, with the aim to correct the specific effects of different groups of mutations.

1.7.6.1. Maintenance therapy

As mentioned above, the maintenance therapy of β thalassemia is represented by a transfusion program associated to iron chelation.

1.7.6.1.1. Transfusion program

In thalassemia major, regular blood transfusions are necessary for the survival. This maintenance therapy corrects the anemia, suppresses erythropoiesis, and inhibits increased

gastrointestinal absorption of iron. The transfusion regimen changes according to patients but is designed to obtain a pre-transfusion Hb concentration of 95-100g/L [Eleftherou, 2003]. To be efficacy, transfusions should start as early as the first months after birth and should continue for life or until the patient undergoes definitive transplantation [Borgna-Pignatti et al., 2004]. Blood is generally given every 2-3 weeks according to Hb levels [Cao & Galanello, 2010].

1.7.6.1.2. Iron chelation tehrapy

Two are the causes of iron overload: the regular blood transfusions and the IE, in fact excessive iron concentration is observed also in NTDT patients [Dong & Rivella, 2017].
Hopefully, the overload can be prevented by proper iron chelation [Mohamed, 2017]. Chelators are drugs that bind iron through covalent bonds and allow its elimination. Deferoxamina, Deferiprone and Deferasirox are, at present, the three active chelator molecules used in clinical practice.
Deferoxamina, also known with the name of Desferal®, is the only chelating drug to have been extensively used in clinic since 1970. This molecule has short half-life, and, also for this reason, it is necessary a slow subcutaneous administration of 8/24 hours each day, resulting in a poor compliance in some patients [Gananello, 2001; Rivella & Rachmilewitz, 2009].
Deferiprone, also known as Ferriprox®, approved in Europe in 1999 for the treatment of patients for whom deferoxamina is contraindicated, has the advantage to have an oral uptake. Combination studies have shown that the co-administration of Deferoxamina and Deferiprone allows a synergic effect in the iron elimination in urine [Choen et al., 2004]. In addition, Deferiprone plays a best action to protect heart, while Deferoxamina has a greater effect on the liver [Cao & Galanello, 2010]. The drug Deferasirox, also known as Exjade®, like Deferiprone has an oral uptake, and is adopted in patients with a moderate cardiac and hepatic iron accumulation [Alavi et al., 2014]. With the aim of improve adherence to iron, new pharmaceutical formulations of Deferasirox have been investigated: a film coated tabled has the potential to improve adherence offering greater flexibility in administration [Shah, 2017].
New iron chelators are being developed in order to gain better efficacy with lesser adverse effects. Among the new iron chelators, the compounds known as SSP-004184, a desferrithiocin derivate, Amplopidine and LJPC-401 are in clinical trials for TDT patients treatment [Manglani & Kini, 2017; clinicaltrials.gov].

The current therapy for the treatment of β thalassemias allows the survival and the life quality amelioration but is not a definitive cure.

1.7.6.2. Definitive therapy

Although transfusions and iron chelation treatment have significantly improved over the years and, thus, improving the quality of life, they do not provide a definitive cure. At present, the only realistic and clinically definitive cure is bone marrow transplantation using hematopoietic stem cells (HSCs) from an allogenic donor with the wt *HBB* gene, preceded by the destruction by chemotherapy of patients stem cells [Cai et al., 2018].

Initial transplant experiences were limited to high risk thalassemia patients, those who were relatively young with limited morbidities and from matched sibling donors (MSD) [Sodani et al., 2004; Manglani & Kini, 2017]. Recent developments have advanced the technique from both siblings and alternate donors [Mohamed, 2017]. Although many progresses have been made in this therapeutic field, there are transplant-related complications such as infections, rejection, acute and chronic graft versus host disease (GVHD), which occurs when the donor antibodies recognize the patient tissue as non-self, that can limit the success of transplantation [Silvestroni, 1998]. Other important limitations are age and lack of donors availability [Cai et al., 2018].

1.7.6.3. Innovative diagnostic approaches

Considering the non-definitive treatment and the numerous problems associated to the bone marrow transplantation, the research is highly active, and progress has been made towards the development of new drugs, including biological products, some of which have recently reached the clinical trial stage.

Novel approaches are being developed to correct the β thalassemia resulting α/β globin chains imbalance, in an effort to move beyond the palliative management of this disease and the complications of its treatment which impose high coasts on healthcare systems [de Dreuzy et al., 2016].

Studies of genotypic and phenotypic heterogeneity among patients have provided new targets for designing novel agents that can be tailored to individual patient needs [Soni, 2017].

Different potential innovative approaches for treatment of β thalassemia are been investigated.

1.7.6.3.1. Fetal globin genes chemical reactivation

One of the possible therapeutic strategies, for the treatment of β thalassemia, is the reactivation of γ globin genes in adult life (after birth), decreasing the α/β chains imbalance and leading to increased levels of HbF, functionally similar to HbA molecule. Into adulthood, the amount of HbF is very low, less than 1% of total Hb, but in some individuals HbF levels may exceed this threshold. It is well known that subjects with HPFH (hereditary persistence of HbF) phenotype are characterized by the continuous production of HbF in adult life, resulting in a very good clinical picture and do not require transfusion therapy [Thein & Wood, 2009; Sankaran & Orkin, 2013]. From this evidence, the attraction of therapeutic approaches reactivating HbF.

Different compounds have been investigated for their potential to be HbF inductors in thalassemia syndromes. Erythropoietin (EPO) is a recombinant growth factor stimulating the proliferation of erythroid cells that has given variable results in β thalassemia treatment with increase of HbF levels in some patients [El-Beshlawy et al., 2013]. Another HbF inducer is the 5-azacytideine compound thanks to its function of DNA methylation inhibitor: in fact, during fetal development, γ globin promoters result physiologically unmethylated [El-Beshlawy et al., 2013; Sankaran & Orkin, 2013]. Sodium butyrate and its derivatives act partially inhibiting histone deacetylase, so they are very effective with a low risk in the treatment of β thalassemia [Fard et al., 2013]. The main problem of these HbF inducers is the mutagenic and carcinogenic activity, for this reason new DNA-binding drugs are been discovered that interact with specific DNA sequences altering the binding of specific transcription factors; some of these drugs are angelicin [Bradai et al., 2003], mithramycin [Fathallah & Atweh, 2006], chromomycin [Fibach et al., 2006], cisplatin [Gambari & Fibach, 2007]. However, these compounds do not shown clinical relevant effects, necessitating search for alternative drugs. In this regard, recently, the rapamycin compound, also known as sirolimus, has been designated orphan drug by the food and drug administration (FDA) and the european medicine agency (EMA) [www.rarepartners.org].

1.7.6.3.2. Gene therapy

Gene therapy, using patient own hematopoietic stem and progenitor cells (HSPCs) represents an alternative and potential definitive cure because the aim is to correct the mutated *HBB* gene or add back a functional copy of β or γ globin, recovering directly the Hb protein function.

For gene addiction, researchers delivery a functional copy of the gene *ex vivo* into human hematopoietic stem cells [Cai et al., 2018].

Initially, studies of gene addition were done with oncoretroviral vectors but their success was limited, for this reason, nowadays, the attention is shifted to lentiviral vectors [Dong & Rivella, 2017]; these are based on the human immunodeficiency virus (HIV) and they are efficiently able to encompass large therapeutic transgene cassettes, have an high transduction efficiency and are able to infect both dividing and non-dividing cells, important characteristics for hematological gene therapy purposes [Breda et al., 2009].

Different lentiviral vectors have been designed containing regulatory elements, promotes, enhancers, insulators, and β globin or γ globin, and have shown success in correction of mouse models of thalassemia [May et al., 2000; Rivella et al., 2003], and of human CD34+ peripheral blood cells [Roselli et al., 2010; Nienhuis & Persons, 2012].

In addition to the numerous studies to add back β and γ globin genetic sequences, there are additional studies bases on adding other genetic elements which can modify the gene expression, like the well-known γ globin repressor BCL11A [Wilber et al., 2011].

Cavazzana-Calvo and colleagues developed the first successful gene therapy trial for β thalassemia in 2010. The vector used for the trial (HGB-204) is βT87Q LentiGlobin (LentiGlobin® HPV569). LentiGlobin works by inserting a functional human *HBB* gene into patients own hematopoietic stem cells outside the body (*ex vivo*) and then transplanting those modified cells into the patients blood stream through infusion, also known as autologous stem cell transplantation [www.bluebirdbio.com]. This small clinical trial has engaged only two patients: the first one filed to engraft due to technical issues unrelated to the vector, instead the second (heterozygote β^E/β^0) has been transfusion independent now for several years [Cavazzana-Calvo et al., 2010; Alexis et al., 2014].

Subsequently, the drug was slightly modified to improve the gene expression and other clinical trials have been approved for the molecule to study safety and efficacy. EMA and FDA have designated LentiGlobin as an orphan drug for the TDT treatment, as well as priority medicine and breakthrough therapy. In August 2018 Blubird Bio, a pharmaceutical company specialising in genetic and orphan diseases, has announced that the CHMP (committee for medicinal products for human use) of EMA has awarded an accelerated assessment of the LentiGlobin gene therapy for β^0/non β^0 TDT adolescent and adult patients to present the marketing authorisation application (MAA). The accelerate assessment is supported by the several clinical trials on the drug: completed Northstar Phase I/II study (HGB-204), in progress Phase I/II study (HGB-205), Northstar-2 Phase III study (HGB-207), long term follow-up LTF-303 [www.osservatorimalattierare.it].

Given the numerous studies in the field of HbF chemical inducers and the more than promising approach of lentivirus vectors, another innovative strategy is combining them. The combined treatment induces an increase of both HbA (by gene addition) and HbF (by chemical HbF induction) to achieve clinical benefits not achievable with either strategy alone [Finotti et al., 2015].

1.7.6.3.3. Personalized therapy

The personalized therapy is defined as all treatments targeted to the needs of individual patients based on genetic, biomarker and phenotypic characteristics that distinguish a given patient from other patients with similar clinical presentations. This therapy aims to improve clinical outcomes for individual patients and minimizing unnecessary side effects for those less likely to have response to a particular treatment [Jameson & Longo, 2015].

β thalassemia is characterized by over 300 mutations [Soni, 2017], therefore patients can be stratified into clinically distinct subgroups with the objective of reaching a dedicated therapy. For instance, the first step for an optimized personalized therapy protocol might differ between patients with β^0 genotype and those with β^+ genotypes, in order to understand which kind of approach can be applied depending on the residual levels of HbA. The next step in personalized therapy is define the gene therapy for specific mutations.

1.7.6.3.3.1. Antisense approach

An example of personalized therapy is represented by the antisense approach; this kind of strategy is useful, specifically, for a subgroup of β thalassemia defects creating new cryptic splice sites and lead to an incorrect splicing. The most common splice mutations are IVSI (including β^0IVSI-1, β^+IVSI-6, and β^+IVSI-110) and IVSII, which create a splice site in intron 1 and 2 of the *HBB* gene respectively. Precisely, because the original splice site is intact, approaches have been made to create splice switching oligonucleotides, which cover the aberrant splice site and restore splicing to the original site [Dong & Rivella, 2017]. The antisense oligonucleotides, introduced into thalassemic cells, shall be short (15-20 nucleotide residues maximum) and complementary to a portion of the mRNA target sequence containing the specific mutation [Suwanmanee et al., 2002].

To improve affinity for the target, resistance to nucleases and pharmacokinetics, numerous modified nucleotides have been developed: for example peptide nucleic acids (PNA), synthetic analogues of DNA in which repetitive units of N-(2-aminoethyl) glycine replace the phosphodiester backbone [Pallavi et al., 2009]; N3'-P5' phosphoroamidates (NP), compounds in which the 3'hydroxyl of deoxyribose is replaced by an amine group

[Gryaznov, 1999]; 2'-deoxy-2'fluoro-β-D-arabino nucleic acids (FANA), having a modified sugar moiety; locked nucleic acids (LNA), in which a methyl bridge connects the oxygen in 2' of the ribose with the carbon in 4'; morpholino phosphoroamidate (MF), in which a morpholino group replaces the ribose; cyclohexene nucleic acids (CeNA), for which a pentatomic ring is substituted with a hexatomic one; and tricycle DNA (tcDNA) with increased capacity of interaction to complementary sequences [Kurreck, 2003].

In recent studies, manipulation of splicing patterns has been applied to β thalassemia. To give some examples: Suwanmanee and colleagues developed a morpholino oligomer, conjugated to a cell-penetrating peptide, targeting the aberrant splice site in the IVSII-654 mutation, improved Hb synthesis in mice [Suwanmanee et al., 2002; Svasti et al., 2009; Bauman et al., 2009]; Derakhshan and colleagues developed an antisense oligonucleotide with a 2'-O-methyl backbone system, targeting the aberrant splice site in the IVSI-110 mutation and restoring the correct splicing in K562 cell model [Derakhshan & Khaniani, 2017].

1.7.6.3.3.2. Read-through approach

For the treatment of genetic disorders due to PTCs, such as nonsense mutations causing β thalassemia, a promising approach is the use of compounds to force stop codon read-through and, therefore, protection from nonsense-mediated mRNA decay (NMD) for full length protein production. Aminoglycoside antibiotics, inducing a conformational change in ribosomes, have shown to decrease the accuracy in the codon-anticodon base pairing, inducing a ribosomal read-through of PTC [Rivella & Rachmilewitz, 2009]. These discoveries have introduced the development of a pharmacologic approach and new drugs to treat disease due to nonsense mutations, such as β thalassemia, for which the most frequent mutation in Italy is the $\beta^0$39 nonsense mutation [Huang, 2001; Thein, 2015].

The potential of read-through strategy has been tested in several pre-clinical models of genetic human diseases [Karijolich & Yu, 2014]: cystic fibrosis [Maiuri et al., 2017], ataxia telangiectasia [Gatti & Perlman, 2016], retinitis pigmentosa [Zheng et al., 2015], Rett syndrome [Brendel et al., 2011], and Duchenne muscular dystrophy [Guiraud & Davies, 2017].

Although several clinical trials stared, only few of them have shown an increase of full-length protein. The only read-through drug has been conditionally approved is ataluren (Translarna™) for Duchenne muscular dystrophy nonsense mutations [clinicaltrial.gov].

An accurate diagnosis of β thalassemia mutations is required to prescribing the specific treatment to allow a personalized therapy.

1.7.7. Diagnosis

In order to be eligible for a possible personalized therapy for β thalassemia, a molecular analysis is required to detect the mutation on the β globin gene that may cause the disease or may be transmitted to children.

Today genetic screening can be defined as any type of test performed for the systematic and early identification or exclusion of a hereditary disease, to know the predisposition to some diseases or to determine a predisposition which may produce a hereditary disease in offspring.

Nowadays, the diagnosis of β thalassemia can be performed at various levels.

At the *first level* the most important test is the complete blood count, that provides an estimation of the average level of Hb, the RBC count, the Mean Cell Volume (MCV), the Mean Hemoglobin (MCH) and RBC morphology [Clarke & Higgins, 2000].

The separation and quantification of the different Hb fractions in RBC may be performed in order to define the percentage of HbA_2 and HbF and allow a diagnosis of thalassemia major or intermediate. To date, a variety of analytical methods have been reported for separation of globin chains, including immunoassays, gel electrophoresis, HPLC (high performance liquid chromatography) and RP-HPLC (reversed phase high performance liquid chromatography) [Wan et al., 2012; Prajantasen et al., 2013; Greene et al., 2015].

Sometimes these techniques are not sufficient, because in infants different fractions of HbA_2 and HbF are normal. Therefore, *second level* analyses may be performed, consisting in evaluation of globin biosynthesis *in vitro*. Reticulocytes, isolated from a blood sample, are incubated with a mixture of aminoacids comprising tritiated lysine, for 1-2 hours at a temperature of 37°C. This allows to identify the types and quantities of globin chains that are produced, including those abnormal and unstable [Silvestroni, 1998].

Anyway, recently *third level* DNA analyses aimed to detect the pathogenic molecular defect have been increasingly employed, both in cases where prior techniques have not provided a reliable diagnosis, both in view of a personalized therapy. Different methods are used according to the need of searching a point mutation or a deletion.

2. AIM OF THE THESIS

Commonly, invasive procedures (for instance amniocentesis and chorionic villus sampling) are employed for the diagnosis of fetal disorders in pre-natal stage. The problem associated with these procedures is the risk of miscarriage of 1%; in addition, they cannot be performed until 11 weeks of gestation.

Therefore, after the recent discovery of circulating cfDNA in maternal blood, non-invasive pre-natal diagnosis techniques are widely under investigation for detecting pre-natal disorders and pregnancy monitoring, based on fetal DNA analysis starting from a simple maternal peripheral blood sampling avoiding impairment of the unborn child and the pregnant woman health associated to conventional invasive techniques withdrawal.

In particular, the fetal sex determination is necessary for an early conventional therapy and a timely targeted personalized therapy employment, and for the possible decision of pregnancy interruption.

Against this background, the principal purpose of the first part of the research proposed in this thesis, is the development of rapid, sensitive, and cost-effective non-invasive diagnostic methods for the fetal sex determination, in particular at early gestational ages.

Duchenne muscular dystrophy and haemophilia are just two of the more than 100 X-linked inherited human disorders that have been identified until now. In order to predict the pathological phenotype for the unborn, the fetal sex identification is a crucial point. In fact, for recessive X-linked diseases, the possibility of pathological phenotype for female fetuses is excluded, while for males the risk persists.

β thalassemia is caused by nearly 300 mutations. This autosomal recessive inherited blood disorder has a high incidence in the Mediterranean area and is associated with the absence (β^0) or reduction (β^+) of adult hemoglobin causing anemia. The most severe form of the disease is β thalassemia major: patients are unable to survive into adulthood without a therapeutic maintenance transfusion plan associated with iron chelation. The only definitive treatment, for this condition, is the bone marrow transplantation, but it hides transplant-related complications (for instance infections, rejection and GVHD).

In this context, innovative therapeutic approaches are been investigated in order to employ a personalized therapy based of the patient. Some examples of β thalassemia personalized therapies are the antisense approach, targeting splicing mutations such as β^+IVSI-110, β^0IVSI-1 and β^+IVSI-6, and the read-through approach, specifically studied for nonsense mutations such as $\beta^0$39.

For this reason, the pathogenic mutation detection, causing the disease, is of great significance for the employment of targeted and personalized therapy for the patients. Clearly, also in this case, an early diagnosis is also need to start a maintenance therapy as soon as possible, in order to improve the quality of life of the patients. Even more, a pre-natal β thalassemia diagnosis provides the basis for the timely personalized therapy application.

Over the years, several molecular techniques for the detection of point mutations have been developed: DNA sequencing is an example of routinely technology employed to identify molecular alterations. In addition, no commercial assays are available to recognize point mutations, indeed, the non-invasive screening tests offered can detect only aneuploidies, fetal sex, small deletions or insertions. However, all those techniques are costly, labour intensive and technically demanding.

Against this background, the main aim of the second part of the research proposed in this thesis, is the development of rapid, sensitive and cost-effective post-natal and pre-natal diagnostic approaches for the identification of the four most common mutations causing β thalassemia in the Mediterranean area ($\beta^0$39, β^+IVSI-110, β^0IVSI-1, β^+IVSI-6).

3. MATERIALS AND METHODS

3.1. Samples collection

Blood samples collected from healthy subjects, healthy carriers and β thalassemia patients (before routine transfusion) were obtained after informed consent with the collaboration of the Thalassemic Day Hospital (DHT) of the University Hospital *Sant'Anna* (Cona, Ferrara) and of *Hospital Santa Maria della Misericordia* (Rovigo). About 25 mL of peripheral blood, were collected in Vacutainer LH treated tubes (Becton Dickinson), containing EDTA as anticoagulant.

For pre-natal diagnosis experiments, about 18-20 mL of whole peripheral blood of pregnant women were collected, after approval by Ethical Committee of *Sant'Anna* Hospital (Cona, Ferrara), by using test tubes containing EDTA as anticoagulant. This was done in collaboration with the Laboratory of Chemical and Clinical Analysis and Microbiology, University Hospital *Sant'Anna* (Cona, Ferrara).

When possible, also 6 mL of blood or two buccal swabs were obtained by future fathers, if carriers of β thalassemia, as well. In addition, buccal swabs were collected by newborn babies, by contacting the mothers after delivery. In all cases informed consent was obtained. All the experiments were conducted in agreement with the Declaration of Helsinki.

Depending on whether blood should be used to prepare plasma or to extract genomic DNA, it was promptly processed or stored at -80°C until extraction, respectively. On the contrary, buccal swabs were stored at -20°C and processed for DNA purification within a few days from sampling.

3.2. Extraction of genomic DNA

Genomic DNA was extracted either by 0.5 ml of blood, or by two buccal swabs, by using the QIAamp® DNA Blood Mini Kit (Qiagen), according to manufacturer's instructions.

For the two different starting materials the protocol differed only in the first part. In case of blood, 50 µL of QIAGEN Protease and 500 µL of buffer AL were added to 500 µL of blood; the mixture was then mixed by vortex for 15 sec and incubated at 56°C for 10 min. After briefly centrifuging, 500 µL of 96% ethanol were added to the sample and this was mixed by vortexing for 15 sec. On the contrary, in case of buccal swabs, the cotton swab was cut with a scalpel, and then 400 µL of PBS (phosphate buffered saline), 20 µL of QIAGEN Protease and 400 µL of buffer AL were added; the mixture was mixed by vortex for 15 sec

and incubated at 56°C for 1 hour. After a brief centrifugation, 400 µL of 96% ethanol were added to the sample and it was mixed by vortex for 15 sec.

At this point, the two protocols converged. The mixture was applied to a QIAamp® Mini spin column, which was centrifuged at 6000 x g for 1 min to allow DNA binding to the column membrane and to remove contaminants; then two washes were performed, with 500 µL of AW1 buffer and 500 µL of AW2 buffer, respectively, and finally the column was centrifuged at full speed for 1 min, to eliminate the possible AW2 buffer carryover. Two DNA elutions were performed, each with 60 µL of buffer AVE: after incubation at room temperature for 5 min and centrifugation at 6000 x g for 1 min, collected DNA was checked by agarose gel electrophoresis, spectrophotometrically quantified, and stored at -20°C.

3.3. Agarose gel electrophoresis

The agarose gel for electrophoresis was prepared by melting the desired amount of agarose in 50 mL of TAE buffer (0.04 M tris-acetate, 0.001 M EDTA pH 8), adding Green Gel Plus™ (FMB, Fisher Molecular Biology), and pouring in a suitable horizontal tray, where solidification occurred in about 30 min. 1 µL of loading dye (0.25% orange G, 50% glycerol in TE (10 mM Tris-Cl, pH 7.5 1 mM EDTA) buffer) was added to each sample, before loading into wells. A suitable molecular weight marker was also loaded in a lateral well: 1Kb DNA Ladder (10000, 8000, 6000, 5000, 4000, 3500, 3000, 2500, 2000, 1500, 1000, 750, 500, 250 bp) (MBI Fermentas) or GeneRuler™ 50 bp DNA Ladder (1000, 900, 800, 700, 600, 500, 400, 300, 250, 200, 150, 100, 150 bp) (Thermo Fisher Scientific). The electrophoresis was performed at 80 volt. The DNA was visualized by using ChemiDoc™ MP System (Bio-Rad) and the image was digitally captured.

3.4. Spectrophotometric quantification

Genomic DNA extracted by blood or buccal swabs was quantified by UV spectrophotometric measurement, by using the SmartSpec™ Plus (Bio-Rad) spectrophotometer. Briefly, samples were prepared in UV-suitable cuvettes, in a 50 µL final volume, and then the absorbance at 260 nm wavelength was measured. As $1OD_{260}$ corresponds to a double-stranded DNA concentration of 50 µg/mL, it was possible to determine the sample concentrations.

In addition, the DNA purity was evaluated by calculating the ratio between absorbance measurements made at 260 and 280 nm, respectively.

3.5. Plasma preparation

Plasma was prepared within 3 hours from blood collection, according the protocol described by Legler [Legler et al., 2007]. After mixing tubes in a rotator for 5-10 min, all the blood was collected in a clean polypropylene 50 mL tube and centrifuged at 1200 x g for 10 min at 4°C without brake. Plasma was then carefully transferred to a clean 15 mL polypropylene tube and centrifuged again at 2400 x g for 20 min at 4°C in order to completely remove platelets and precipitates. The resulting supernatant was collected and stored at -80°C into 1 mL aliquots, so as not to thaw them more than once.

3.6. Extraction of circulating DNA

Circulating DNA was extracted from 2 mL of maternal plasma, not thawed more than once, by using the QIAamp® DSP Virus Spin Kit (Qiagen), according to the manufacturer's instructions. DNA elution was performed in 60 µL of AVE buffer (Qiagen).

3.7. Synthetic oligonucleotides

Synthetic oligonucleotides used as primers in sequencing reactions and PCR (**Table 3.1** and **Table 3.2**) were purchased from Sigma Genosys. On the contrary, genotyping assays used in qRT-PCR and ddPCR, including the two differently-labeled normal and mutated probes for each mutation (**Table 3.3**), were purchased by Thermo Fisher Scientific and Bio-Rad. Finally, primers and TaqMan® probes employed in qRT-PCR and ddPCR to specifically detect β globin gene or EIF2C1 gene or SRY gene (**Table 3.4**) were purchased from IDT (Integrated DNA Technologies).

Synthetic oligonucleotides used as targets or probes in SPR-based experiments (**Table 3.5**) were purchased from IDT (Integrated DNA Technologies). The biotinylated probes were purified using high performance liquid chromatography (HPLC).

3.8. PCR

Each PCR, aimed to amplify a specific DNA sequence, was prepared in a final volume of 50 µL, containing ExTaq Buffer (Takara) with 2 mM $MgCl_2$, 0.8 mM dNTPs, 150 ng PCR primers, 1.25 U/reaction ExTaq DNA polymerase (Takara).

After a first denaturation step at 94°C for 2 min, 35 amplification cycles included denaturation at 94°C for 30 sec, annealing at 1-2 degrees lower than the melting temperature of primers for 30 sec, and elongation at 72°C for a time proportional to the product length,

Name	Sequence	Tm (melting temperature)	Length (bp)
	5'-GTGCCAGAAGAGCCAAGGACAGG-3'	72.1°C	641
	5'-AGTTCTCAGGATCCACGTGCA-3'	67.1°C	
	5'-GCCTGGCTCACCTGGACA-3'	67.9°C	954
	5'-GTTGCCCAGGAGCTGTG-3'	67.2°C	
	5'-ACAATCCAGCTACCATTCTGCTTT-3'	65.7°C	436
	5'-CACTGACCTCCCACATTCCCTTTT-3'	69.9°C	

Table 3.1. List of primers employed to amplify and sequence the β globin gene. The name, the nucleotide sequence and the melting temperature are reported for each oligonucleotide. For every pair of primers, the length of the respective PCR product is indicated as well.

Name	Sequence	Tm (melting temperature)	Length (bp)	Mutation
BG8F	5'-TTAGGCTGCTGGTGGTCTA-3'	55.5°C	81	$\beta^0$39
BG6R	5'-CCATAACAGCATCAGGAGTGG-3'	55.4°C		
IVS110BF	5'-AGAGAAGACTCTTGGGTTTCTGATAG-3'	55.7°C	74	β^+IVSI-110
IVS110BR	5'-GCAGCCTAAGGGTGGGAAA-3'	57.6°C		
IVS16R	5'-CATGCCCAGTTTCTATTGGTCTC-3'	55.7°C	96	β^+IVSI-6
IVS16F	5'-CAAGGTGAACGTGGATGAAGTT-3'	55.5°C	96 105	β^+IVSI-6 β^0IVSI-1
IVSR	5'-CTGTCTCCACATGCCCAGTTT-3'	57.6°C	105	β^0IVSI-1

Table 3.2. List of primers employed to amplify ccfDNA in non-invasive pre-natal diagnosis experiments. The name, the nucleotide sequence and the melting temperature are reported for each oligonucleotide. For every pair of primers, the length of the respective PCR product and the relative mutation are also indicated.

Name	Sequence	Primer/Probe	Mutation
Beta-39 F	5'-CTTAGGCTGCTGGTGGTCTAC-3'	Forward primer	$\beta^0$39
Beta-39 R	5'-AGTGGACAGATCCCCAAAGGA-3'	Reverse primer	
Beta-39 N	5'-AAGAACCTCTGGGTCCAA-3'	VIC® probe	
Beta-39 M	5'-CAAAGAACCTCTAGGTCCAA-3'	FAM™ probe	
Beta-110 F	5'-GGGTTTCTGATAGGCACTGACT-3'	Forward primer	β^+IVSI-110
Beta-110 R	5'-GCAGCCTAAGGGTGGGAAA-3'	Reverse primer	
Beta-110 N	5'-CTCTGCCTATTGGTCTAT-3'	VIC® probe	
Beta-110 M	5'-TCTCTGCCTATTAGTCTAT-3'	FAM™ probe	
Beta-6 F	5'-GTGAACGTGGATGAAGTTGGT-3'	Forward primer	β^+IVSI-6
Beta-6 R	5'-CTATTGGTCTCCTTAAACCTGT-3'	Reverse primer	
Beta-6 N	5'-CTTGTAACCTTGATACCAACC-3'	VIC® probe	
Beta-6 M	5'-TGTAACCTTGATGCCAACC-3'	FAM™ probe	
Beta-1 F	5'-GTGAACGTGGATGAAGTTGG-3'	Forward primer	β^0IVSI-1
Beta-1 R	5'-CCCAGTTTCTATTGGTCTCCTTA-3'	Reverse primer	
Beta-1 N	5'-TGGGCAGGTTGGTA-3'	VIC® probe	
Beta-1 M	5'-TGGGCAGATTGTAT-3'	FAM™ probe	
b39 ddPCR assay	dHsaMDS696192379 (Bio-Rad)	FAM™/HEX™ assay	$\beta^0$39

Table 3.3. List of oligonucleotides used as primers or probes for genotyping experiments based on qRT-PCR and ddPCR. The name, the nucleotide sequence and the relative investigated mutation are reported. If the nucleotide sequence is unknown the ID number of the assay is indicated.

Name	Sequence/ID number	Primer/Probe	Target
Beta probe	5'-TAGTGATGGCCTGGCTCACCTGGAC-3'	FAM™ probe	β globin gene
Beta F	5'-GCACCCGGTGCCCTTGAGGT-3'	Forward primer	
Beta R	5'-CAAGAAAGTGCTCGGTGCCT-3'	Reverse primer	
SRY probe	5'-AGCAGTAGAGCAGTCAGGGAGGCAGA-3'	FAM™ probe	SRY gene
SRY F	5'-CCCCCTAGTACCCTGACAATGTATT-3'	Forward primer	
SRY R	5'-TGGCGATTAAGTCAAATTCGC-3'	Reverse primer	
EIF2C1 assay	dHsaCp2500349 (Bio-Rad)	HEX™ assay	EIF2C1

Table 3.4. List of oligonucleotides employed as primers or probes in qRT-PCR and ddPCR experiments aimed to detect total (target: β globin or EIF2C1 gene) and fetal (target: SRY gene) ccfDNA in non-invasive pre-natal diagnosis experiments. The name and the sequence are reported for each oligonucleotide. If the nucleotide sequence is unknown the ID number of the assay is indicated.

Name	Biotinylated probes sequence	β^N/β^M	Length (bp)	Mutation
Bio-N-beta39	5'-CTCTGGGTCCAA-3'	β^N	12	$\beta^0$39
Bio-M-beta39	5'-CTCTAGGTCCAA-3'	β^M	12	$\beta^0$39
Bio-N-beta(I)110	5'-AGACCAATAGGC-3'	β^N	12	β^+IVSI-110
Bio-M-beta(I)110	5'-AGACTAATAGGC-3'	β^M	12	β^+IVSI-110
Bio-N-beta(I)6	5'-CCTTGATACCA-3'	β^N	11	β^+IVSI-6
Bio-M-beta(I)6	5'-CCTTGATGCCA-3'	β^M	11	β^+IVSI-6
Bio-N-beta(I)1	5'-CCAACCTGCCC-3'	β^N	11	β^0IVSI-1
Bio-M-beta(I)1	5'-CCAATCTGCCC-3'	β^M	11	β^0IVSI-1
Name	**Targets sequence**	β^N/β^M	**Length (bp)**	**Mutation**
N-beta39c	5'-TTGGACCCAGAG-3'	β^N	12	$\beta^0$39
M-beta39c	5'-TTGGACCTAGAG-3'	β^M	12	$\beta^0$39
35mer-β^{N39}	5'-CTTAGGCTGCTGGTGGTCTACCCTTGGACCCAGAG-3'	β^N	35	$\beta^0$39
35mer-β^{M39}	5'-CTTAGGCTGCTGGTGGTCTACCCTTGGACCTAGAG-3'	β^M	35	$\beta^0$39
N-beta(I)110c	5'-GCCTATTGGTCT-3'	β^N	12	β^+IVSI-110
M-beta(I)110c	5'-GCCTATTAGTCT-3'	β^M	12	β^+IVSI-110
N-beta(I)6c	5'-TGGTATCAAGG-3'	β^N	11	β^+IVSI-6
M-beta(I)6c	5'-TGGCATCAAGG-3'	β^M	11	β^+IVSI-6
N-beta(I)1c	5'-GGGCAGGTTGG-3'	β^N	11	β^0IVSI-1
M-beta(I)1c	5'-GGGCAGATTGG-3'	β^M	11	β^0IVSI-1

Table 3.5. List of normal (β^N) and mutated (β^M) oligonucleotides employed as probes or targets in SPR-based Biacore™ X100. For each oligonucleotide, the name, the nucleotide sequence, the length and the related β thalassemia mutation are reported.

taking into account that the enzyme inserts, on average, 1000 bp/min. At the end, the reactions were maintained at 72°C for 10 min to complete the process of elongation.

In each PCR reaction aimed to DNA sequencing, 100 ng or 30 ng of human genomic DNA obtained from blood or buccal swabs, respectively, were amplified by using three pairs of primers (**Table 3.1)**, in order to include the whole β globin gene. 12.5 μM dNTPs were employed, and 35 amplification cycles were performed. PCR products were analyzed by agarose gel electrophoresis before being purified for DNA sequencing.

On the contrary, in each PCR reaction aimed to pre-amplification for pre-natal diagnosis, 1 μL of ccfDNA extracted from maternal plasma was amplified by using a specific pair of primers, according to the mutation under investigation (**Table 3.2**). 0.4 mM dNTPs were employed, and 30 amplification cycles were performed. Annealing temperature was 61°C for primers BG8F/BG6R, and 55°C for the other pairs. 1 μL of PCR product was used as a template for genotyping assay, without previous analysis.

3.9. Purification of PCR products

PCR products purification was carried out in order to remove the unincorporated PCR primers before sequencing. An equal volume of MicroCLEAN (Microzone Limited) reagent was added to each PCR product, then, after carefully mixing and incubating for 5 min at room temperature to allow DNA precipitation, this was collected by a first centrifugation at 13400 x g for 10 min and then a second one for 1 min to be able to remove all the supernatant. Finally, the desired volume of ultrapure water was added, before incubating at room temperature for 10 min and then mixing to allow the complete DNA dissolution. The purified samples were stored at -20°C until sequencing.

3.10. Sequence reaction

β globin PCR products obtained by using, as target, genomic DNA from healthy donors, healthy carriers, β thalassemia patients, future fathers, or from babies, were sequenced according to Sanger's method [Sanger et al., 1977] with primers reported in (**Table 3.1**). Sequence reactions were performed in a final volume of 20 μL, containing 40-90 ng of PCR template, 3.2 pmol of sequencing primer and ultrapure water to a 12 μL volume. Then 8 μL of Terminator Ready Reaction Mix of ABI PRISM® BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (Thermo Fisher Scientific), were added, containing the four differently-labeled dideoxyribonucleotides (ddNTPs), the AmpliTaq® DNA polimerase, $MgCl_2$ and Tris-HCl buffer at pH 9.0. A total of 45 amplification cycles were performed, as follows: denaturation, 96°C, 10 sec; annealing, 65°C, 5 sec; elongation, 65°C, 3 min.

The reaction products were then purified from unincorporated ddNTPs by using a 96-well MultiScreen™ (Millipore Corporation) plate: after loading on pre-hydrated wells filled with Sephadex™ G-50 Superfine (Amersham Biosciences), samples were recovered by centrifugation at 900 x g for 6 min and dried under vacuum.
Sequencing was finally performed by BMR Genomics (Padua, Italy), while the obtained sequence data were analyzed by the Sequence Scanner, version 1.0 (Applied Biosystems), software.

3.11. Genotyping assays based on qRT-PCR

Genotyping analyses were aimed to detect point mutations causing β thalassemia, in particular $\beta^0$39, β^+IVSI-110, β^0IVSI-1, β^+IVSI-6. For the discrimination of each mutation a specific genotyping assay was employed, containing forward and reverse primers and two different probes: a normal probe, labeled with the fluorochrome VIC® and a mutated probe, labeled with FAM™, in order to distinguish the two different alleles. The nucleotide sequences of primers and probes are reported in **Table 3.3**.
Every reaction had a final volume of 15 µL and consisted of DNA template, amplification genotyping assay and a PCR Master Mix (Thermo Fisher Scientific), that contains: the enzyme AmpliTaq Gold DNA polymerase for the polymerization reaction; the enzyme AmpErase Uracil-N Glicosilase, active at 50°C and inactivated at 95°C, that degrades uracil-containing sequences and so removes possible previously amplified contaminant molecules; deoxyribonucleotides (dNTPs) containing dUTP instead of dTTP; 1 mM MgCl2; the reference ROX™ for the normalization of data. In particular, TaqMan® Universal PCR Master Mix (Thermo Fisher Scientific) was employed in post-natal analyses, whereas the genotyping pre-natal reactions required PCR Master Mix Genotyping, that contains optimized mix components in order to provide better specificity for discrimination between alleles (Thermo Fisher Scientific). 1 ng of genomic DNA was employed as a template for post-natal analyses, while, at pre-natal level, 1 µL of pre-amplification reactions of ccfDNA from maternal plasma, was used.
All the reactions were performed in duplicate. For each analysis, no-template controls were prepared as well.
The reactions were carried out on a StepOne™ Real-Time PCR System (Applied Biosystems-Thermo Fisher Scientific), by using the StepOne Software (Applied Biosystems-Thermo Fisher Scientific). After a first 2-min cycle at 50°C to activate the enzyme Uracil-N Glycosylase and a second 10-min cycle at 95°C to inactivate it, equal for all assays, the amplification cycles were different for each specific genotyping assay: 50

amplification cycles with denaturation at 95°C for 15 sec, and annealing and elongation at 62°C for 1 min for $\beta^0$39, β^+IVSI-110 and β^+IVSI-6 assays; 60 amplification cycles with denaturation at 95°C for 15 sec, and annealing and elongation at 60°C for 1 min for β^0IVSI-1 assay.

For analysis of post-natal data, the end-point fluorescence produced by VIC® and FAM™ was normalized with the ROX™ reference, according to the following equations: VIC® Rn = VIC®/ROX™; FAM™ Rn = FAM™/ROX™. Since each reaction was performed in duplicate, the average Rn value was calculated for each sample. For each genotyping assay, final VIC® and FAM™ Rn values were plotted in an allelic discrimination plot.

3.12. Prediction of secondary structures

The expected secondary structures of target oligonucleotides or single-stranded unbalanced PCR products were determined by using *The mfold Web Server* (http://mfold.rit.albany.edu/?q=mfold/) [Zuker 2003], after setting the temperature at 25°C and the ionic concentrations as $[Na^+]$ = 150 mM and $[Mg^{++}]$ = 1.8 mM, corresponding to the hybridization conditions.

3.13. Unbalanced PCR

For PCR reactions aimed to the SPR-based detection of $\beta^0$39, β^+IVSI-110, β^+IVSI-6 and β^0IVSI-1 thalassemia mutations, different pairs of primers were designed, specific for each alteration (**Table 3.6**). First, a balanced reaction was performed by amplifying 200 ng of genomic DNA extracted from blood or salivary swabs. Each reaction had a final volume of 50 µL, containing ExTaq Buffer (Takara) with 2 mM $MgCl_2$, 0.2 mM dNTPs, 150 ng PCR primers, 1.25 U/reaction ExTaq DNA polymerase (Takara). After a first denaturation step at 94°C for 2 min, the 50 amplification cycles were as follows: denaturation at 94°C for 30 sec, annealing at a temperature 1-2 degrees lower than the melting temperature of primers for 30 sec, and elongation at 72°C for 10 sec; at the end, the reactions were maintained at 72°C for 10 min to complete the process of elongation. Finally, for the unbalanced amplification required to produce single-stranded PCR products for bio-specific interaction analysis with Biacore™ X100, 5 µL of the balanced PCR products were employed as templates and only the forward primers were added. The experimental conditions were the same just described.

3.14. Bio-specific interaction analysis with Biacore™ X100

The Biacore™ X100 analytical system (GE Healthcare) was used in all experiments, together with the Biacore™ X100 Control Software (GE Healthcare). SA sensor chips,

Name	Forward/reverse	Sequence	Tm (melting temperature)	Length (bp)	Mutation
BG8F	Forward	5'-TTAGGCTGCTGGTGGTCTA-3'	55.5°C	81	$\beta^0$39
BG6R	Reverse	5'-CCATAACAGCATCAGGAGTGG-3'	55.4°C		
BG8F	Forward	5'-TTAGGCTGCTGGTGGTCTA-3'	55.5°C	53	$\beta^0$39
BG8R	Reverse	5'-CCCAAAGGACTCAAAGAACCTC-3'	55.6°C		
IVS16F	Forward	5'-CAAGGTGAACGTGGATGAAGTT-3'	55.5°C	96	β^+IVSI-6 β^0IVSI-1
IVS16R	Reverse	5'-CATGCCCAGTTTCTATTGGTCTC-3'	55.7°C		
IVS110BF	Forward	5'-AGAGAAGACTCTTGGGTTTCTGATAG-3'	55.7°C	74	β^+IVSI-110
IVS110BR	Reverse	5'-GCAGCCTAAGGGTGGGAAA-3'	57.6°C		

Table 3.6. List of primer pairs employed to produce targets for SPR-based Biacore™ X100. The name, the orientation, the nucleotide sequence and the melting temperature are reported for each oligonucleotide. For every pair of primers, the length of the respective PCR product and the relative mutation are indicated.

precoated with streptavidin, and the running buffer HEPES-buffered saline-EP+ (HBS-EP+), containing 0.01 mM HEPES, pH 7.4, 0.15 M NaCl, 3 mM EDTA, and 0.05% Surfactant P20, were purchased from GE Healthcare. Buffers were filtered before use. The experiments were conducted at 25°C temperature and at 5 µL/min flow rate.

In order to immobilize the biotinylated DNA probe on the SA sensor chip surface, the well-documented streptavidin-biotin interaction was employed. After pre-treatment with three 10 µL pulses of 50 mM NaOH - 1 M NaCl, an injection of 80 µL of HBS-EP+ containing the oligonucleotide probe at the concentration of 10 ng/µL, were administered in the flow cell 2 of the sensor chip.

The analysis of bio-specific interaction with target DNA oligonucleotides was carried out by injecting the targets in HBS-EP+ buffer for 4 min, and then washing with the running buffer alone for 3 min. The target concentrations were 1 µM for the 11mer and 12mer oligonucleotides, and 15 µM for the 35mer-β^N and 35mer-β^M oligonucleotides.

Instead, the interaction analysis with unbalanced PCR products was performed by injecting 90 µL of HBS-EP+ buffer containing the target for 12 min, and then carrying out a 3 min washing step with the running buffer alone.

After hybridization, the sensor chips were regenerated by a 5 µL pulse of 50 mM NaOH.

Sensorgrams were analyzed with the Biacore™ X100 Evaluation Software, version 2.0.1 (GE Healthcare). A subtraction of the background signal possibly recorded by the analyte injection on the empty flow cell 1, as a control, was automatically produced. In addition, suitable blank control injections with running buffer were performed, and the resulting sensorgrams subtracted from the experimental results.

3.15. qRT-PCR

6 µL of ccfDNA extracted from maternal plasma were analyzed by using qRT-PCR amplification assays specific for the β globin and the SRY gene (**Table 3.4**), in order to detect total and fetal (in case of male fetus) DNA, respectively. For each reaction, some standards containing known amounts of male genomic DNA were prepared as well, to make a calibration line for the absolute quantification of samples.

Each reaction had a final volume of 15 µL and consisted of DNA template, amplification assay and TaqMan® Universal PCR Master Mix (Thermo Fisher Scientific) that contains: the enzyme AmpliTaq Gold DNA polimerase for the polymerization reaction; the enzyme AmpErase Uracil-N Glicosilase, active at 50°C and inactivated at 95°C, that degrades uracil-containing sequences and so removes possible previously amplified contaminant molecules; deoxyribonucleotides (dNTPs) containing dUTP instead of dTTP; 1 mM $MgCl_2$; the

reference ROX™ for the normalization of data. All the reactions were performed in duplicate. For each analysis, no-template controls were prepared as well.

The reactions were carried out on a StepOne™ Real-Time PCR System (Applied Biosystems-Thermo Fisher Scientific), by using the StepOne Software v2.3 (Applied Biosystems-Thermo Fisher Scientific). The amplification program included 2 min at 50°C, 10 min at 95°C, and 50 cycles of amplification, as follows: denaturation, 15 sec, 95°C; annealing and elongation, 1 min, 60°C.

To determine the concentration of template DNA, an absolute quantification was used based on calibration lines produced by amplification of DNA standards at known concentrations. For the β globin assay, standard amounts corresponding to 33 ng, 6,6 ng, 3,3 ng and 660 pg copies of genomic DNA were employed; whereas, in the case of the SRY assay, the standard used concentrations were lower, corresponding to 660 pg, 330 pg, 165 pg and 66 pg genomic DNA copies.

3.16. SspI enzymatic digestion

The enzymatic digestion of the genomic DNA was carried out using SspI as restriction enzyme (Thermo Fisher Scientific), in order to simulate the ccfDNA.

Each reaction had a final volume of 20 µL and consisted of 50 ng of genomic DNA, 1 U of SspI enzyme, SspI 1x buffer. The reaction was incubated at 37°C for 16 hours and at the end, the enzyme was inactivated at 65°C for 10 min.

3.17. ddPCR

DNA was detected and quantified using QX200™ Droplet Digital™ PCR system (Bio-Rad). Briefly, for fetal sex determination, 11 µL of 2x ddPCR™ Supermix for Probes (Bio-Rad), 1 µL of 20x EIF2C1 Bio-Rad assay (**Table 3.4**), 1 µL of 62.5x SRY assay (**Table 3.4**) were mixed with 8 or 9 µL of ccffDNA template, according to gestation weeks, or with 1 ng of circulating or genomic DNA, in a reaction volume of 22 µL.

To generate the droplets, 20 µL of ddPCR reaction and 70 µL of Droplet Generation Oil for Probes (Bio-Rad) were inserted in an eight-well cartridge using QX200™ AutoDG™ Droplet Generator (Bio-Rad) according to manufacturer instructions. Then, 40 µL of the generated droplet emulsion were transferred to a new 96-well PCR plate (Eppendorf) and amplified in the GeneAmp®PCR System 9700 (Applied Biosystems). Amplification conditions started with 10 min of activation of DNA polymerase at 95 °C, followed by 45 cycles of a two-step thermal profile of 30 sec at 94 °C for denaturation, and 1 min at 60 °C

for annealing and extension. A final hold of 10 min at 98 °C was used for the enzyme inactivation.

After thermal cycling, plates were transferred to QX200^{TM} Droplet Reader (Bio-Rad). The software provided with the ddPCR system (QuantaSoft 1.3.2.0; Bio-Rad) was used for data acquisition to calculate the absolute concentration of target DNA in copies/µL of reaction using Poisson distribution analyses [Pinheiro et al., 2012; Hindson et al., 2011].

For $\beta^0$39 and β^+IVSI-110 mutations detection, 2x ddPCR^{TM} Supermix for Probes (Bio-Rad), 1x β^+IVSI-110 or 0.75x of $\beta^0$39 assay (**Table 3.3**), were mixed with 1 ng of genomic DNA, 10 ng of genomic DNA mixtures or 8 µL of ccffDNA template, in a reaction volume of 22 µL.

The amplification reaction was carried out using IQ^{TM}5 Multicolor Real-Time PCR Detection System (Bio-Rad). Amplification conditions started with 10 min of activation of DNA polymerase at 95 °C, followed by 40, for $\beta^0$39, or 50, for β^+IVSI-110, cycles of a two-step thermal profile of 30 sec at 94 °C for denaturation, and 1 min at 58 °C for annealing and extension for the β^+IVSI-110 assay, or 1 min at 59°C or 57°C for the $\beta^0$39 optimization and samples analysis, respectively. A final hold of 10 min at 98 °C was used for the enzyme inactivation.

3.18. Statistical analysis

Statistical differences between groups were compared by the Student's t-test, selecting a 95% or 99% confidence level. Statistical significance was assumed at $p<0.05$ or $p<0.01$, according to the analysis.

4. RESULTS AND DISCUSSION

Currently more than 100 X-linked inherited human disorders have been identified, such as hemophilia and Duchenne muscular dystrophy, taking a relevant place in the genetic counseling [Germain, 2006].

Therefore, the fetal sex identification is fundamental in order to predict the pathological phenotype for the unborn. In the specific case, for recessive X-linked diseases, the possibility of pathological phenotype for female fetuses is excluded, while for males the risk persists [Mandieh et al., 2013].

Routinely, the pre-natal diagnosis is obtained using invasive procedures such as amniocentesis and chorionic villus sampling, but these procedures are associated with a 1% risk of miscarriage and cannot be performed until 11 weeks of gestation [Sillence et al., 2015], so, recently non-invasive pre-natal diagnosis techniques are widely under investigation.

Non-invasive pre-natal diagnosis started after the discovery by Lo et al. [Lo et al., 1997] of ccffDNA in maternal circulation, and is based on fetal DNA analysis starting from a simple maternal peripheral blood sampling without disrupting or endangering the health of the unborn child and the pregnant woman, thus eliminating the risks associated with conventional invasive techniques [D'Aversa et al., 2018].

The possibility to known the fetal sex is necessary for: (1) early conventional therapy application, (2) timely possible personalized therapy for the newborn and (3) possible decision of pregnancy interruption from the parents.

β thalassemia is an autosomal recessive inherited disease, with high incidence in the Mediterranean area [Cao, 2010], originates from quantitative or qualitative changes of the adult haemoglobin molecule, responsible for the physiological oxygen transport [Sankaran & Orkin, 2013]. Indeed, this genetic blood disorder, caused by nearly 300 mutations, is associated with absence (β^0) or reduction (β^+) of adult hemoglobin causing a severe anemia [Silvestroni, 1998; Cao & Galanello, 2010]. β thalassemia major, also defined as Cooley's anemia, is the most severe form of the disease, and patients are unable to survive into adulthood without life-long periodic blood transfusion and iron chelation therapies [Weatherall, 1980]. These therapies are just for maintenance, the only definitive treatment, for this condition, is the bone marrow transplantation, which hides transplant-related complications such as infections, rejection and GVHD [Silvestroni, 1998; Cai et al., 2018].

On this basis, novel approaches, including biological drugs, are been investigated as personalized therapy based on genetic, biomarkers and phenotypic characteristics of the patient [Jameson & Longo, 2015]. Some examples of β thalassemia personalized therapies, that are being developed, are the antisense approach targeting splicing mutations, such as β^{+}IVSI-110, β^{0}IVSI-1 and β^{+}IVSI-6 [Suwanmanee et al., 2002], and the read-through approach applied to nonsense mutations [Doronina et al., 2006], such as β^{0}39, caused by a nonsense codon mutation causing premature termination of β globin chain synthesis [Orkin & Goff, 1981].

For this reason, the detection of the pathogenic molecular alterations, causing the disease, could be of substantial importance for the employment of targeted and personalized therapy for the patients. Clearly, an early diagnosis is also need to start a maintenance therapy as soon as possible, in order to improve the patients' life quality.

As has just been discussed, β thalassemia diagnosis is the key for the personalized therapy applicability, both in post-natal and in pre-natal phases. In the latter case, the possibility to diagnose the mutation transmittable to offspring, in a very early stage, could be extremely useful for carrier parents in view of a targeted timely therapeutic plan.

Several molecular techniques for the detection of point mutations, causing β thalassemia, have been developed over the years, such as RFLP-PCR or ARMS-PCR, and currently the genomic DNA sequencing is routinely employed to identify molecular alterations [Mahdieh & Rabbani, 2013]. However, all those techniques are costly, labour intensive and technically demanding, and required trained staff for performing the assays and analyzing the results. Therefore, new diagnostic approaches aimed to overcome such drawbacks are highly needed.

In this context, the primary aim of the research (**Figure 4.1**), proposed in this thesis, was the development of rapid, sensitive, and cost-effective non-invasive diagnostic methods for the determination of fetal sex, in particular at early gestational weeks, and for the identification of the most common mutations causing β thalassemia in the Mediterranean area (β^{0}39, β^{+}IVSI-110, β^{0}IVSI-1, β^{+}IVSI-6) [Cao & Galanello, 2010], from genomic DNA of patients (post-natal diagnosis) and ccffDNA extracted from plasma of pregnant women (pre-natal diagnosis). The principal purpose of both diagnosis is the potential application of a personalized therapy, which in case of pre-natal diagnosis can be timely.

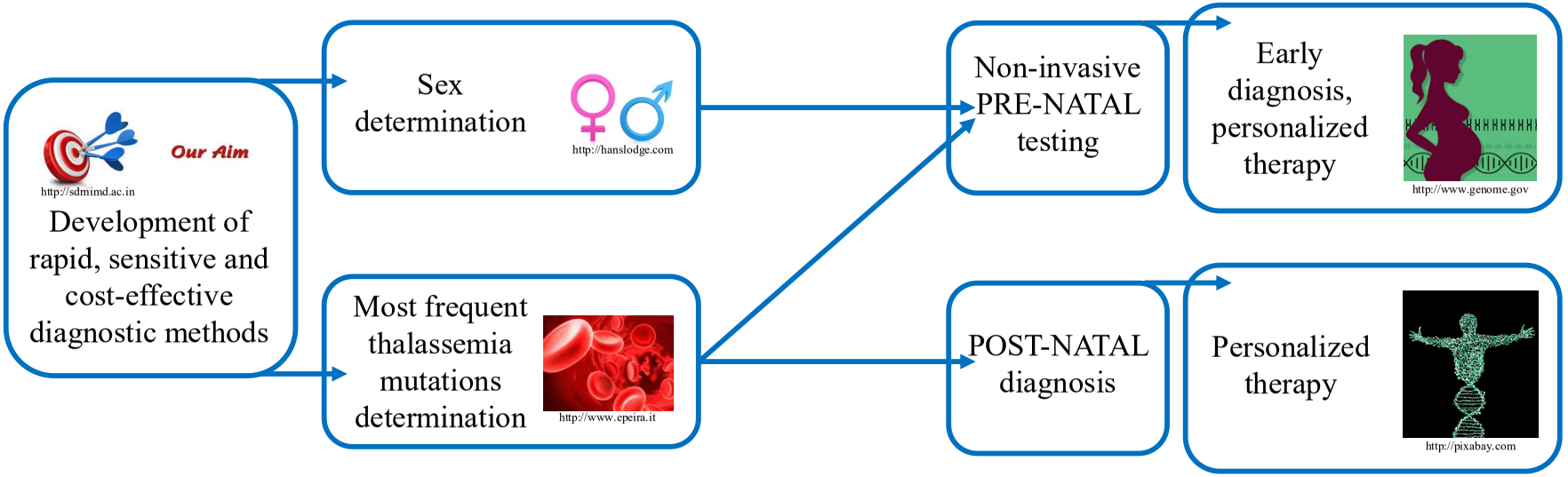


Figure 4.1. Schematic representation of the research primary purpose. The main aim of the research thesis, is the development of rapid, sensitive and cost-effective non-invasive diagnostic methods for the determination of fetal sex (pre-natal diagnosis) and for the identification of the most common mutations causing β thalassemia in the Mediterranean area ($\beta^0$39, β^+IVSI-110, β^0IVSI-1, β^+IVSI-6) from genomic DNA of adult patients (post-natal diagnosis) and ccffDNA extracted from plasma of pregnant women (pre-natal diagnosis). The ultimate goal of both pre- and post-natal diagnosis is the personalized therapy, whose can be applied timely in case of early stage.

4.1. Fetal sex pre-natal diagnosis from ccffDNA

In recent years, ccffDNA has been extensively investigated for detecting pre-natal disorders and pregnancy monitoring [Galbiati et al., 2005; Tounta et al., 2011; Edlow & Bianchi, 2012; Pescia et al., 2017]. Non-invasive pre-natal diagnosis was born with the discovery of ccffDNA within the maternal plasma and serum [Lo et al., 2003], and since then an increasing number of studies has been performed with the aim to develop experimental non-invasive diagnostic approaches [Lo et al., 1999; Lun et al., 2008; Chiu & Lo, 2013; Breveglieri et al., 2017; D'Aversa et al., 2018].

Non-invasive pre-natal diagnosis has become increasingly important, because, although it retains only a predictive/probabilistic value, it allows to study the fetal health without any risk for both fetus and mother [Webb et al., 2001].

In particular, as previously underlined, the fetal sex determination is fundamental in the non-invasive pre-natal diagnosis field for sex-related diseases, such as Duchenne muscular dystrophy and haemophilia [Germain, 2006], as it allows predicting the pathological phenotype for the unborn, thus avoiding invasive diagnostic procedures for pregnant women, for therapeutic early intervenction.

In this context, the first purpose of this research thesis was the development of non-invasive pre-natal fetal sex diagnostic methods based on ccffDNA.

4.1.1. Previously published results

Already reported data, in this field, had been developed by our research group [Breveglieri et al., 2016].

This study, based on ccffDNA obtained from maternal plasma, was aimed to the final development of an experimental non-invasive method of pre-natal diagnosis to determine the fetal gender.

With this aim, 26 blood samples from pregnant women with a wide variability in terms of weeks of gestation and sex of the future newborn were collected, after approval by the Ethical Committee of University Hospital *S.Anna*, Ferrara (Italy).

4.1.1.1. Experimental strategy

The experimental strategy is displayed in **Figure 4.2**. 18 mL of peripheral blood samples were collected from pregnant women (**Figure 4.2.A**); plasma aliquots are immediately prepared and stored at -80°C (**Figure 4.2.B**) until the extraction of total circulating cfDNA (**Figure 4.2.C**). Subsequently, the circulating DNA was amplified in order to determine the

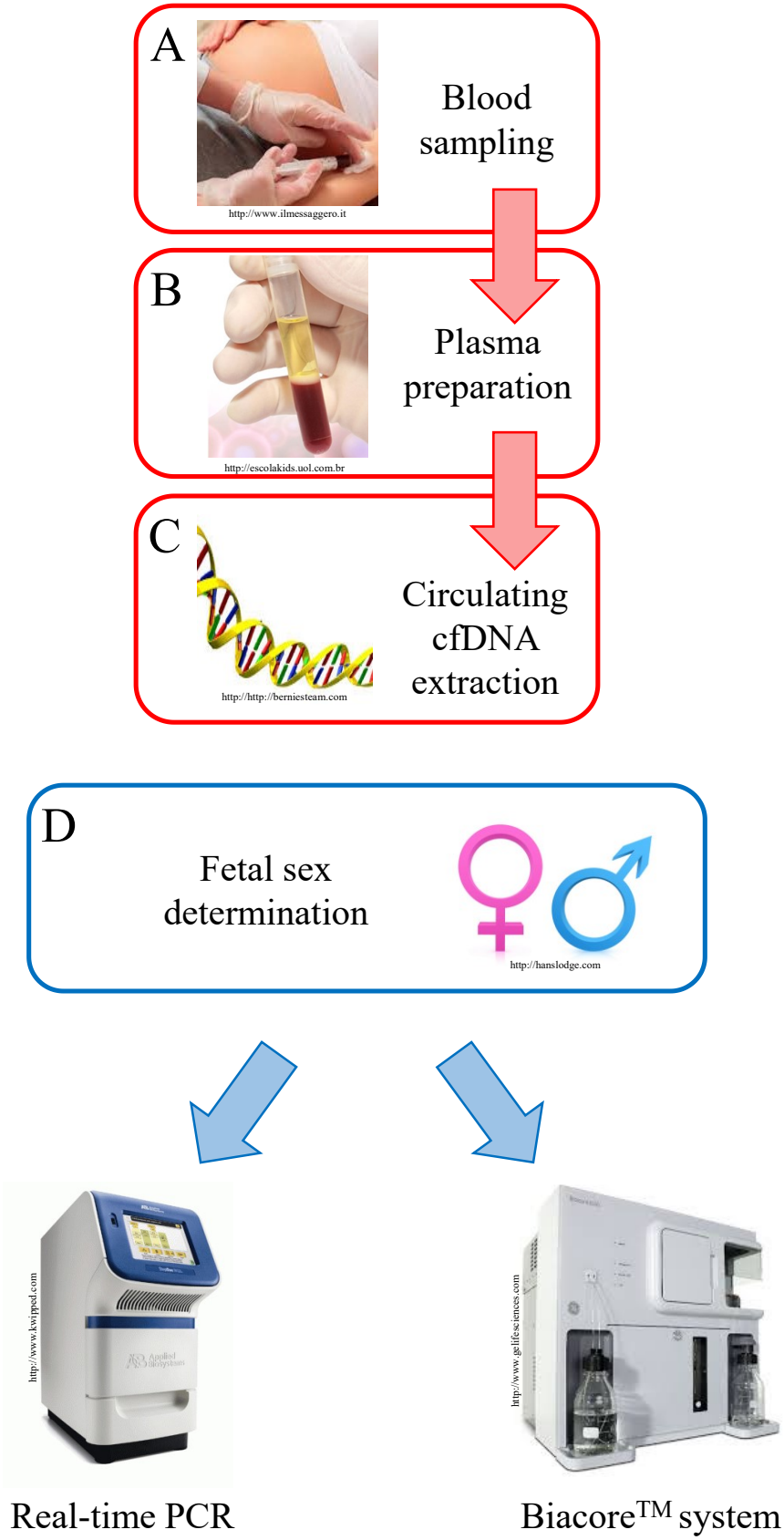


Figure 4.2. Experimental strategy for the development of non-invasive pre-natal sex diagnosis methods. After collecting peripheral blood samples for pregnant women (A), plasma aliquots are immediately prepared and stored at -80°C (B), until the extraction of the total circulating cfDNA (C). Then, the fetal sex is determined (D) following two different approaches: qRT-PCR technique or SPR-based Biacore™ system.

fetal sex (**Figure 4.2.D**). The fetal gender diagnosis was employed using two different approach: qRT-PCR and SPR-based Biacore™ system.

4.1.1.2. Detection of fetal Y chromosome sequences by qRT-PCR

Currently, the most frequently employed molecular technique to identify the fetal gender is the qRT-PCR [Lo et al., 1998]. For this reason, the first molecular approach had provided qRT-PCR employment. Accordingly, my research group adopted a TaqMan® amplification assay specific for the SRY gene, located on the Y chromosome, allowing the detection of fetal male DNA, designed according to literature [Lo et al., 1998]. Its applicability was tested using a wide range of samples with particular attention to early gestational ages, where generally pre-natal diagnosis should be required.

After the extraction of circulating DNA from 2 mL of plasma specimens, using the QIAamp® DSP Virus Spin Kit (Qiagen), derived from 26 pregnant women at different gestational weeks, the detection of total and fetal DNA was carried out using specific TaqMan® amplification assays for the β globin and the SRYhuman genes, respectively, both FAM™ labeled. The β globin assay was used as reference gene to amplify both the fetal and the maternal DNA.

The **Figure 4.3** shows examples of SRY (**Figure 4.3.A**) and β globin (**Figure 4.3.B**) amplification curves obtained from male (on the left side) and female (on the right side) circulating DNA extracted from maternal plasma. The male curves were obtained from pregnant woman at 33th week of gestation, instead, the female curves were obtained from pregnant woman at 37th week of gestation. In the considered graphs, the fluorescence intensity (ΔRn) is a function of the number of cycles.

The fetal gender was considered male or female according to the generation or not of a SRY-specific amplification plot. The β globin amplification curve is useful to confirm the DNA presence, in order to avoid false negative.

In addition, the amplification curves relative to the SRY gene are shifted to the right compared to the β globin ones, due to the higher concentration of total circulating DNA, mainly maternally derived, than the fetal one.

The actual sex of the future newborns and the diagnostic outcomes are reported in **Table 4.1** for all samples, listed in a decreasing order according to the gestational week.

These results demonstrated that the formulated diagnoses were correct and a proper fetal gender diagnosis could be performed by qRT-PCR starting from the 9th gestational week. For some samples at 7th and 6th week of gestation (#22 and #25), the diagnosis was not clear

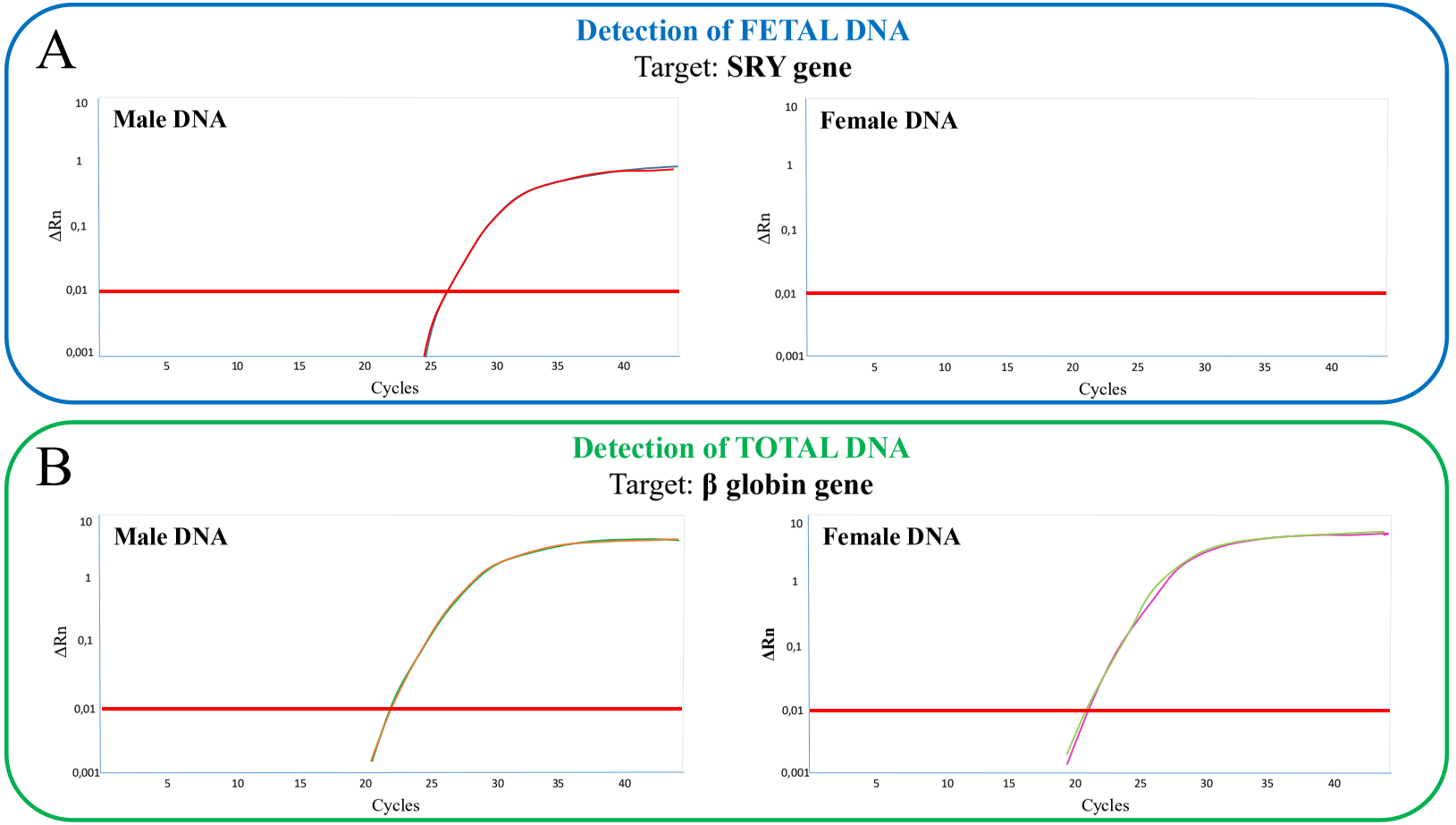


Figure 4.3. Fetal and total circulating DNA detection from maternal plasma using qRT-PCR. SRY gene (A) and β globin gene (B) specific amplification assays are employed for the detection of fetal (in blue) and total (in green) circulating DNA, respectively, from plasma of pregnant women. (A, B) Examples of amplification curves obtained from male (on the left side) and female (on the right side) circulating DNA. The plots show the fluorescence intensity (ΔRn) as a function of the amplification cycles, while the threshold line is drawn in red. The amplification was performed by using the StepOne™ Real-Time PCR System.

# sample	Gestational weeks	Result at birth	Result by qRT-PCR
1	37	M	M
2	36	M	M
3	31	M	M
4	30	F	F
5	25	M	M
6	24	F	F
7	21	F	F
8	17	F	F
9	17	M	M
10	17	F	F
11	16	F	F
12	16	M	M
13	16	M	M
14	16	M	M
15	15	M	M
16	14	M	M
17	14	M	M
18	11	M	M
19	10	M	M
20	10	M	M
21	9	M	M
22	7	M	*n.d.*
23	7	F	F
24	7	F	F
25	6	M	*n.d.*
26	6	F	F

Table 4.1. qRT-PCR fetal sex outcome. The actual fetal sex and the diagnostic outcome after fetal sex determination by qRT-PCR are reported for circulating cfDNAs obtained from 26 pregnant woman at different gestational age. M, male. F, female. *n.d.*, not detectable. The amplification was performed by using the StepOne™ Real-Time PCR System [Modified from: Breveglieri et al., 2016].

and generate an uncertain outcome, suggesting the need of alternative approaches with higher sensitivity and suitable for molecular efficient diagnosis of non-invasive detection of fetal gender. The reason of wrong outcomes is probably due to the fetal DNA amounts, if fact the fetal DNA is present in maternal circulation at very low concentration and increases during the progression of pregnancy [Zhou et al., 2015]. The proportion of ccffDNA grows by 0.1% every seven days between the 10th and the 21st week of gestation, then increases faster after the 21st week, reaching almost 1% increment every week [Zhou et al., 2015; Drury et al., 2016]. In addition, the ccffDNA amount depends on other factors, such as maternal diseases and body weight, aneuploidies and twin pregnancies [Vora et al., 2012; Zhou et al., 2015].

In order to obtain a better sensitivity in male fetal DNA identification, our research group decided to employ the SPR-based Biacore^{TM} technology, already known for its low detection limit, user-friendliness, reproducibility, low cost and automation [Feriotto et al., 2001; Feriotto et al., 2002; Karlsson, 2004; Brambilla et al., 2010; Stravalaci et al., 2011], for a new efficient and high sensitive non-invasive pre-natal diagnostic strategy.

4.1.1.3. Detection of fetal Y chromosome sequences by Biacore^{TM} X100

Optical biosensors based on SPR are widely used to study in real-time and in label-free mode bio-molecular interactions [Perera et al., 2014; Ahn et al., 2018]. After ligand immobilization on the sensor chip surface and injection of analytes, their possible interaction produces an increment in mass resulting in a change of SPR angle, which is monitored in real-time as resonance signal in function of time in a sensorgram [Gambari, 2001].

The Biacore^{TM} X100 instrument (GE Healthcare) was chosen to carry out the hybridization analyses thanks to its numerous advantages: (1) simple handling, (2) availability of validated buffers injection, (3) rapid real-time analysis, (4) high reproducibility, (5) full automation and (6) low costs related to the fact that the sensor chip can be reused up to 80-100 times.

In the experiments, based on Biacore^{TM} X100, a biotinylated oligonucleotide probe specific for the SRY gene, located on the Y chromosome, was immobilized on a streptavidin-coated sensor chip. Then the hybridization with an injected unbalance PCR product obtained by circulating DNA purified from maternal plasma was evaluated. The target PCR product was obtained in two different steps. In the first, the SRY gene of the target DNA, if present, was amplified with the two SRY primers, generating a 73 bp product containing a 15 nucleotides region corresponding to the sequence of the immobilized probe. The second step consisted in a second unbalance amplification of the first amplicon with the use of only the forward

primer, in order to obtain a single-stranded SRY target sequence complementary to the probe, for an efficient hybridization with the immobilized probe. The expected secondary structure of this single-stranded product, predicted by *The mfold Web Server* (http://mfold.rit.albany.edu/?q=mfold/) [Zuker, 2003], showed that a major portion of the sequence complementary to the probe was expected to be available for possible hybridization with the ligand DNA (data not shown). This is a key result, because heavy secondary structure of the sequence to be analyzed can deeply interfere with the probe hybridization as elsewhere reported [Feriotto et al., 2001].

The sensorgram on **Figure 4.4** shows two representative examples of the resulting interaction SPR curves. The curve relative to the pregnant woman bearing a female child represents an example of what we observed in case of injection of samples enriched of female fetal DNA. In this case, the increase of the SPR signal was not observed because, as expected, hybridization did not occur between the PCR product and the SRY-specific probe immobilized on the sensor chip. On the contrary, the curve showing a ligand-analyte interaction were obtained by sample derived from circulating DNA of pregnant woman bearing a male child. In particular, the male curve was obtained from pregnant woman at 36^{th} week of gestation, instead, the female curve was obtained from pregnant woman at 21^{th} week of gestation.
After injection of the unbalance PCR product (**a**), an interaction with the immobilized DNA probe was observed. The following washing with HBS-EP+ buffer (**b**) did not cause a significant decrease of the signal, because the difference between the RUfin (measured after the analyte injection) and RUres (measured after the washing step) was very small, demonstrating that the generated hybridization complexes were quite stable. The results obtained were very encouraging, because they demonstrated a highly specific hybridization.

All the analyzed pregnancies with female fetuses did not produce a significant increase of the SPR-generated signal, because of absence of interactions with the SRY probe (**Table 4.2**). On the contrary, by the injection of the analyzed specimens, deriving from pregnant women expecting a male, clearly detectable signals were produced, showing final RU values higher than the previous group, corresponding to an effective interaction with the probe. A certain degree of heterogeneity was observed depending on the ccffDNA amounts related to gestational age (data not shown). These data suggest that there is a statistically significant difference between RU values obtained by pregnancies with male fetuses and with female fetuses, so this technique resulted suitable to detect the fetal gender.

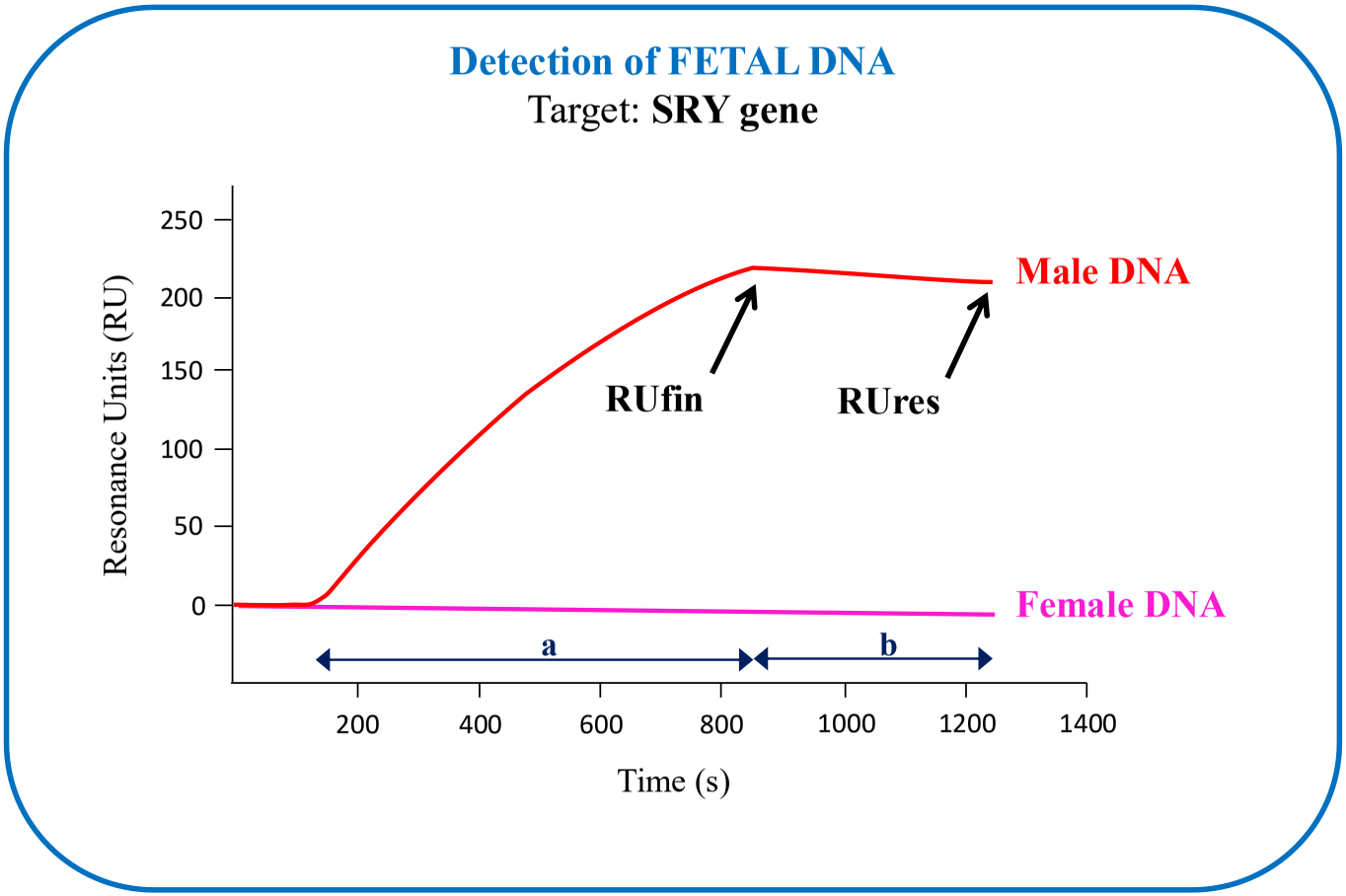


Figure 4.4. Fetal sex discrimination using Biacore™ X100 system. Sensorgrams obtained by injection on the immobilized SRY probe of unbalanced SRY-SB PCR products obtained by male and female samples of circulating DNA extracted from plasma of pregnant woman. a, sample injection for 12 min. b, washing step with HBS-EP+ buffer. RUfin (final resonance units) and RUres (residual resonance units) were measured after the sample injection and the washing step, respectively. The assay was performed by using the Biacore™ X100 instrument, at 25°C and 5 µL/min flow rate; the running buffer was HBS-EP+; the results were analyzed by the Biacore™ X100 Evaluation Software [Modified from: Breveglieri et al., 2016].

# sample	Gestational weeks	Result at birth	Result by Biacore™ analysis after double SRY pre-amplification
1	37	M	M
2	36	M	M
3	31	M	M
4	30	F	F
5	25	M	M
6	24	F	F
7	21	F	F
8	17	F	F
9	17	M	M
10	17	F	F
11	16	F	F
12	16	M	M
13	16	M	M
14	16	M	M
15	15	M	M
16	14	M	M
17	14	M	M
18	11	M	M
19	10	M	M
20	10	M	M
21	9	M	M
22	7	M	M
23	7	F	F
24	7	F	F
25	6	M	M
26	6	F	F

Table 4.2. Biacore™ X100 analysis of circulating DNAs obtained from 26 pregnant women at different gestational weeks. The result was obtained after injection on an immobilized SRY probe of double pre-amplified unbalanced SRY-SB PCR products. For each sample the gestational weeks, the Biacore™ X100 outcome and the actual fetal sex are indicated. M, male. F, female [Modified from: Breveglieri et al., 2016].

Samples at early gestational age, such as #22 specimen, not detectable by qRT-PCR, were able to generate a positive SPR signal using this strategy, demonstrating that this approach, based on pre-amplification of PCR products injected onto sensor chip flow cells, permitted to identify the fetal sex with high accurancy until to the 7th gestational week.
For samples at the 6th week of gestation, such as #25, the same approach is not enough, in fact a double pre-amplification was performed, in order to increase the amount of the fetal DNA for the analysis. The results demonstrated that this second approach could solve uncertain outcomes and increase the possibility to assess fetal sex from specimens collected from pregnant women at the 6th gestational week (**Table 4.2**).

In conclusion, despite the fact that the number of cases analyzed was low, the data strongly suggest the Biacore^{TM} X100 allows to detect male fetal DNA even in earlier gestation periods compared to the qRT-PCR technique, so, it was found to be an efficient fetal sex pre-natal diagnosis method.

These previously published results discussed in this section are the basis of the future developments, which will be presented in this thesis.

4.1.2. Fetal sex pre-natal diagnosis from circulating ccffDNA: further studies

Despite the excellent results obtained using SPR-based Biacore^{TM} X100, the limitation of the technique is that it is possible to detect the fetal sex up to the 7th gestational week. Furthermore, for detecting the gender at the 6th gestational week a double pre-amplification, before performing the unbalanced PCR reaction, was required, in order to increase the amount of template fetal sequences. In fact, the low amount of ccffDNA, relative to circulating maternal DNA, is a crucial issue.
Consequently, in order to correctly determinate the fetal sex for gestational weeks previous to the 7th, and prevent the sample further handling, a technology with a higher sensitivity and specificity is required: the digital droplet PCR (ddPCR).

4.1.2.1 Experimental strategy

The experimental strategy, displayed in **Figure 4.5**, has the aim to develop methods of non-invasive pre-natal diagnosis for the fetal sex determination. 18 mL of peripheral blood samples were collected from pregnant women (**Figure 4.5.A**); plasma aliquots are immediately prepared and stored at -80°C (**Figure 4.5.B**) until the extraction of total circulating cfDNA (**Figure 4.5.C**). Subsequently, the circulating DNA was amplified in

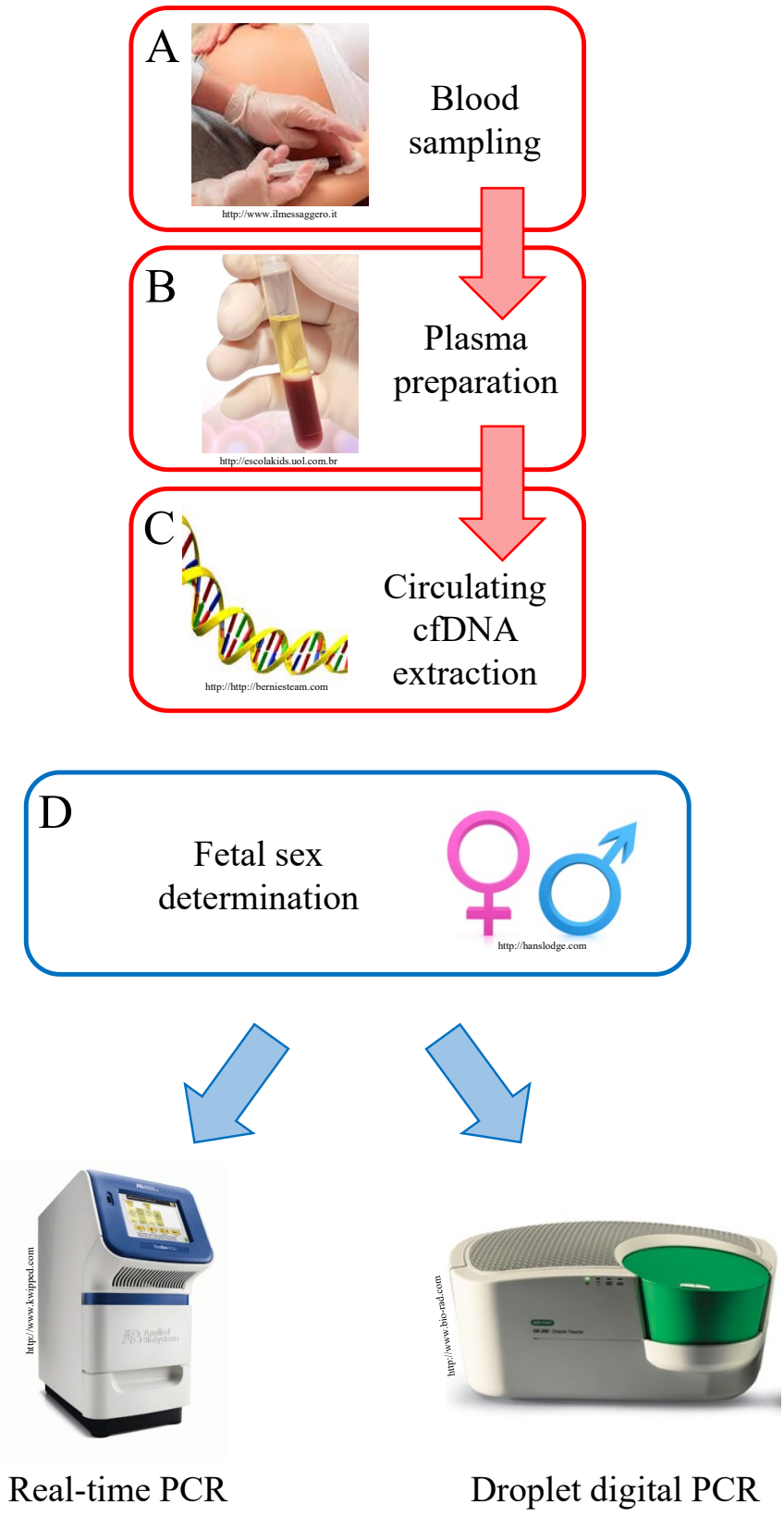


Figure 4.5. Experimental strategy for the development of non-invasive pre-natal sex diagnosis methods. After collecting peripheral blood samples for pregnant women (A), plasma aliquots are immediately prepared and stored at -80°C (B), until the extraction of the total circulating cfDNA (C). Then, the fetal sex is determined (D) following two different approaches: qRT-PCR technique or ddPCR advanced technology.

order to determine the fetal sex (**Figure 4.5.D**). The fetal gender detection has been developed using two different approach: qRT-PCR and ddPCR.

4.1.2.2 Samples collection and stratification

With the aim to develop a non-invasive pre-natal diagnosis method, 139 samples, of whole peripheral blood from pregnant women, are collected, after informed consent. This was performed in collaboration with the *Laboratory of Chemical and Clinical Analysis and Microbiology* of *S. Anna* University Hospital (Cona, Ferrara), directed by Dr. Giovanni Guerra. In particular, Dr. Patrizia Pellegatti has been responsible for addressing pregnant women and for promptly carrying collected blood samples to the laboratory. A progressive number was assigned to each specimen, to keep the donors anonymous.

Within a few hours from blood sampling, plasma was prepared, and stored in single-use aliquots at -80°C. Lo et al. demonstrated that fetal DNA concentration was very similar in maternal plasma and serum but in serum there is a larger maternal DNA contribution causing a less robust fetal DNA detection [Lo et al., 1998]. For this reason, plasma is the source of election for the best recovery of ccffDNA.

So a biobank was established including all plasma samples obtained by pregnant women, which show a wide variability in terms of gestational weeks.

The **Table 4.3** lists the 139 plasma samples: for each specimen the assigned progressive number, the gestational week and the actual sex, result at birth, are indicated. For same samples the actual fetal sex was unknown (*u.k*), due to a miscarriage or no feedback from the mother after birth.

As previously underlined, the samples show a wide variability in terms of gestational age, from 39 to 4 weeks. So, in order to better discriminate the late from the early gestational ages, the samples were arbitrarily divided in four gestational ranges: from 39 to 30 (in pink), from 29 to 20 (in yellow), from 19 to 10 (in green) and from 9 to 4 (in light blue) gestational weeks, respectively (**Table 4.3**).

The same partition, in terms of gestational age, was found in **Figure 4.6**, whose show better the sample stratification. The specimens are equally spread in the four gestational ranges, as indicated by the percentages and the sample number.

# sample	Gestational weeks	Result at birth
1	39	M
2	39	M
3	39	M
4	38	F
5	38	M
6	37	M
7	37	M
8	37	M
9	37	F
10	37	F
11	36	M
12	36	M
13	36	M
14	35	M
15	35	M
16	35	M
17	35	M
18	34	M
19	33	F
20	33	F
21	33	M
22	33	M
23	33	M
24	33	F
25	32	M
26	32	M
27	32	M
28	31	M
29	31	M
30	30	M
31	30	M
32	30	F
33	30	M
34	30	M
35	29	M
36	29	F
37	28	F
38	28	M
39	28	M
40	27	F
41	26	F
42	26	F
43	25	M
44	25	M
45	25	M
46	24	F
47	24	F

# sample	Gestational weeks	Result at birth
48	23	F
49 B	23	*u.k.*
50	23	M
51	22	M
52	22	F
53	21	F
54	21	F
55	21	M
56	21	M
57	20	M
58	20	M
59 B	20	F
60	20	F
61	19	M
62	18	M
63	18	M
64	18	M
65	17	M
66	17	F
67	17	F
68	17	M
69	17	M
70	16	M
71	16	M
72	16	M
73	16	F
74	16	M
75	15	F
76	15	M
77	15	F
78	15	F
79	14	M
80	14	M
81	14	*u.k.*
82	14	F
83	13	M
84	13	M
85 B	12	M
86	12	M
87	11	M
88	11	*u.k.*
89	11	F
90	11	M
91	11	M
92	10	F
93	10	M
94	10	M

# sample	Gestational weeks	Result at birth
95	10	F
96	10	M
97	10	M
98	10	F
99	10	M
100	10	M
101	9.5	M
102	9	M
103	9	M
104	9	F
49 A	8	*u.k.*
105	8	*u.k.*
106	8	M
107	8	M
108	8	M
109	8	M
110	8	M
111	8	F
112	8	*u.k.*
113	8	*u.k.*
114	8	F
115	8	M
116	7	M
117	7	M
118	7	F
119	7	F
120	7	F
85 A	7	M
121	7	M
122	7	M
123	7	M
124	7	M
125	7	F
126	7	M
127	6	F
128	6	M
129	6	*u.k.*
130	6	M
59 A	5	F
131	5	F
132	5	M
133	5	*u.k.*
134	5	F
135	4.5	M
136	4	*u.k.*

Table 4.3. List of 139 blood samples collected from pregnant women. For each sample the assigned serial number, the gestational age and the actual fetal sex are reported. *u.k.*, unknown fetal sex. The different gestational ranges are indicated with different colour: from 39 to 30 in pink, from 29 to 20 in yellow, from 19 to 10 in green and from 9 to 4 gestational weeks in light blue. The samples marked A and B refer to the same pregnant woman at different gestational ages.

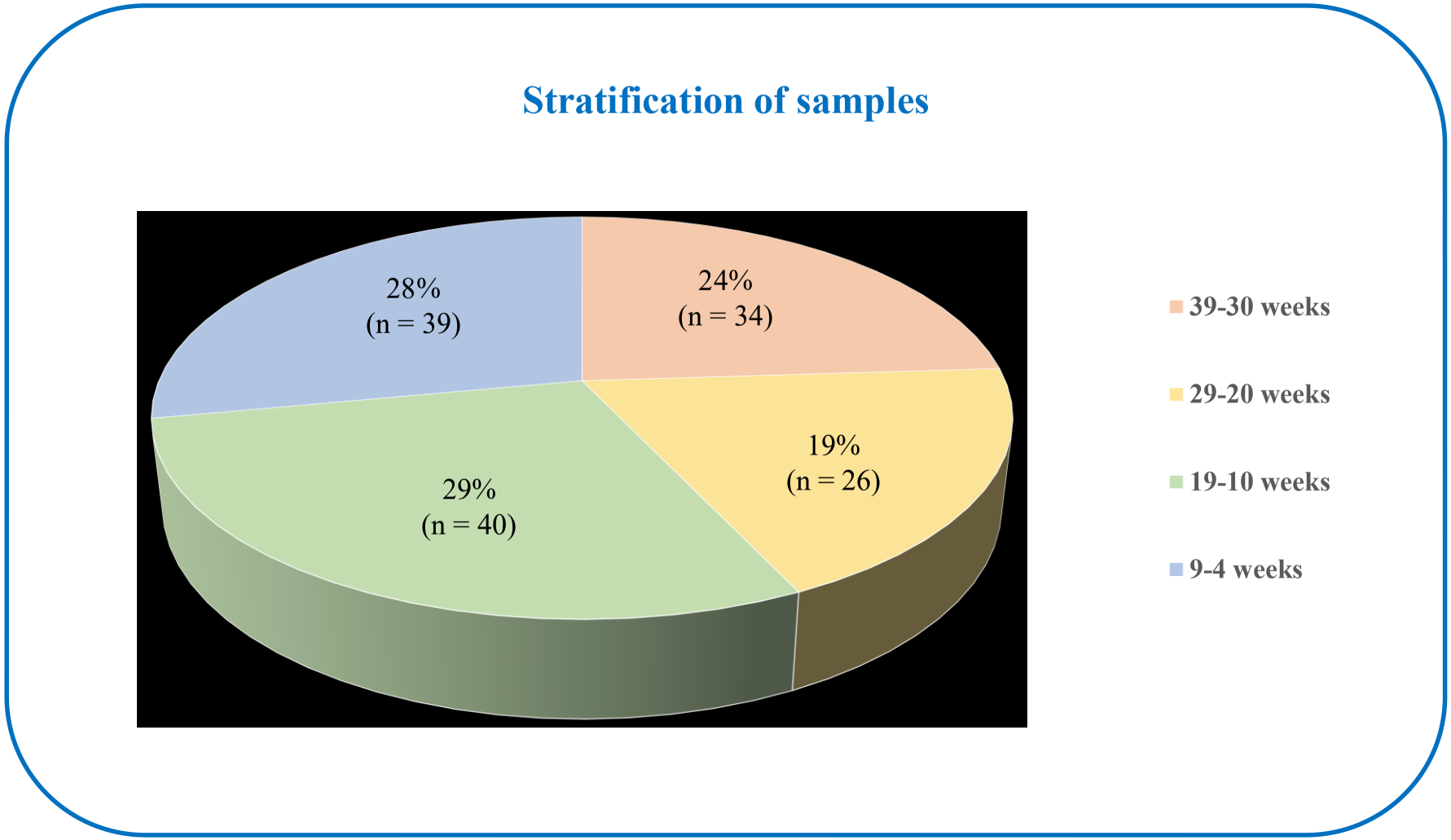


Figure 4.6. Stratification of the 139 blood samples from pregnant women based on gestational age. The samples are equally spread in 4 gestational ranges: 34 samples (24%) belong to the range 39-30 weeks (in pink), 26 samples (19%) belong to the range 29-20 weeks (in yellow), 40 samples (29%) belong to 19-10 weeks' range (in green) and 39 samples (28%) belong to the range 9-4 weeks (in light blue).

4.1.2.3. Detection of fetal Y chromosome sequences by qRT-PCR: an extension study

As previously underlined, the most common used molecular technique employed for detecting male fetus-specific DNA in maternal plasma is represented by qRT-PCR [Lo et al., 1990; Lo et al., 1998; Stanghellini et al., 2006], due to its good specificity and easiness of application [Lo et al., 1998].

In the work discussed in the section of previously published results [Breveglieri et al., 2016], the qRT-PCR was employed for non-invasive fetal sex determination, amplifying a region of the SRY gene. The obtained results showed that diagnosis could be performed by this technique starting from the 9th gestational week.

Considering the low number of samples analyzed in that work [Breveglieri et al., 2016], we firstly decided to extend the number of plasma samples to analyze with the qRT-PCR technique, with the aim to confirm the gestational limit of the molecular technology employed.

Therefore, all the 139 plasma samples were analyzed using qRT-PCR and following the same approach indicated in the previous section. The circulating cfDNA was estracted using the QIAamp® DSP Virus Spin Kit (Qiagen). An SRY assay was used to amplify the fetal male DNA, as the SRY gene is located only on the Y chromosome. Instead, the human β globin gene was used as a reference gene for the quantification of both fetal and maternal DNA, confirming the presence of extracted circulating DNA.

The fetal sex diagnosis was unreliable for all the 139 samples analyzed. The qRT-PCR outcomes are reported in **Figure 4.7**: the samples are divided in the four gestational ranges; for each group the percentages of determined (in blue) and not determined (in red) fetal sex were reported. The failure gender identification seems to be related to the gestational age, in fact with decreasing of gestational weeks, the undetermined percentage increases.

As regard the two highest gestational ranges (Figures **4.7.A** and **4.7.B**) the fetal sex was determined in the 100% of the time. The determined percentage of the samples belonging to the 19-10 gestational weeks is 78%, while that of the samples belonging to the 9-4 gestational weeks is 43%, and just for plasma samples bearing female fetuses.

These results confirmed the data reported in literature pointing out that the qRT-PCR testing is not reliable and accurate for fetal sex diagnosis when performed using blood withdrawn at early gestational period, in particular prior to 7th week of gestation [Devaney et al., 2011; Breveglieri et al., 2016].

The difficulty of qRT-PCR-based methods to identify male fetal sex at early gestational stages is due to (A) absence of double amplification curves for the SRY gene, creating false

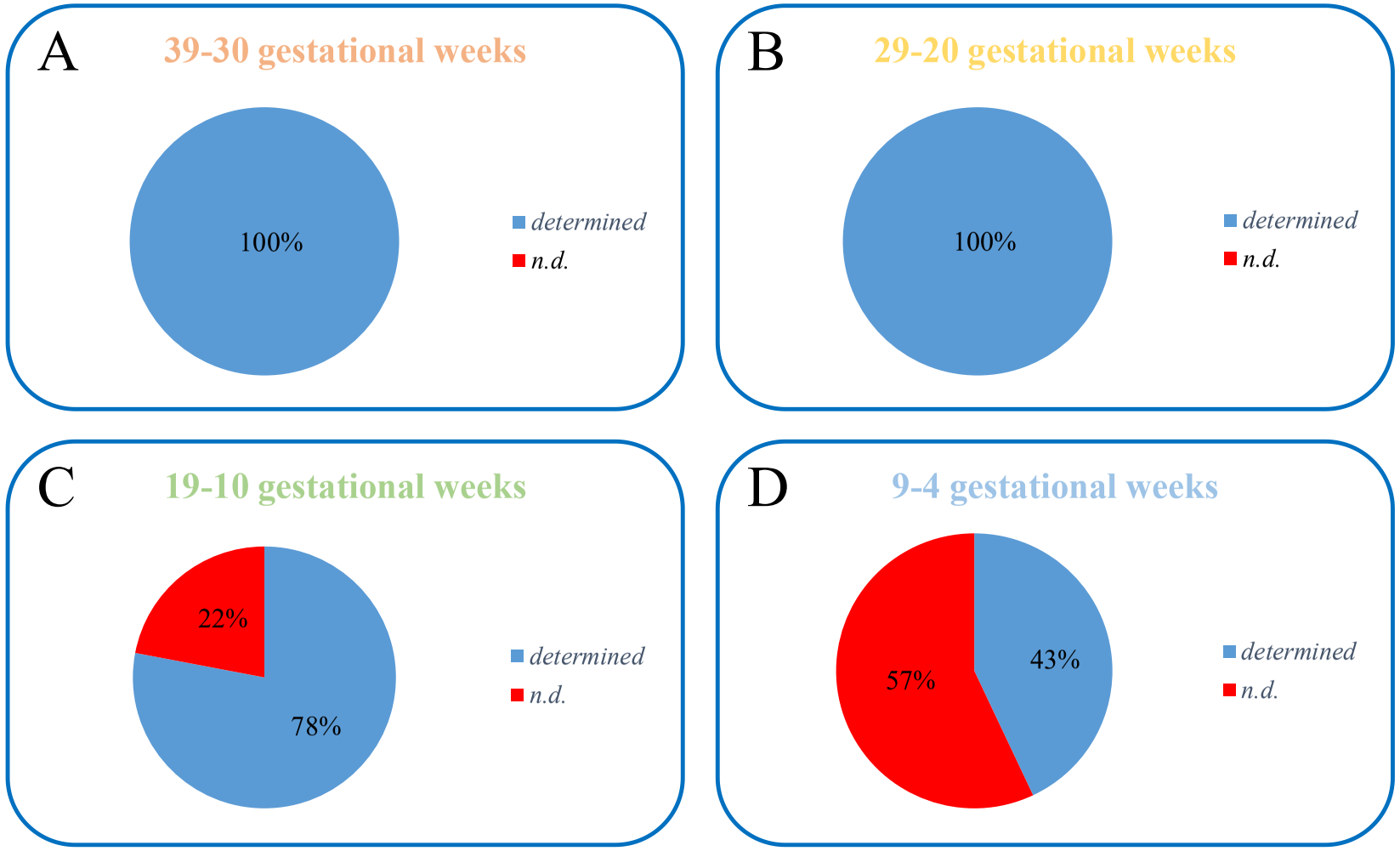


Figure 4.7. Fetal sex outcome distribution by using qRT-PCR. The obtained results, expressed as a percentage, for the fetal sex diagnosis of the 139 analyzed samples, are divided in the 4 gestational ranges: 39-30 (A) (in pink), 29-20 (B) (in yellow), 19-10 (C) (in green) and 9-4 (D) (in light blue) weeks, respectively. The different colours of the charts indicate that the fetal sex was determined (blue) or not determined (*n.d.*) (red). The amplification was performed by using the StepOne™ Real-Time PCR System.

negative (**Figure 4.8.A**); (B) generation of only one amplification curve caused by SRY amplification or contamination, preventing a robust and correct diagnosis (**Figure 4.8.B**); (C) presence of double amplification curves not superimposed with ΔCt>0.5 (**Figure 4.8.C**) [Yuan et al., 2006], generating an unreliable quantification.

Anyway, in all the cases, the described problems are due to the insufficient amount of ccffDNA present in maternal plasma in early gestational weeks (12 weeks or less) [Zhou et al., 2015]. In conclusion, despite the sensitivity and specificity of the qRT-PCR method, this technique has limitations for robust reliable diagnosis of fetal sex when a reduced amount of template is available. Therefore, for earlier prenatal diagnosis of fetal sex, we hypothesized that more sensitive, specific and efficient technologies, such as ddPCR, could be a valid and alternative approach.

The technique allows to amplify a single DNA template from minimally diluted samples, therefore generating amplicons that are exclusively derived from one template and can be detected with different fluorophores or sequencing to discriminate different alleles [Pohl & Shih, 2004; Hudecova, 2015]. For example, Lun et al. used microfluidic digital PCR to quantify the male fetal DNA in first-, second- and third- trimester maternal plasma amplifying two 87-bp amplicons of the ZFX and ZFY loci [Lun et al., 2008].

4.1.2.4. Determination of fetal sex from maternal plasma by ddPCR

Hence, in this part of the study the ddPCR advanced technology was employed in order to detect SRY gene target from ccffDNA at the earliest gestational ages. The ddPCR system partitions nucleic acid samples in thousands of nanoliter-sized water-oil emulsion droplets, permitting the precise quantification of rare target nucleic acids in the sample [Pinheiro et al., 2012]. The massive partitioning is a key aspect of the ddPCR technique, whose, therefore, present several advantages: (A) absolute quantification, (B) high precision and accuracy, (C) reduction in cost due to the nanoliter-range, (D) precious sample preserving, (E) high tolerance to inhibitors [Hindson et al., 2011].

4.1.2.4.1. Set-up of ddPCR conditions

In order to identify the fetal and total circulating DNA, two amplification assays were used in ddPCR, both consisting of two primers (forward and reverse) and a hydrolysis probe each with a fluorophore. The first assay, labeled with the FAM™ fluorophore, is specific for the SRY gene, and is exactly the same assay previously used in qRT-PCR [Breveglieri et al., 2016], applied to ddPCR. Fetal sex was considered male or female depending on whether or not positive FAM™ fluorescence events, corresponding to the SRY gene, were detected.

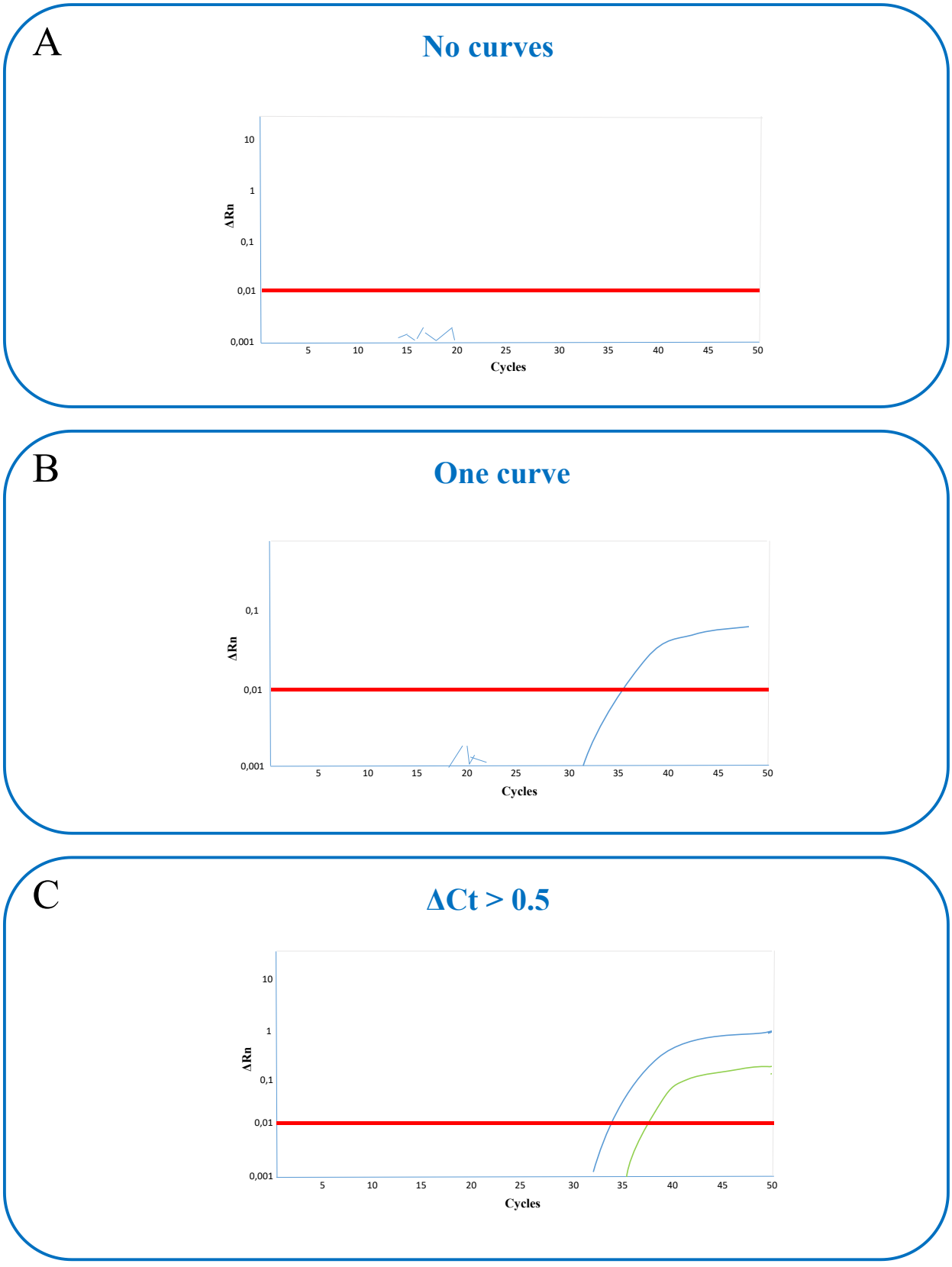


Figure 4.8. qRT-PCR problems related to fetal sex determination at early gestational weeks. The graphs (A, B, C), in which the fluorescence intensity (ΔRn) correlates the amplification cycles, represent 3 clear examples of qRT-PCR technique limitations: (A) absence of both amplification curves refering to duplicate, (B) presence of one of the two duplicate amplification curves, (C) presence of both the two duplicate amplification curves that come up at different amplification cycles (ΔCt>0.5). The threshold line is drawn in red. The amplification was performed by using the StepOne™ Real-Time PCR System.

The second assays, labeled with the HEX™ fluorophore, specific for the EIF2C1 gene, is located on the chromosome 1 coding for an argonaute protein and considered as reference gene in order to confirm the actual presence of circulating DNA in the absence of positive events for SRY.

Since the assays are marked with two different fluorophores, both amplifications were performed in the same reaction, with the advantage to increase the precision of the analysis and using half of the precious starting material.

To test the correct performance of the two assays and to identify the optimum amplification conditions, a preliminary set-up experiment was performed (**Figures 4.9** and **4.10**). In this experiment, genomic DNAs and circulating DNAs from male and female adults (obtained from three different subjects for each category) were analyzed.

In **Figure 4.9** ddPCR representative graphs are dyspalyed: the upper graphs (**Figures 4.9.A-4.9.D**) show the events in which the SRY amplification reaction gene occurred correlating the FAM™ fluorescence intensity with the number of observed events (blue dots); while the lower graphs (**Figures 4.9.E-4.9.H**) identify the events in which the EIF2C1 gene was amplified relative to the HEX™ fluorescence intensity with the number of events (green dots). Male genomic DNA (**Figure 4.9.A** and **4.9.E**) was considered as a positive control showing positive events for both the SRY gene and the EIF2C1 gene, as expected, and with a predicted ratio of 1:2, since the SRY gene is present in a single copy relative to the duplicate EIF2C1 gene. Female genomic DNA (**Figure 4.9.C** and **4.9.E.G**) was considered the negative control reporting no positive events for the SRY gene (**Figure 4.9.C**), and only negative events (black dots), since female subject does not possess this gene. The detected positive events relative to the HEX™ fluorescence intensity (**Figure 4.9.G**) confirmed the template presence in the reaction. Finally, the male (**Figure 4.9.B** and **4.9.F**) and female (**Figure 4.9.D** and **4.9.H**) circulating DNAs were used as a further positive and negative control respectively, since they are more similar in terms of fragmentation to ccffDNA extracted from maternal plasma than genomic DNA, obtaining, also in these cases, the expected results.

Figure 4.10 shows the absolute quantification obtained, applying the Poisson statistic, using the described samples (three specimen for each category) and the SRY target (blue triangle) and EIF2C1 reference (green square) genes.

The SRY gene for male genomic DNA was calculated to be 1387.68 copies/µL of plasma, whereas the EIF2C1 content was 2884.15 copies/µL of plasma with a ratio 1:2, due to the fact that the EIF2C1 gene is present in duplicate, while the SRY gene, being present only on the male sexual Y chromosome, is present as a single copy. This ratio is not maintained in

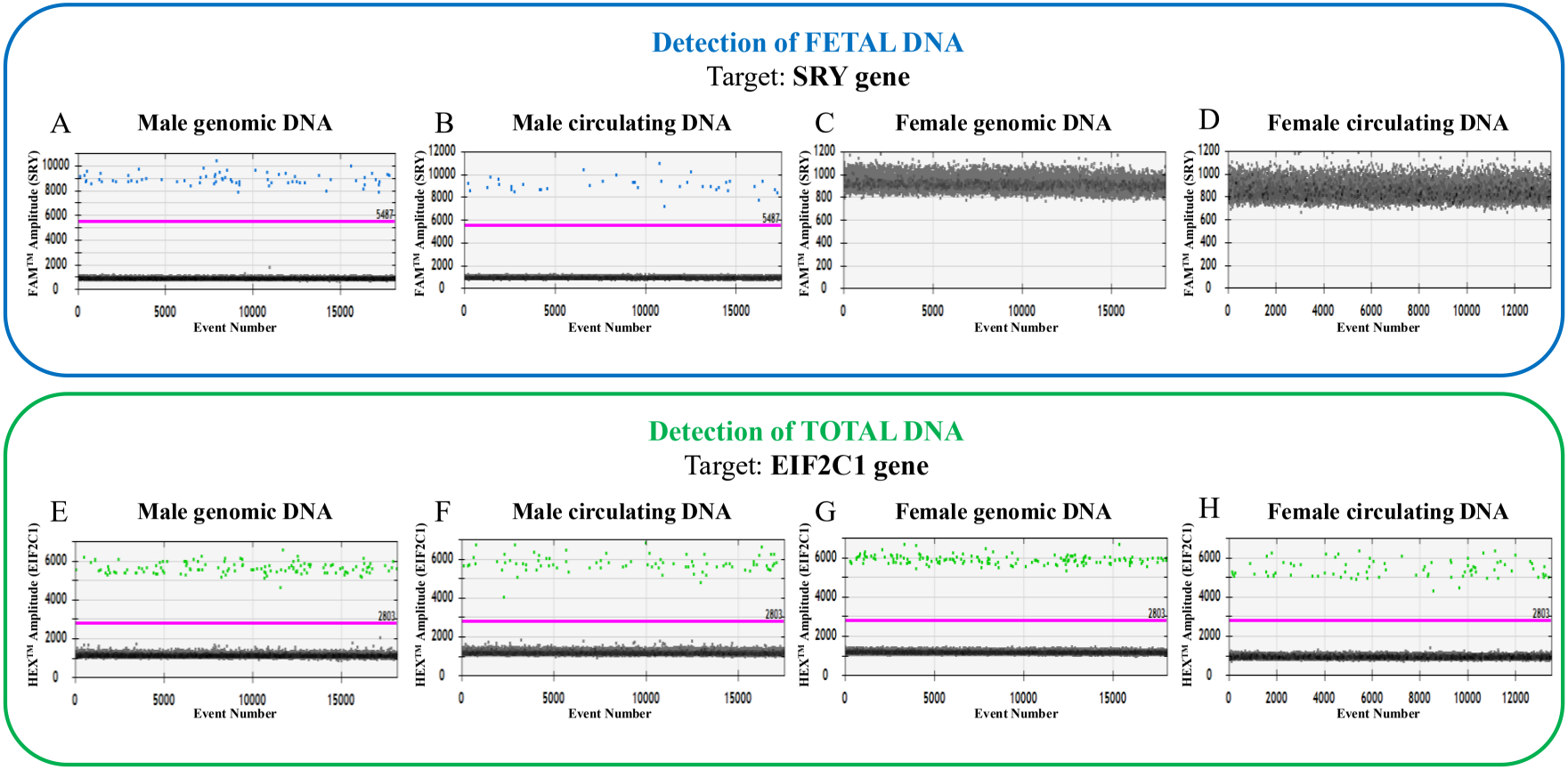


Figure 4.9. ddPCR representative graphs for male and female circulating and genomic DNA samples in order to set-up the SRY assay. With the aim to set-up the ddPCR experimental conditions, male genomic (A,E) and circulating (B,F), and female genomic (C,G) and circulating (D,H) DNA samples were analyzed in ddPCR for SRY (in blue) and EIF2C1 (in green) gene targets, for the detection of fetal and total DNA, respectively. For all samples, the upper graphs (A, B, C, D) correlate the FAM™ fluorescence intensity, corresponding to the SRY gene (blue dots), to the number of positive events; the lower graphs (E, F, G, H) report the HEX™ fluorescence intensity, relative to the EIF2C1 reference gene (green dots), to the number of positive events. Black dots indicate negative events (no amplification events). The threshold lines are coloured in fuchsia.

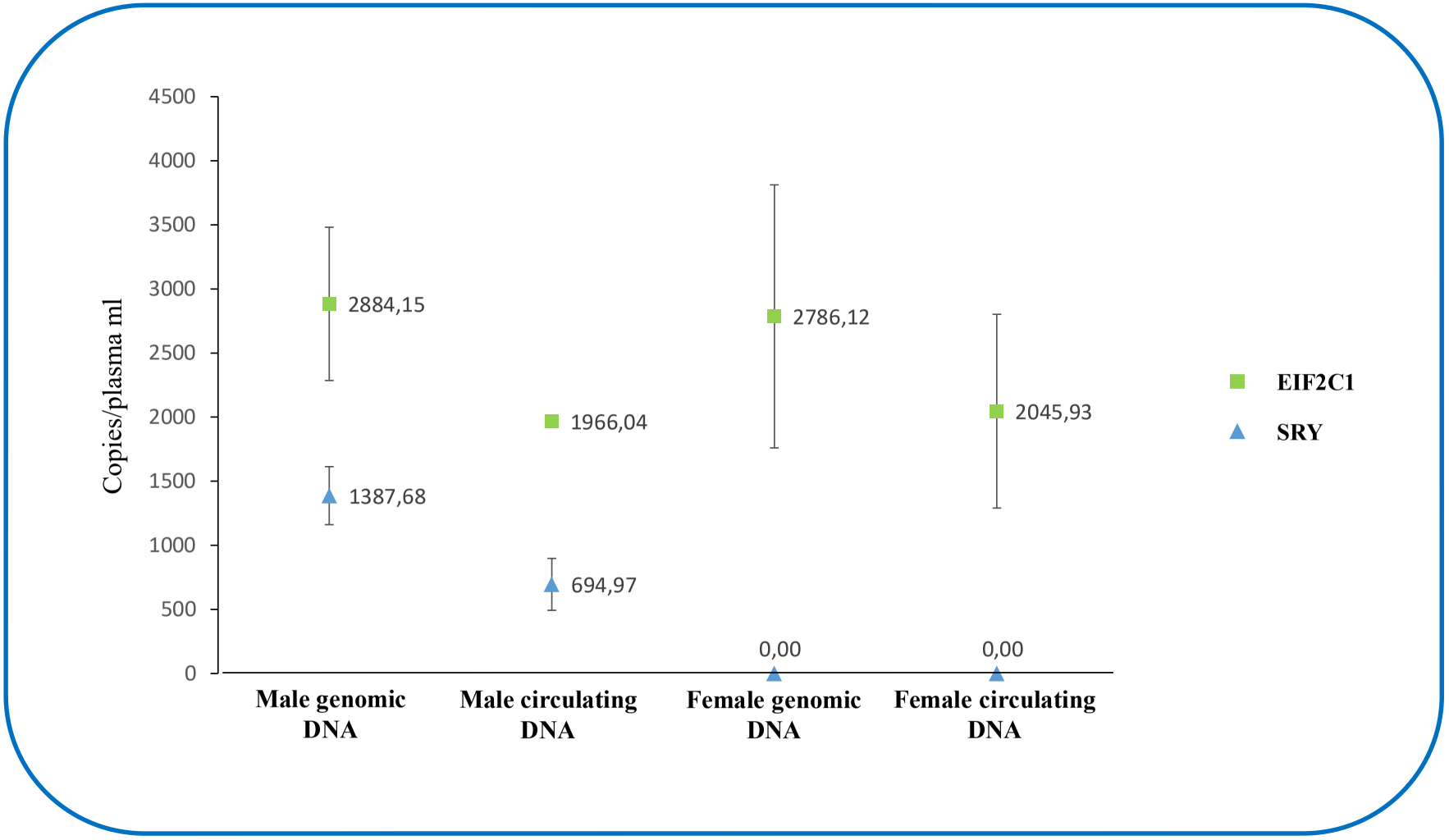


Figure 4.10. ddPCR analysis on male and female circulating and genomic DNA samples in order to set-up the SRY assay. The concentration average±SD (from three different subjects for each category) of SRY (blue triangles) and EIF2C1 (green squares) was expressed in copies/plasma mL, obtained by QuantaSoft software, based on Poisson's statistics [Modified from: D'Aversa et al., 2018].

the circulating DNA of an adult male. In fact, EIF2C1 quantification of 1966.04 copies/μL of plasma and 694.97 copies/μL of plasma for SRY were observed. The apparent inability to maintain the 1:2 ratio is caused by the quality of the sample, which, being circulating DNA, is extremely fragmented, with small size and easy degradation [Drury et al., 2016].
On the other hand, in the case of female genomic and circulating DNAs, SRY is not present and its concentration is equal to 0, demonstrating that sex can be discriminated in these experimental conditions. Furthermore, the EIF2C1 quantification is similar to the corresponding male samples, confirming the different nature and variability of genomic and circulating DNAs.
In any case, we would to point out that, as previously discussed, for the diagnostic identification of fetal sex, it is not essential to obtain the same amount of circulating DNA present in maternal plasma, but it is required only the confirmation of its presence or absence.

4.1.2.4.2. ddPCR analysis of plasma samples from pregnant women at early gestational ages

After validating ddPCR experimental conditions, 29 maternal plasma samples at early gestational stages (12-4.5 weeks), previously analyzed by qRT-PCR (**Table 4.4**), with negative outcome for most of them, were chosen and quantified for SRY gene target using ddPCR in order to identify the fetal sex.
Figure 4.11 shows the representative graphs obtained from maternal plasma samples at the 7^{th} gestational week carrying male (**Figures 4.11.A** and **4.11.D**) or female (**Figures 4.11.C** and **4.11.F**) fetus, respectively, and at the 4.5^{th} week having male fetus (**Figure 4.11.B** and **4.11.E**).
The upper graphs (**Figures 4.11.A-4.11.C**) show the events in which the SRY amplification reaction gene occurred correlating the FAM™ fluorescence intensity with the number of observed events (blue dots); while the lower graphs (**Figures 4.11.D-4.11.F**) identify the events in which the EIF2C1gene was amplified relative to the HEX™ fluorescence intensity with the number of events (green dots).
In **Figures 4.11.A** and **4.11.B**, the limited number of positive events related to the SRY gene observed for samples #122 and #135 (7^{th} and 4.5^{th} gestational week, respectively) are still sufficient to perform a robust and reliable analysis being more than three. These data are due to the decrease in the concentration of fetal DNA circulating in maternal plasma at decreasing gestational time [Zhou et al., 2015]. While, for the sample #125 at 7^{th} week carrying a female fetus (**Figure 4.11.C**), no positive events of SRY amplification have been observed reporting only negative events (black dots). In the lower charts (**Figures 4.11.D-**

# sample	Gestational weeks	Result at birth	Result by qRT-PCR	Result by ddPCR
85 B	12	M	*n.d.*	M
86	12	M	*n.d.*	M
91	11	M	*n.d.*	M
96	10	M	*n.d.*	M
98	10	F	F	F
99	10	M	M	M
100	10	M	*n.d.*	M
101	9.5	M	*n.d.*	M
104	9	F	F	F
105	8	F	F	F
106	8	M	*n.d.*	M
107	8	M	*n.d.*	M
108	8	M	*n.d.*	M
109	8	M	*n.d.*	M
110	8	M	*n.d.*	M
114	8	F	F	F
115	8	M	M	M
116	7	M	*n.d.*	M
117	7	M	*n.d.*	M
85 A	7	M	*n.d.*	M
121	7	M	*n.d.*	M
122	7	M	*n.d.*	M
124	7	M	*n.d.*	M
125	7	F	F	F
126	7	M	*n.d.*	M
128	6	M	*n.d.*	M
130	6	M	*n.d.*	M
132	5	M	*n.d.*	M
134	5	F	F	F
135	4.5	M	*n.d.*	M

Table 4.4. Fetal sex determination of circulating cfDNAs by qRT-PCR and ddPCR. The actual fetal sex and the diagnostic outcome after fetal sex determination by qRT-PCR and ddPCR were determined for ccfDNAs obtained from 29 pregnant women at different gestational ages. The samples 85 A and 85 B were obtained from the same pregnant woman at two different gestational weeks, as reported. M, male. F, female. *n.d.*, not determinable [Modified from: D'Aversa et al., 2018].

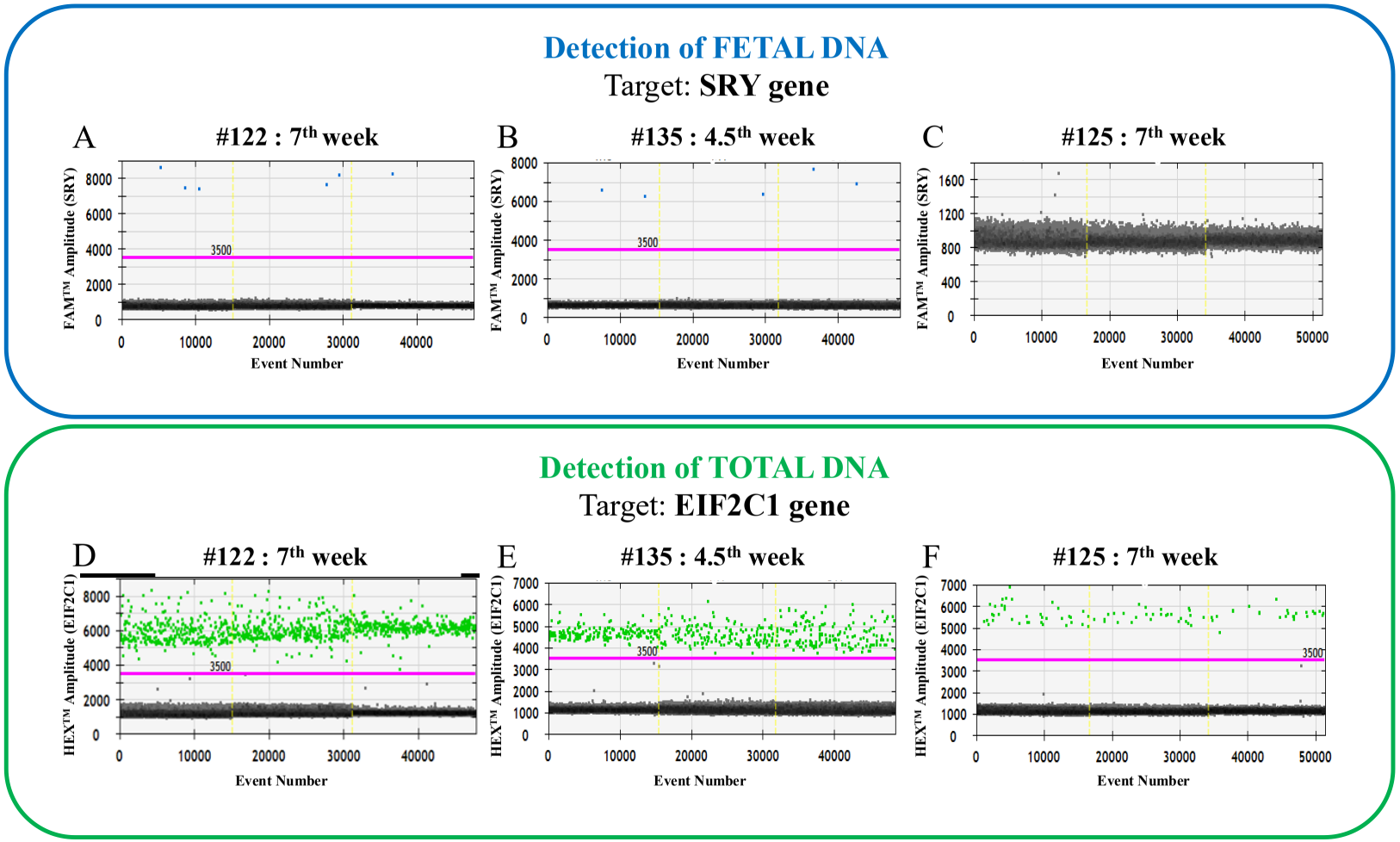


Figure 4.11. Representative graphs for circulating DNA samples at different gestational ages analyzed by ddPCR. Three circulating DNA samples, extracted from maternal plasma at different gestational weeks and carrying male (A and D, #122: 7th week; B and E, #135: 4.5th week) of female fetus (C and F, #125: 7th week), were analyzed in ddPCR for SRY and EIF2C1 gene targets, for the detection of fetal and total DNA, respectively. For all three samples, the upper graphs (A, B, C) correlate the FAM™ fluorescence intensity, coresponding to the SRY gene (blue dots), to the number of positive events; the lower graphs (D, E, F) report the HEX™ fluorescence intensity, relative to the EIF2C1 reference gene (green dots), to the number of positive events. Black dots indicate negative events (no amplification events). The threshold lines are coloured in fuchsia [Modified from: D'Aversa et al., 2018].

4.11.F), the positive events, relative to the HEX™ fluorescence intensity (green dots) correlated to the EIF2C1 gene amplification, were detected for all three samples and were significantly than the SRY gene quantification confirming the template presence in the reactions.

Table 4.4 summarizes the results of fetal sex determination obtained by ddPCR for the 29 maternal plots analyzed, in comparison with those obtained by qRT-PCR. For all the samples analyzed in ddPCR, it was possible to determine and confirm the birth sex, also in the case of pregnant carrying a male fetus at earlier gestation weeks, not determine in qRT-PCR.

For the first time, we have demonstrated that ddPCR technology can be used for non-invasive pre-natal diagnosis of Y chromosome at early gestation ages (prior to 7th week). In particular, all maternal plasma samples were determined correctly for SRY gene presence using ddPCR even at earliest gestational age (4.5 weeks), achieving 100% accuracy. To our knowledge, previous to this work, the earliest reliable ccffDNA detection time point was 7th week in pregnancy using Biacore™ system [Breveglieri et al., 2016], but no studies are reported for ddPCR at so early week pregnancy.

All these interesting results have been recently published in *Molecular Medicine* [D'Aversa et al., 2018].

In conclusion, the ddPCR is a robust, sensitive, efficient and reliable technology for the earliest possible fetal sex determination from maternal plasma.

As far as other biomedical applications, considering ddPCR capability [Hindson et al., 2011], demonstrated in this study, it should be verified when non-invasive pre-natal diagnosis is applied to the detection of monogenic diseases, such as β thalassemia, in the carrier fetus.

About this, the detection of β thalassemia point mutations, both maternally and paternally inherited, from circulating cfDNA by ddPCR, is another issue of the present thesis (**Section 4.2.4**). Since, the great relevance of β thalassemia diagnosis in gestational period, the study regarding the fetal sex determination was a good starting point for the non-invasive pre-natal diagnosis of monogenic diseases.

In addition, the technique could be extend its applicability to other very important field of biomedicine, such as liquid biopsies in tumor patients (where the presence of tumor-free circulating DNA content is similar to fetal circulating DNA) [Wu et al., 2017].

4.2. β thalassemia diagnosis of point mutations

As previously underlined, the identification of the pathogenic β thalassemia alteration could be of great importance in view of a timely start of a maintenance therapy, and of a targeted and personalized therapy. In fact, the specific early intervention for a particular mutation could change the quality of life of many β thalassemic patients. The diagnosis can be employed both in post-natal and pre-natal ages. In the latter case, the possibility to known the mutation possibly transmittable to offspring, in a very early stage, could be useful for carrier parents in view of a targeted timely therapeutic plan.

Various molecular techniques for post-natal and pre-natal detection of point mutations have been developed, such as ARMS-PCR and nested-PCR, but they all are labour-intensive and technically demanding [Kho et al., 2013].

For this reason, the second part of this work was aimed to detect β thalassemic point mutations, in post-natal and pre-natal ages, developing diagnostic approaches based on simple, sensitive, rapid and cost-effective molecular techniques, such as Biacore^{TM} X100, genotyping assays and ddPCR.

The β thalassemic mutations selected for the analysis were $\beta^0$39, β^+IVSI-110, β^+IVSI-6 and β^0IVSI-1 because they are the most common in the Mediterranean area [Cao & Galanello, 2010].

4.2.1. Post-natal diagnosis of β thalassemia mutations by using Biacore^{TM} X100

Optical biosensors based on SPR are widely used to study in real-time and in label-free mode bio-molecular interactions, including those allowing the identification of single point mutations responsible of genetic diseases [Breveglieri et al., 2018]. After ligand immobilization on the sensor chip surface and injection of analytes, their possible interaction produces an increment in mass resulting in a change of SPR angle, which is monitored in real-time as resonance signal in function of time in a sensorgram [Gambari, 2001].

In spite of the large and increasing number of SPR-based bio-molecular interaction studies, reports on the identification of single point mutations causative of genetic diseases are limited [Feriotto et al., 1999; Feriotto et al., 2001; Breveglieri et al., 2016]. Studies of single point mutation detection based on the format in which an allele-specific probe is immobilized on sensor chip and a PCR product is injected, are not available. To the best of our knowledge, the only paper on a similar strategy reported a label-free efficient and accurate detection of cystic fibrosis mutations using an azimuthally rotated GC-SPR platform [Meneghello et al., 2014].

On this background, in this part of the study, the attention was focused on the development of a new efficient and high sensitive post-natal diagnostic strategy based on the real-time analysis of bio-specific interactions by affinity biosensors based on SPR. In particular, this technology has been applied to the molecular diagnosis of β thalassemia, through the detection of the four most common point mutations in the Mediterranean area: $\beta^0$39, β^+IVSI-110, β^+IVSI-6 and β^0IVSI-1 [Cao & Galanello, 2010]. The Biacore™ X100 instrument (GE Healthcare) was chosen to carry out the hybridization analyses, as SPR-based system had already demonstrated to be sensitive enough to efficiently detect point mutations [Wang et al., 1997; Feriotto et al., 1999].

The advantages of the Biacore™ X100 instrument are the following: (1) simple handling, (2) availability of validated buffers injection, (3) real-time analysis, (4) high reproducibility and (5) low costs related to the fact that the sensor chip can be reused up to 80-100 times. All these characteristics make the instrument a promising candidate for our analysis.

4.2.1.1. Experimental strategy

The experiments aimed to develop a detection approach for point mutations affecting the β globin gene, were carried out using Biacore™ X100, an affinity biosensor where the signal transduction is based on the phenomenon of SPR [Karlsson, 2004]. The latter allows to monitor in real-time, as a result of the refractive index change near the surface of the sensor chip, the bio-specific interactions between an immobilized ligand and an analyte that flows in solution [Torreri et al., 2005].

The detection method was based on DNA-DNA interactions, where a pair of oligonucleotide probes was required for each mutation, one complementary to the normal DNA sequence and the other carrying the mutation, in order to discriminate the normal allele from the mutated one after hybridization with the target sequence.

The experimental strategy, shown in **Figure 4.12**, was divided into three steps:

- Immobilization of a pair of biotinylated oligonuclotide probes on different flow cells of streptavidin-coated (SA) sensor chips (**Figure 4.12.A**). They were single-stranded DNA oligonucleotides complementary to the normal or the mutated target, and their nucleotide sequences differed only for the position of the mutation.
- Validation of probes, evaluating the binding with normal and mutated exactly

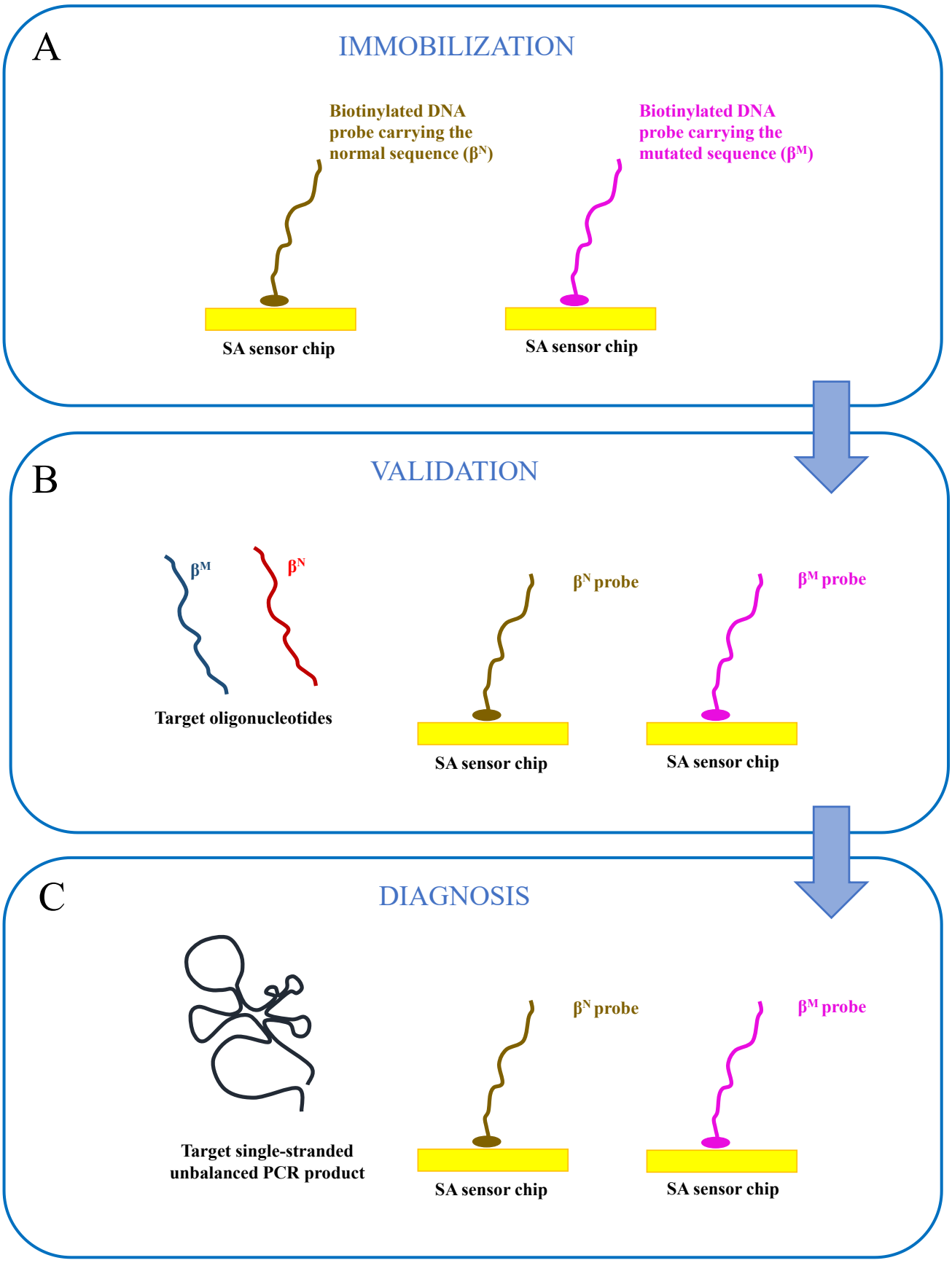


Figure 4.12. Experimental stategy for SPR-based Biacore™ X100 to detect β thalassemia point mutations. (A) Immobilization of two biotinylated oligonucleotide probes, one carrying the normal sequence (β^N, in brown) and the other containing the mutation (β^M, in pink), on the surface of different streptavidin-coated (SA) sensor chips. (B) Probe validation by injection of target complementary normal (β^N, in red) and mutated (β^M, in blue) oligonucleotides. (C) Diagnostic approach by injection of target unbalanced single-stranded PCR products obtained from genomic DNA under investigation.

– complementary oligonucleotides, in order to assess their actual capability of specific and stable recognition of the target sequences (**Figure 4.12.B**).
– Diagnostic approach, analyzing the interaction between the immobilized probes and single-stranded PCR products obtained from genomic DNA under investigation (**Figure 4.12.C**).

The latter experimental step was what differs from the interaction format, extremely expensive, described by Feriotto et al., where on the contrary the target PCR products had been immobilized on the sensor chip flow cells, and the detection probes had been injected over the surface for the interaction [Feriotto et al., 2004].

Specific PCR reactions were performed for each mutation to be analyzed, after designing a primer pair able to amplify a rather short sequence of the β globin gene, reducing the possible target folding and the consequent interference with the interaction. For the development of the diagnostic approach, genomic DNA of different genotypes for every mutation were employed, obtained from normal healthy subjects (β^N/β^N), heterozygous β thalassemia healthy carriers (β^N/β^M), and homozygous β thalassemia patients (β^M/β^M), respectively, whose β globin gene had been previously characterized by DNA sequencing.

4.2.1.2. Probes immobilization on the sensor chip surface

The first step was to design two DNA biotinylated probes, one carrying the normal sequence and the other carrying the mutated one, for each mutation under investigation: $\beta^0$39, β^+IVSI-110, β^+IVSI-6 and β^0IVSI-1.

The nucleotide sequences of the probe pairs were chosen according to those used in previous studies by Feriotto et al. [Feriotto et al., 2004], also aimed at the detection of β thalassemia point mutations. Together with the nucleotide sequence, also the probes length is a crucial issue in terms of sensitivity and specificity of the interaction target recognition and discrimination, especially when a single mismatch has to be detected [Feriotto et al., 2004]. In fact, single-stranded PCR products can give secondary structures in the experimental conditions employed for the hybridization.

Too short oligonucleotide probes can fail in the target binding, while too long probes cannot distinguish the normal and the mutated sequences. Accordingly, 12mer probes were employed for the detection of $\beta^0$39 and β^+IVSI-110 mutations, and 11mer probes for the β^+IVSI-6 and β^0IVSI-1 mutations.

The probes immobilization was performed on SA sensor chips, carrying a dextran matrix where streptavidin has been covalently attached: in this way the high biotin-streptavidin affinity [Lee et al., 2005] allows the capture of biotinylated probes. This immobilization strategy is very fast and neither required other reagents, nor involves chemical reactions. The only requirement is the probe labeled with biotin, currently commercially available.

Only one of the two available flow cells for the probe immobilization was employed, so as to use the empty one as no-ligand negative control for analyte-ligand hybridization.

In **Figure 4.13** the sensorgrams obtained after the immobilization of normal (β^N, in brown) (**Figure 4.13.A**) and mutated (β^M, in pink) (**Figure 4.13.B**) probes for $\beta^0$39 mutation, as an example, are shown. The curve slopes for the other mutations (data not shown) are the same. As an increase in mass (e.g. for ligand immobilization or analyte-ligand binding) takes place on the chip surface, the SPR angle changes and is monitored in real-time as resonance units (RU) in function of time. In all cases, a stable increase of RU was found, showing the efficient capture of all the injected oligonucleotides, after injection of normal and mutated biotinylated probes onto different flow cells of streptavidin-coated sensor chips for 8 minutes.

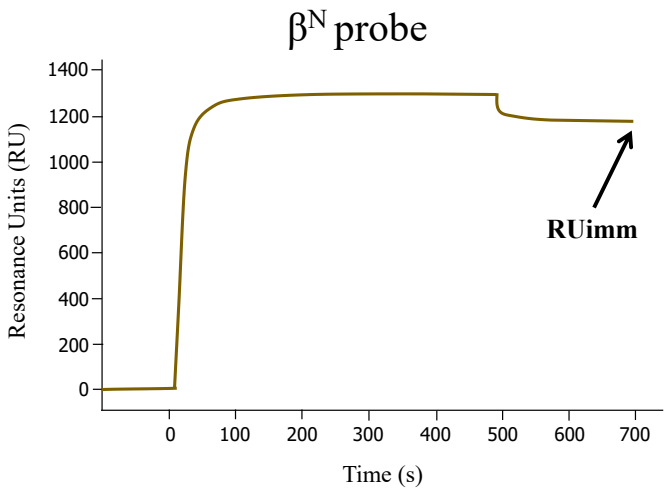
The RU values measured at the end of the immobilization protocol (Ruimm) (**Figure 4.13.A** and **4.13.B**) on sensor chips of normal and mutated probes for each mutation under investigation are reported in **Figure 4.13.C**.

4.2.1.3. Probes validation

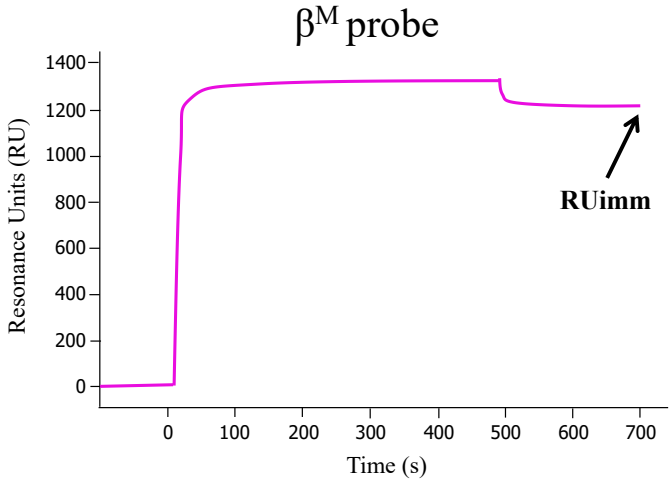
In order to validate the ability of the allele-specific probes to discriminate two DNA sequences only differing for the mutation position, for every alteration under investigation a pair of oligonucleotides exactly complementary to the immobilized probes was designed, one carrying the normal sequence (β^N), the other the mutated one (β^M). Those oligonucleotides were injected as targets on the two sensor chips carrying the respective normal and mutated probes. We expected that each probe would bind differently according to the injected target, in particular preferentially to the complementary oligonucleotide and with greater affinity and stability in comparison with the mismatched target.

The resulting sensorgrams, for $\beta^0$39 mutation, as an example, are shown in **Figure 4.14.A** and **4.14.B**. In both the diagrams it is possible to distinguish an association phase (**a**), where

A



B

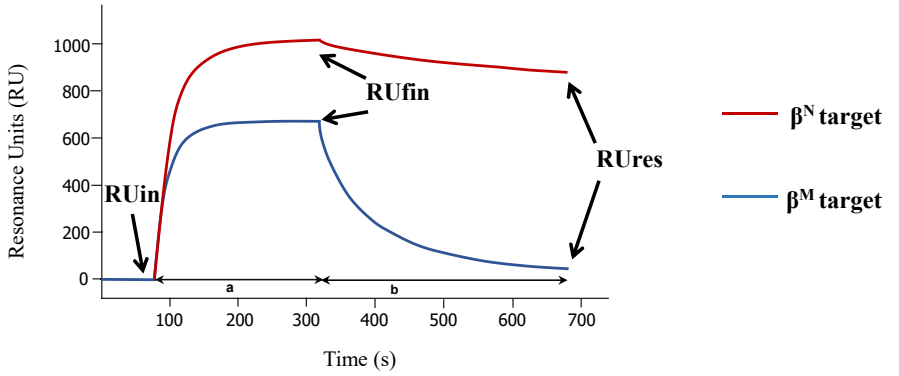


C

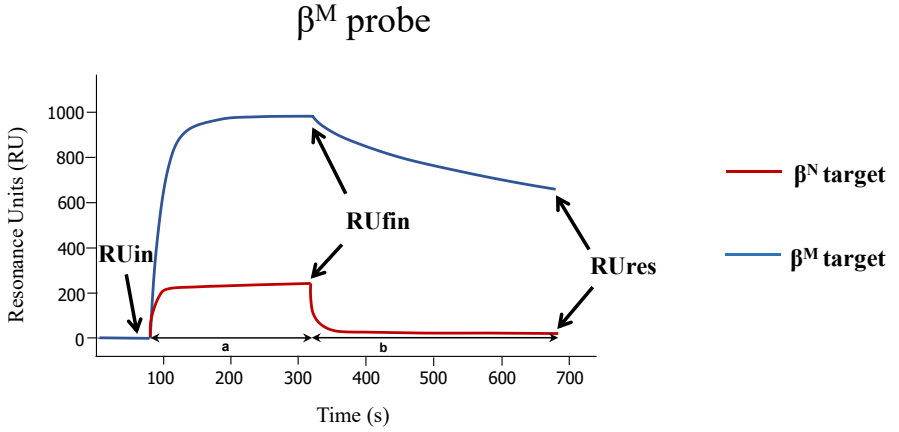
Mutation	Probe	RUimm
β⁰ 39	βN	1179
	βM	1215
β⁺ IVSI-110	βN	1198
	βM	1186
β⁺ IVSI-6	βN	1014
	βM	1117
β⁰ IVSI-1	βN	1241
	βM	1256

Figure 4.13. Normal (β^N) and mutated (β^M) probes immobilization on the sensor chip surface. Sensorgrams obtained after immobilization of biotinylated normal β^N (A) or mutated β^M (B) probes for β^0 39 thalassemia mutation on different flow cells of streptavidin-coated (SA) sensor chips. The resonance unit (RU) values measured at the end of the immobilization protocol (RUimm) are indicated for each probe (A, B). RUimm values obtained after immobilization on sensor chips of normal (β^N) and mutated (β^M) probes for each mutation under investigation are reported in table (C). The immobilizations were performed by using the Biacore™ X100 instrument [Modified from: Breveglieri et al., 2018].

A



B



C

Mutation	Probe	Target	RUfin (mean ± SD)	RUres (mean ± SD)
β⁰ 39	βN	βN	355.67 ± 1.15	294.33 ± 1.53
		βM	230.67 ± 0.58	29.33 ± 2.08
	βM	βN	22.33 ± 1.53	6.33 ± 0.58
		βM	197.33 ± 0.58	131.67 ± 1.15
β⁺ IVSI-110	βN	βN	360.00 ± 8.89	264.00 ± 6.08
		βM	10.00 ± 0.00	6.67 ± 0.58
	βM	βN	80.00 ± 2.00	7.67 ± 1.53
		βM	389.67 ± 1.53	165.00 ± 1.00
β⁺ IVSI-6	βN	βN	372.67 ± 2.31	149.00 ± 3.46
		βM	76.00 ± 1.00	24.67 ± 0.58
	βM	βN	236.00 ± 1.00	36.33 ± 0.58
		βM	440.67 ± 5.03	327.00 ± 1.00
β⁰ IVSI-1	βN	βN	802.00 ± 19.52	713.67 ± 13.01
		βM	510.33 ± 2.52	113.33 ± 1.53
	βM	βN	684.00 ± 6.00	358.33 ± 0.58
		βM	817.33 ± 17.04	692.33 ± 10.07

Figure 4.14. Probes validation using normal (βN) and mutated (βM) complementary target oligonucleotides. Sensorgrams obtained after injection of target normal βN (A, B, red lines) or mutated βM (A, B, blue lines) complementary oligonucleotides on flow cells carrying the normal βN (A) or mutated βM (B) probe for β⁰ 39, respectively (a, target injection; b, washing step with HBS-EP+ buffer). RUin (initial resonance units), RUfin (final resonance units) and RUres (residual resonance units), measured before the analysis, after the sample injection and at the end of the washing step, respectively, are indicated (A, B). In order to compare the sensorgrams, the initial RU signal is arbitrarily set to zero. The assays were performed by using the Biacore™ X100 instrument, at 25°C and 5 µL/min flow rate; the running buffer was HBS-EP+. The binding resulting plots (A, B) were obtained after subtracting the sensorgrams produced by both analyte injection onto an empty flow cell and the running buffer alone injection. The RUfin and RUres values obtained after injection of normal (βN) and mutated (βM) complementary target oligonucleotides on sensor chips of normal (βN) and mutated (βM) probes for each muatation under investigation, are reported in table (C). The RUfin and RUres values are the mean ± stndard deviation (SD) of three indipendent experiments [Modified from: Breveglieri et al., 2018].

the target analyte is injected and the binding step takes place, and a dissociation phase (**b**), which coincides with the end of the injection and a washing with an appropriate buffer (HEPES-buffered saline-EP$^+$ (HBS-EP$^+$)). The latter phase provides useful data on the stability of the analyte-ligand complex.

In order to analyze and quantify the interaction data, it is possible to define some parameters (**Figure 4.12.A** and **4.12.B**): initial RU (RUin) and final RU (RUfin), that are the RU values measured at the beginning and the end of the analyte injection, respectively, while the value of residual RU (RUres) indicates the SPR signal recorded after washing.

In the specific case of the $\beta^0$39 mutation, the normal probe (**Figure 4.12.A**) showed a higher and more stable hybridization with the normal target oligonucleotide (β^N, in red) than with the mutated one (β^M, in blue), that is bound to a lesser extent. In addition, the latter was an unstable binding, as the RU value completely decreased during the washing phase. As expected the mutated probe (**Figure 4.12.B**) bound in a stable and specific manner the mutated oligonucleotide (in blue), sharply distinguishing it from the normal one (in red). Similar sensorgrams were obtained for the other β thalassemia mutations (data not shown).

As a result, all the analyzed probe pairs were validated for their capability to discriminate target sequences differing only for the nucleotide involved in the mutation. The RUfin and RUres values, as mean±standard deviation of three independent experiments, obtained after injection of normal and mutated complementary target oligonucleotides on sensor chips of normal and mutated probes for each muatation under investigation, are reported in **Figure 4.12.C**, confirming these results.

4.2.1.4. Development of diagnostic strategies

After the validation of the probe pairs immobilized on Biacore^{TM} sensor chips for the discrimination of normal and mutated β globin alleles, they were employed with a post-natal diagnostic purpose for the $\beta^0$39, β^+IVSI-110, β^+IVSI-6 and β^0IVSI-1 β thalassemia mutations.

The $\beta^0$39 mutation is the most frequent β thalassemia alteration in the Mediterranean area [Cao, 2010]. It is a non-sense mutation, suitable for a possible correction by the developing read-through approach. On the other hand, the β^+IVSI-110, β^+IVSI-6 and β^0IVSI-1 are

splicing mutations [Huisman, 1997] suitable for a possible correction by the developing antisense approach.

To this aim, the analysis of interactions between the immobilized normal and mutated probes and unbalanced single-strand PCR products, obtained by the patient DNA, was performed.

This interaction format, for the molecular diagnosis of β thalassemia mutations, is very different from that reported by Feriotto et al., where the amplified from patient genomic DNA PCR product was immobilized on the sensor chip and the probes injected in solution [Feriotto et al., 2004], resulting very expensive because one flow cell was required for every sample. On the contrary, the format employed in this work is much cheaper as only one flow cell in needed for analyzing numerous samples.

About the new approach, a very critical issue, to be evaluated, is the possible folding of the target amplicon during injection on the sensor chip surface, thus interfering or preventing the interaction with the immobilized probe. Indeed, the injection conditions (saline concentration, flow rate, temperature) are such as to promote a DNA-DNA binding as the expected analyte-ligand interaction, and not denaturing. As a result, the possible generation of secondary structures may occur in the target, where the DNA sequence complementary to the probe is not completely single-stranded and so may be not available for the hybridization with the specific probe, producing lower signals. In addition, also the amplicon length has to be considered. During the bio-specific interaction analyses, as the possible steric hindrance of very large target molecules may prevent an optimal analyte-ligand hybridization.

So, three steps were sequentially performed for the setup of the diagnostic approach: (1) the use of target oligonucleotides longer than those previously reported; (2) the optimization of the amplification reaction to achieve the best discrimination efficiency; (3) the injection of unbalanced PCR products obtained from genomic DNA of different genotypes to perform the actual diagnosis.

4.2.1.4.1. Assay of 35mer oligonucleotide targets

The first set-up analysis was aimed to assess the capability of the probes to discriminate longer DNA sequences carrying or not the mutation and was performed for the $\beta^0$39

mutation, considering that the PCR products are the real targets of the probes immobilized on the sensor chip surface.
To this purpose, 35mer oligonucleotides carrying the normal or the mutated nucleotide were employed, mimicking targets of different genotypes. The nucleotide sequence was designed based on the previous work of Prof. Spoto and colleagues about the detection of $\beta^0$39 mutation with SPR-I technology [D'Agata & Spoto, 2012].
The possible secondary structures produced by the two target oligonucleotides in the hybridization conditions were determined using *The mfold Web Server* (http://mfold.rit.albany.edu/?q=mfold/) [Zuker, 2003].

The target region containing the mutation position was more accessible for the probe interaction in the oligonucleotide carrying the mutation than in the 35mer target without alterations. So a different hybridization behavior was expected for the two injected targets. Indeed, the mutated target was found to interact with the specific probe with higher affinity than the normal target. However, when the two 35mer oligonucleotides were injected on the two probes a discrimination occurred, even if it was more visible for the β^M probe.

Therefore, the use of longer oligonucleotide targets, for the $\beta^0$39 mutation, confirmed both their detection capability, and the actual binding interfering of possible target folding due to secondary structures. As a consequence, for molecular diagnosis the amplification conditions, for all the β thalassemia mutations taken into consideration, had to be optimized to minimize such interference and allow a proper target recognition.

4.2.1.4.2. Set-up of the amplification conditions

After demonstrating that the normal and mutated 35mer target oligonucleotides were able to be discriminated by the immobilized probes, we started dealing with longer PCR products. These were the real targets of the interaction in order to perform molecular post-natal diagnosis.

In particular, the aim was to find the optimal amplification conditions to allow an efficient and specific hybridization of the obtained single-stranded target PCR products with the immobilized probes.

Indeed, as already mentioned, the most important problem is the possible secondary structures of single-stranded PCR products, as the injection on the sensor chip surface does

not occur in denaturing conditions, rather at room temperature and in the presence of high saline concentrations to promote the hybridization with the probe. In these conditions, an amplicon folding is highly expected, and could significantly affect the specific interaction if the site carrying the mutation is accessible or not to the probe binding. Obviously, the secondary structure generation depends on the length and sequence of the PCR product. In addition, also the analyte steric hindrance on the sensor chip surface may affect the bio-specific interaction with the immobilized ligand. So different amplicons were assayed as possible SPR targets.

To this purpose, two different primer pairs were designed, for $\beta^0$39 mutation, with the aim to produce PCR products of different length, covering the region of the β globin gene carrying the position of the mutation, in order to establish the optimal length of PCR product to be injected on the immobilized probes. The primers BG8F-BG6R give an 81 bp amplification product, instead the primers BG8F-BG8R, give a shorter (53 bp) PCR product aimed to reduce the possible amplicon folding and improve the bio-specific interaction with the probe.

In both cases, the single-stranded target was obtained in two following steps: (1) a balanced PCR amplification employing the same amounts of the forward and reverse primers; (2) an unbalanced PCR using only the forward primer, in order to obtain single-stranded products capable to hybridize with the single-stranded antisense probe immobilized on the sensor chip.

Analyzing the expected secondary structures of the 81 bp and 53 bp amplicons using *The mfold Web Server* (http://mfold.rit.albany.edu/?q=mfold/) [Zuker et al., 1999], in both cases the region of interest was almost partially involved in a secondary structure and so not completely available for the binding with the probe (**Supplementary figure 1**). Moreover, in both cases the $\beta^0$39 nucleotide position was accessible for hybridization only in the mutated sequences, while in the normal ones it was involved in hydrogen bonds. So, only the different steric hindrance, probably related to the amplicon length, was expected to differently affect the interaction efficiency.

The evaluation of the two different PCR conditions was performed by amplifying genomic DNA obtained from 3 normal subjects and 3 β thalassemic patients homozygous for the $\beta^0$39 mutation, and injecting the single-stranded amplicons on the sensor chips where we had previously immobilized the normal and the $\beta^0$39 mutated probes.

The resulting data were analyzed and evaluated by calculating the so called β^{thal} index, a parameter reported by Feriotto et al. for the quantification of SPR-based BIA results in a diagnostic perspective [Feriotto et al., 2004]. It was obtained according to the following formula:

$$\frac{[RU_{fin}\text{-}RU_{in}]\beta\text{-}N}{[RU_{fin}\text{-}RU_{in}]\beta^{0}39\text{-}thal}$$

where RU_{in} and RU_{fin} were the resonance unit (RU) values measured at the beginning of the analysis and the end of the analyte injection, respectively, while *β-N* and *β^{0}39-thal* refer to the normal and mutated probes immobilized on sensor chips. As a consequence, the expected values for β^{thal} index were greater than 1 in the case of a not mutated target, or between 0 and 1 in the case of a $\beta^{0}39$ mutated target.

In the case of the 81 bp amplicon, the β^{thal} index values calculated from normal and mutated targets were very similar, both between 0 and 1, preventing the discrimination of the two different genotypes. Whereas in the case of the 53 bp PCR products, two different groups of values could be clearly distinguished corresponding to the normal (β^{N}/β^{N}) and homozygous (β^{M}/β^{M}) genotypes.

From these data, we concluded that the amplification conditions giving the best discrimination results were those corresponding to the shorter 53 bp amplicons, suggesting the need to employ target amplicons as short as possible. In conclusion, the amplification conditions were so adopted for all the following diagnostic analyses aimed to the development of a diagnostic approach for the $\beta^{0}39$ thalassemia mutation.

The same approach was employed for the β^{+}IVSI-110: in order to produce the target templates, two primers were chosen (IVS110BF-IVS110BR) that amplify a β globin gene sequence of 74 bp, including the region possibly affected by the mutation. In this way, it was possible to firstly amplify the region of interest and then to perform a second unbalanced amplification using only the forward primer, in order to obtain a single-stranded product perfectly complementary to the immobilized probes. The secondary structures of these products (**Supplementary figure 2**) had shown that the region of hybridization, in both cases, was partially involved in hydrogen bonds, and so only in part available for interactions.

As regarding β^{+}IVSI-6 and β^{0}IVSI-1 mutations, the primers IVS16F-IVS16R and IVS11F-IVS11R were chosen, respectively, which amplify, in both cases, a β globin gene region of 96 bp, including the mutations of interest. The expected secondary structures of single-stranded tragets, for both mutations (**Supplementary figure 3** and **figure 4**), had shown that the regions of base paring with the complementary probes are not very accessible, and so in these cases too, some difficulties in the specific interactions are expected.

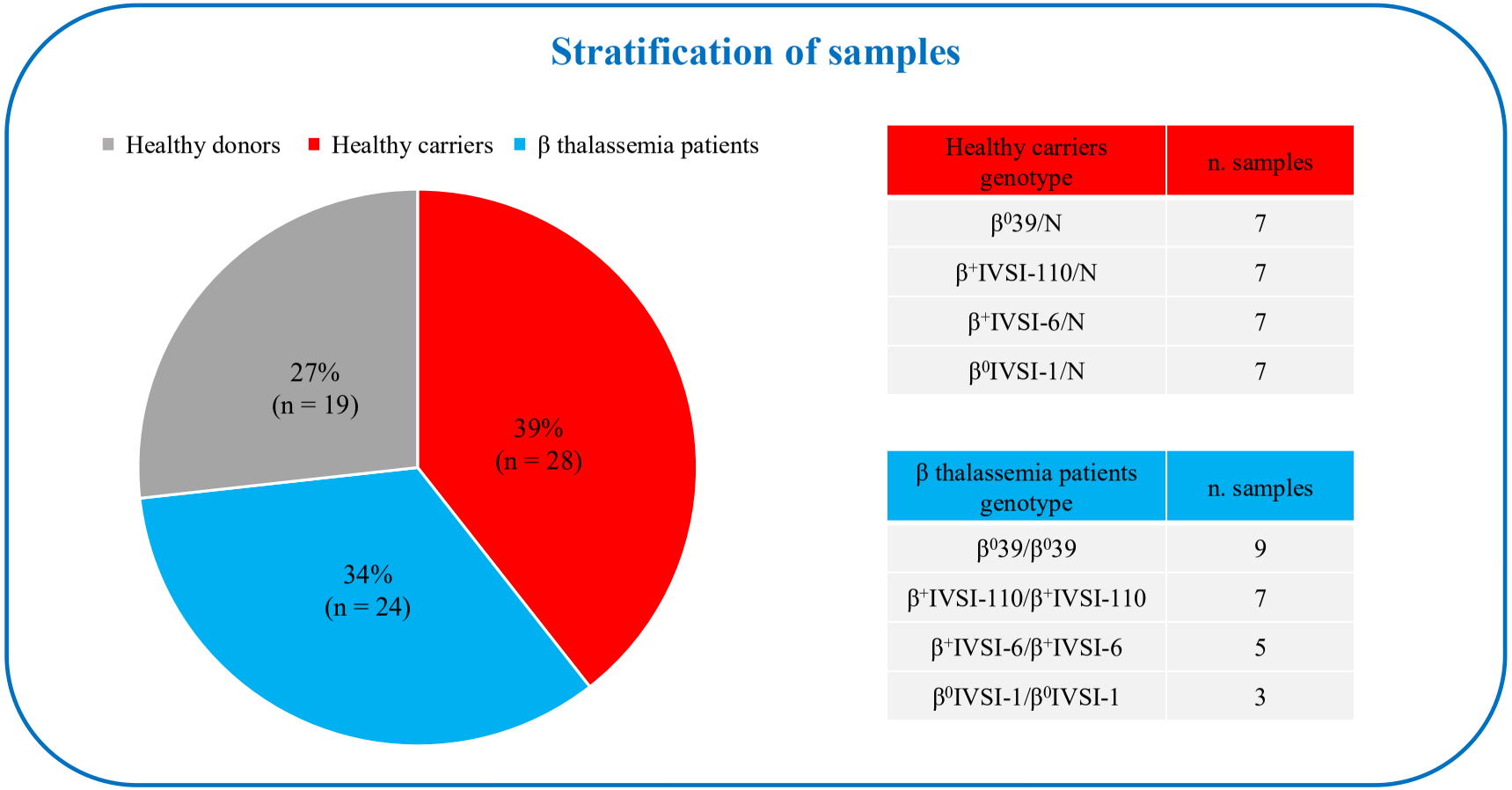
4.2.1.4.3. Detection of β thalassemia mutations in genomic DNA

Once developed the suitable amplification conditions for the hybridization with the immobilized probes, the detection approach was applied to unbalanced PCR products obtained by genomic DNA of different genotypes extracted from blood and buccal swab samples. The latter DNA source allows a sampling technique much less invasive than blood collection for the post-natal diagnosis of β thalassemia.

The stratification of the 71 analyzed samples of genomic DNA is reported in **Figure 4.15**. In particular, specimens from 19 healthy donors (27%), 24 homozygous patients (34%) and 28 (39%) heterozygous healthy carriers were employed. All the samples had been undergone to genotypic characterization by DNA sequencing. Of the 28 heterozygous samples 7 were heterozygous for the $\beta^{0}39$ mutation ($\beta^{0}39$/N), 7 were β^{+}IVSI-110/N, 7 were β^{+}IVSI-6/N and the last 7 were β^{0}IVSI-1/N, so they were equally distributed. From the DNA sequencing it was found that 9 patients are homozygous for the $\beta^{0}39$ mutation ($\beta^{0}39$/ $\beta^{0}39$), instead the patients homozygous for the mutations β^{+}IVSI-110, β^{+}IVSI-6, β^{0}IVSI-1 were 7, 5, 3, respectively.

The genomic DNA was extracted from blood and buccal swabs samples, and then 200 ng were used for a first balanced amplification, and a second unbalanced PCR, to obtain the single-stranded products for the injection on the normal and mutated probes.

In the sensorgrams of **Figure 4.16** three representative cases, for the $\beta^{0}39$ mutation, are shown, obtained by a healthy subject (**Figure 4.16.A**), a heterozygous healthy carrier (**Figure 4.16.B**) and a homozygous thalassemic patient (**Figure 4.16.C**). As clearly appreciable, the expected results were obtained. In the case of the healthy subjects the normal probe (β^{N}, in red) bound in a specific manner the normal DNA, while the mutated probe did not hybridize with the target (**Figure 4.16.A**). In the case of genomic DNA from homozygous patients, the mutated probe (β^{M}, in blue) hybridized with the target DNA, while



Healthy carriers genotype	n. samples
$\beta^0$39/N	7
β^+IVSI-110/N	7
β^+IVSI-6/N	7
β^0IVSI-1/N	7

β thalassemia patients genotype	n. samples
$\beta^0$39/$\beta^0$39	9
β^+IVSI-110/β^+IVSI-110	7
β^+IVSI-6/β^+IVSI-6	5
β^0IVSI-1/β^0IVSI-1	3

Figure 4.15. Stratification of the samples analyzed for SPR-based Biacore™ X100 to detect β thalassemia point mutations. On the left side, the distribution of the 71 genomic DNA samples belonging to healthy donors (in grey), healthy carriers (in red) and β thalassemia patients (in light blue) is dysplayed. For each group the sample number and the percentage are indicated. On the right side, the list of β globin allele genotyes, resulting from sequencing analyisis, for the healthy carriers and patient is reported [Modified from: Breveglieri et al., 2018].

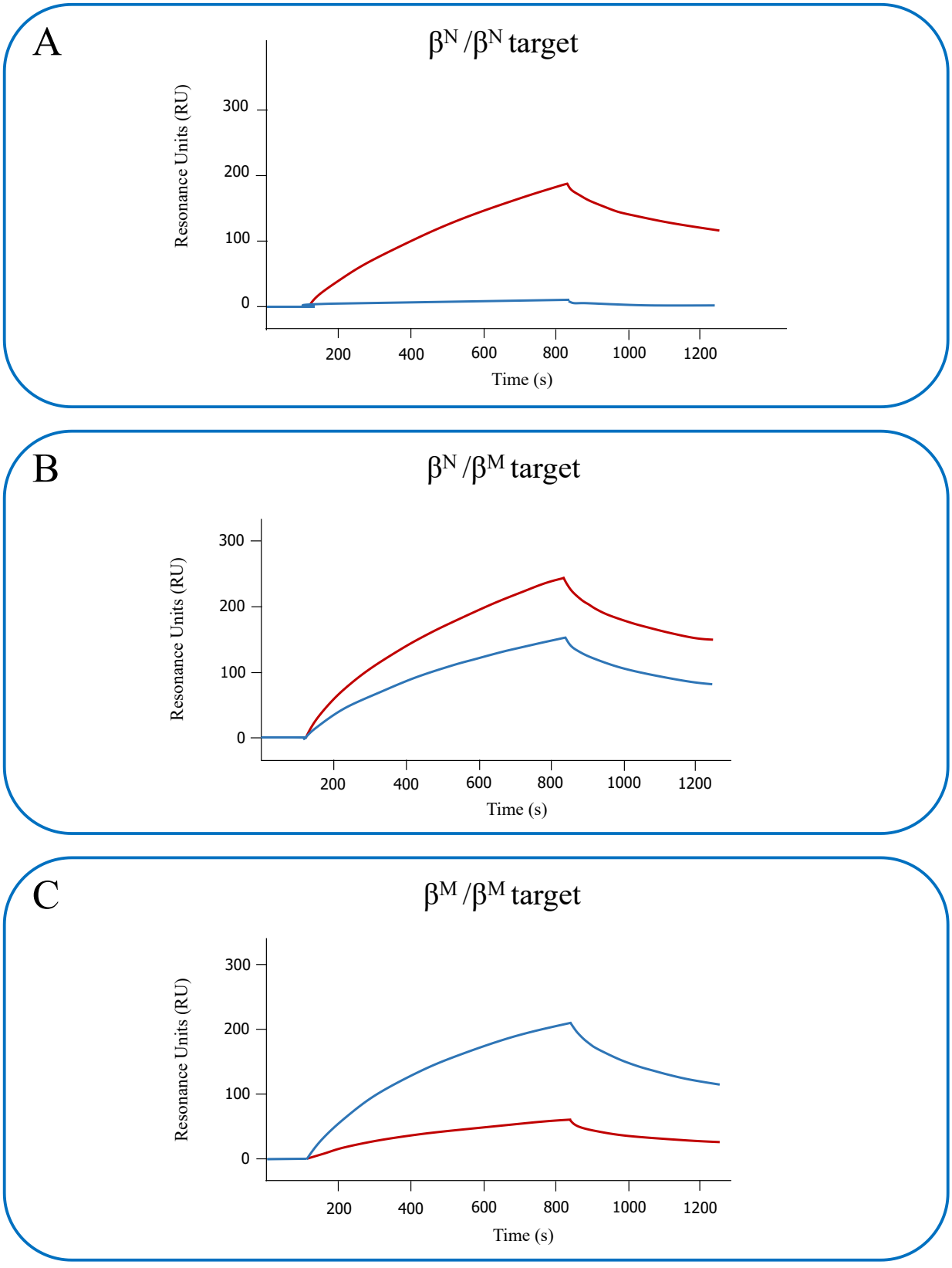


Figure 4.16. Representative sensorgrams for genotype discrimination. Examples of sensorgrams obtained after injection on sensor chips carrying the normal β^N (red line) or the mutated β^M (blue line) probe for $\beta^0$39 thalassemia mutation, of unbalanced PCR products from genomic DNA of different genotypes: normal (β^N/β^N) (A), heterozygous (β^N/β^M) (B) or homozygous (β^M/β^M) (C) for $\beta^0$39 mutation, respectively. The assays were performed by using the Biacore™ X100 instrument, at 25°C and 5 µL/min flow rate; the running buffer was HBS-EP+. The resulting plots were obtained after subtracting the sensorgrams produced by both analyte injection onto an empty flow cell and the running buffer alone injection. In order to compare the sensorgrams, the initial RU signal was arbitrarily set to zero [Modified from: Breveglieri et al., 2018].

the normal probe generated a very little signal (**Figure 4.16.C**). Finally, in the case of heterozygous genotypes, both probes bound the specific sequences in the target amplicons (**Figure 4.16.B**).

Approximately the same trend was found for the sensorgrams obtained for the other three β thalassemia mutations.

The final data were analyzed and reported in **Table 4.5** summarizing the mean values of β^{thal} index±SD obtained by the injection of PCR products from the DNA samples of different genotypes, from each mutation. For each sample, almost three injections on the sensor chip surface were performed, and then a mean value of β^{thal} index was calculated. By examining the obtained results, it was possible to quantitatively validate the discrimination technique, confirming the observations made about sensorgrams. In fact, high significant values of β^{thal} index were always obtained for normal DNA (β^N/β^N); while for the homozygous mutated DNA (β^M/β^M), the β^{thal} indexes were always equal or lower than 1; finally, in the case of heterozygous β^N/β^M DNAs, the values of calculated β^{thal} index were, as expected, intermediate.

The **Figure 4.17** shows the distribution of all β^{thal} index values obtained for the mutations $\beta^0$39 (**Figure 4.17.A**), β^+IVSI-110 (**Figure 4.17.B**), β^+IVSI-6 (**Figure 4.17.C**) and β^0IVSI-1 (**Figure 4.17.D**) according to the different genotypes, normal homozygous (violet), heterozygous (green) and mutated homozygous (orange).

Despite some variability in the obtained values, for the three different genotypes it is possible to distinguish three different value populations not overlapping each other. Also the statistical analysis confirmed that the differences between the values obtained from different genotypes were statistically significant, and that these groups were clearly distinct, demonstrating the suitability of the developed SPR-based approach to correctly discriminate the different allelic conditions for the β thalassemic mutations taken into consideration.

Therefore, the obtained results were in line with our expectations, confirming that the assayed molecular probes were able to recognize the specific targets and so were useful for the identification of the possible presence of $\beta^0$39, β^+IVSI-110, β^+IVSI-6 or β^0IVSI-1 thalassemia mutations in genomic DNA.

No differences have been observed between the two DNA sources; in this way we demonstrated that the developed method of diagnosis based on SPR may be efficiently

Mutation	Target genotype	n. analyzed samples	β^{thal} index (mean ± SD)
β^0 39	β^N/β^N	8	32.90 ± 26.68
	β^N/β^M	7	1.54 ± 0.13
	β^M/β^M	9	0.18 ± 0.07
β^+ IVSI-110	β^N/β^N	7	179.37 ± 103.18
	β^N/β^M	7	1.37 ± 0.20
	β^M/β^M	7	0.04 ± 0.03
β^+ IVSI-6	β^N/β^N	7	2.05 ± 0.66
	β^N/β^M	7	1.01 ± 0.22
	β^M/β^M	5	0.49 ± 0.14
β^0 IVSI-1	β^N/β^N	8	3.69 ± 0.76
	β^N/β^M	7	1.55 ± 0.09
	β^M/β^M	3	1.00 ± 0.23

Table 4.5. β^{thal} index mean values from genomic DNA carrying different genotypes. β^{thal} index values calculated after SPR-based biosensor analysis of genomic DNA extracted from blood samples or salivary swabs, normal (β^N/β^N), heterozygous (β^N/β^M), or homozygous (β^M/β^M) for each mutation under investigation, respectively, expressed as mean values ± standard deviations (SD). The number of analyzed samples for each genotype is indicated [Modified from: Breveglieri et al., 2018].

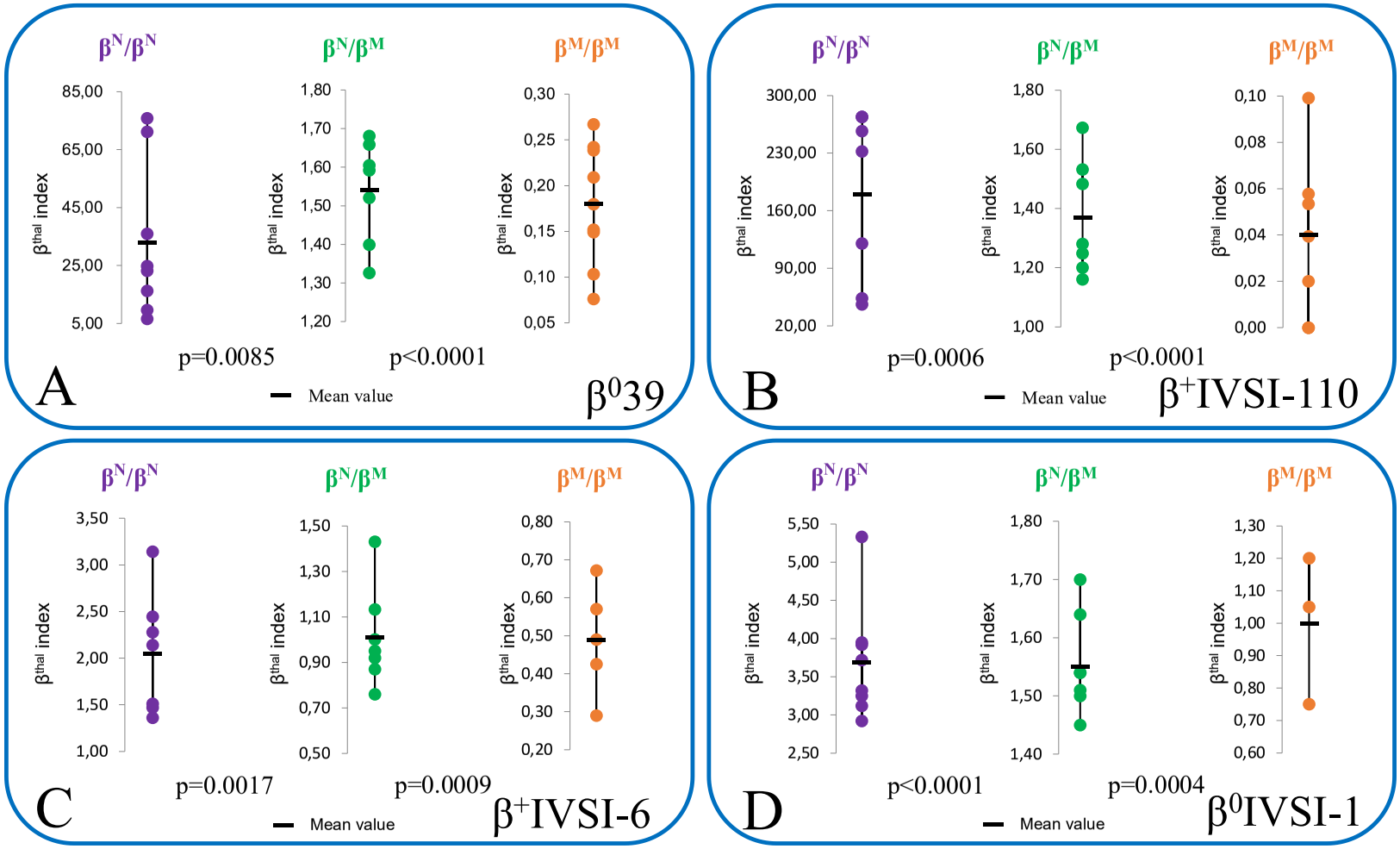


Figure 4.17. β^{thal} index values distribution through SPR-based Biacore™ X100 to detect β thalassemia point mutations. Distribution of β^{thal} index values obtained after $\beta^0$39 (A), β^+IVSI-110 (B), β^+IVSI-6 (C) and β^0IVSI-1 (D) SPR-based Biacore™ X100 analysis of normal (β^N/β^N) (violet), heterozygous (β^N/β^M) (green), or homozygous (β^M/β^M) (orange) genomic DNAs extracted from blood samples or salivary swabs. The mean value of the distribution and the statistical significance of the analysis are also indicated. Statistical differences between grups were compared by the Student's *t*-test, selecting a 95% confidence level [Modified from: Breveglieri et al., 2018].

applied both to blood samples and to the much less invasive salivary swabs, the latter certainly preferable in order to achieve patients compliance.

Interestingly, this diagnostic procedure, finalized to obtain rapidly data on β^{thal} indexes, does not require pre-analysis determination of the amount of injected asymmetric PCR products. Fully in agreement with this hypothesis, we demonstrated that no relationship does not exist between the amount of the injected normal PCR products and the calculated β^{thal} indexes. This is shown by the representative experiment in **Table 4.6**, in which different amounts of asymmetric β^N/ $\beta^0$39 PCR products were injected, obtaining very similar β^{thal} index values allowing in all cases a univocal diagnostic interpretation.

PCR quantity	Normal probe		Mutated probe		β^{thal} index
	RUfin	RUres	RUfin	Rures	
1 x	113.9	70.7	56.6	27.9	2.01
0.5 x	69.9	44.3	32.4	12.2	2.16
0.25 x	46.5	29.0	20.4	10.7	2.28
0.125 x	25.9	15.7	13.8	9.2	1.88
0	-3.4	-1.7	-1.1	-6.1	

Table 4.6. Correlation between β^{thal} index values and PCR quantity. β^{thal} index values calculated after SPR biosensor analysis of different amounts of asymmetric PCR products obtained from $\beta^0$39 heterozygous (β^N/β^M) genomic DNA [Modified from: Breveglieri et al., 2018].

In conclusion, this method based on Biacore^{TM} X100 is a new diagnostic SPR-protocol for thalassemia single point mutations simple, fast, automated, reproducible, and sensitive, and, therefore, it can be apply to other genetic diseases.

The discussed results have been recently published in *Sensors and Actuators B: Chemical* [Breveglieri et al., 2018].

4.2.2. Post-natal diagnosis of β thalassemia mutations by using genotyping assays

This part of the study was aimed to set-up genotyping assays to identify β thalassemia point mutations. The importance of a post-natal molecular diagnosis is related both to the possibility to take advantage of developing personalized therapies targeting specific classes of DNA mutations, and to discover alterations possibly transmittable to offspring to make pre-natal diagnosis easier.

4.2.2.1. Experimental strategy

The experimental strategy for the development of a diagnostic technique based on genotyping assays, shown in **Figure 4.18**, first required the extraction of genomic DNA collected from blood or buccal swabs samples (**Figure 4.18.A**). Both blood and buccal swabs DNAs were stored at -20°C. The second part of the experimental strategy has provided for optimize and validate genotyping assays in order to detect the four most frequent β thalassemia point mutations in the Mediterranean area (**Figure 4.18.B**). In this regard, a certain number of samples from healthy donors, healthy carriers and β thalassemia patients was selected, and their β globin gene genotypes were characterized by DNA sequencing. For each identified point mutation, a genotyping assay was then optimized, and validated analyzing normal heterozygous and homozygous DNA samples for that alteration.

Once developed the protocol of genotyping assays for each specific mutation, molecular diagnosis was performed for unknown samples (**Figure 4.18.C**): 25 DNA samples from patients affected by β thalassemia were selected to be analyzed by developed genotyping assays and the diagnostic outcome was finally verified by β globin gene sequencing.

4.2.2.2. Samples collection and genomic DNA extraction

In order to develop a versatile, fast, simple and economical method for post-natal diagnosis of β thalassemia, we have collected (**Figure 4.19**) a total of 72 blood samples (of which 11% healthy donors, in grey, 31% healthy carriers, in red, and 58% patients of β thalassemia, in light blue) and 30 buccal swab samples (of which 57% healthy donors, in grey, 37% healthy carriers, in red, and 7% patients of β thalassemia, in light blue).

Blood sampling was preferred as the usual source of genomic DNA, confirmed by the higher number of blood samples than buccal swab samples, and was performed during blood collection for routinely laboratory analyses, mainly coming from patients (**Figure 4.19**, in light blue). Buccal swab samples were collected especially from healthy subjects and healthy carriers (**Figure 4.19**, in grey and red, respectively). The decision to choose as starting material also buccal swabs was due to the purpose to verify the suitability of an alternative non-invasive sampling technique to obtain genomic DNA.

This was done after informed consent, in collaboration with *Thalassemic Day Hospital* of *S. Anna* University Hospital (Cona, Ferrara) and *S. Maria della Misericordia* Hospital (Rovigo).

After collection of blood or buccal swab samples (**Figure 4.18.A**), these were readily subjected to extraction of genomic DNA in order to determine the β globin gene genotype of the subjects, using QIAamp® DNA Blood Mini Kit (Qiagen).

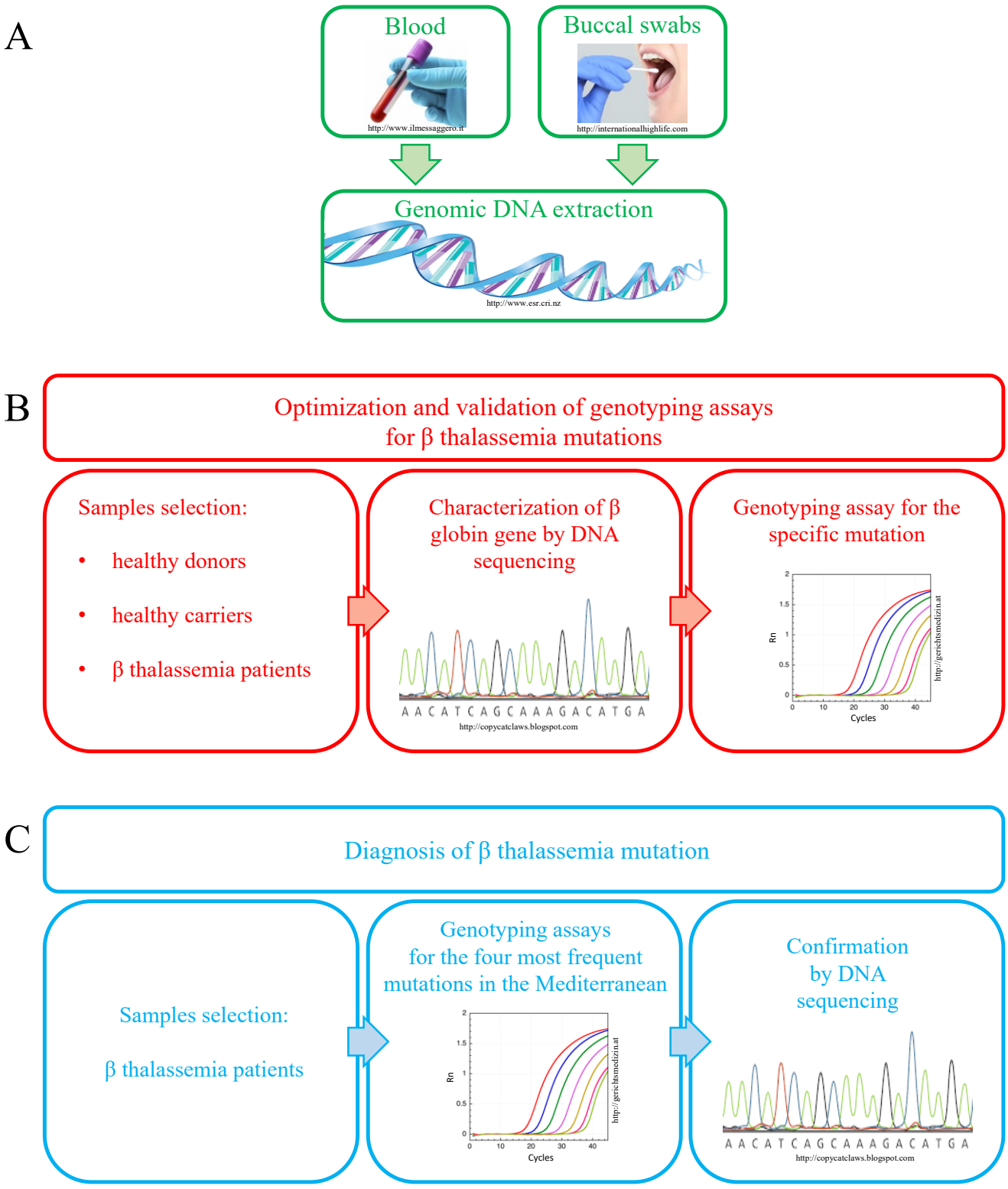


Figure 4.18. Experimental strategy for the development of the post-natal diagnosis technique based on genotyping assays. After collecting blood and buccal swab samples, genomic DNA was extracted (A). Then genotyping assays were optimized and validated to detect β thalassemia point mutations (B), and finally applied to diagnosis for the four most frequent β thalassemia mutations in the Mediterranean area.

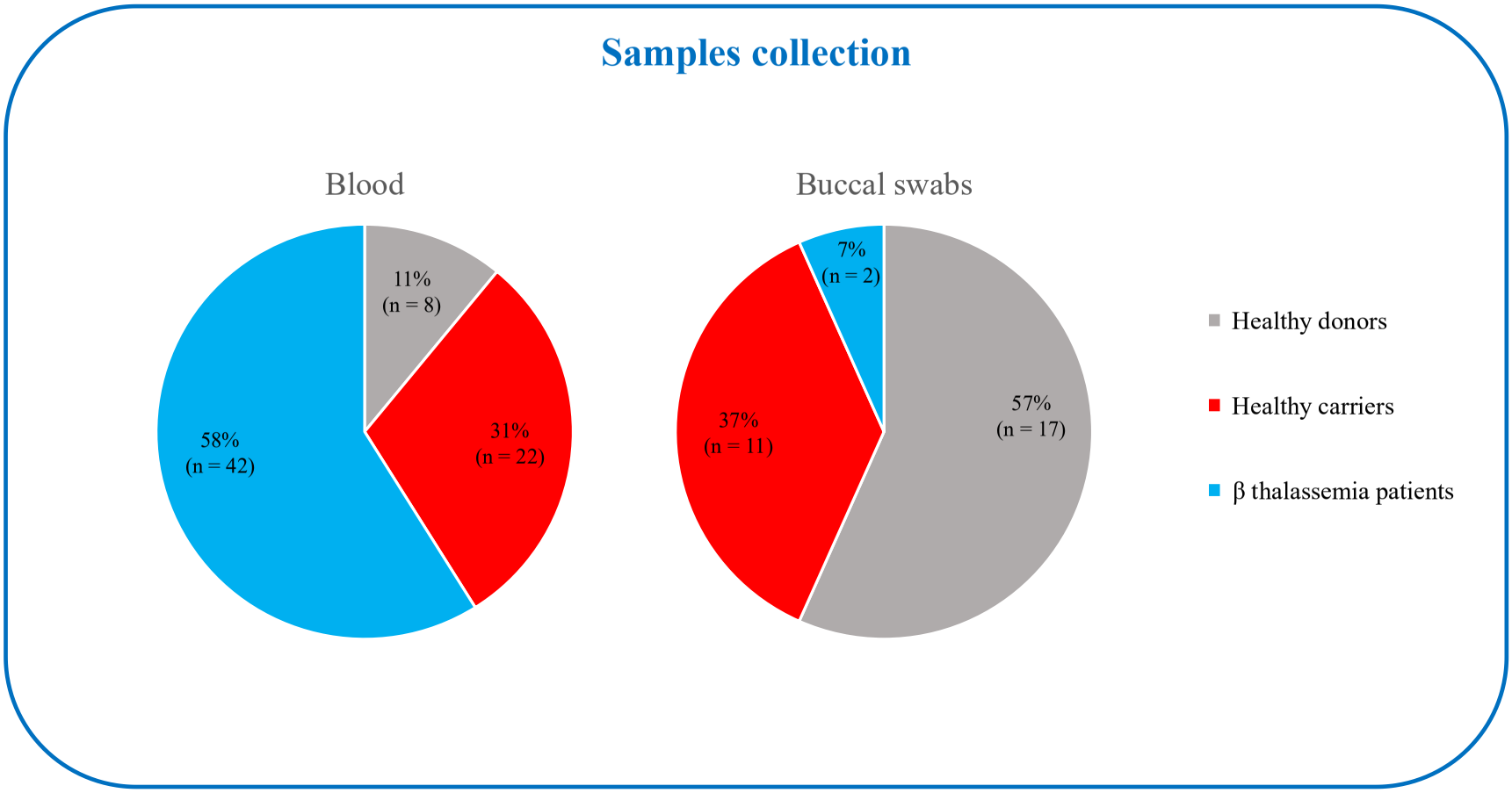


Figure 4.19. Distribution of blood and buccal swab samples collected for the development of the post-natal dignosis technique based on genotyping assays. For each sample category, the number of samples and the percentage belonging to healthy donors (in grey), healthy carriers (in red) and β thalassemia patients (in light blue) are indicated.

If it was not possible to immediately proceed with DNA extraction from blood samples, they were stored at -80°C until purification. On the contrary, buccal swabs were processed within a few days from sampling, to avoid degradation of a few genomic DNA, and in the meantime they were stored at -20°C.

Subsequently, the extraction efficiency and the genomic DNA integrity, purified from blood samples, were assayed by electrophoresis in 0.8% agarose gel. The DNA purified from buccal swabs samples did not show band, because of the few quantity of cells available on the swab. Anyway, the presence of DNA was demonstrated as well by the final spectrophotometric analysis, performed for all samples in order to establish the concentration of the purified genomic DNA.

4.2.2.3. Optimization and validation of genotyping assays

As previously underlined, the first purpose of this part of the work was to develop a post-natal approach for molecular diagnosis of β thalassemia mutations, based on genotyping assays specific for the four most frequent β thalassemia mutations in the Mediterranean area [Cao & Galanello, 2010]. Therefore, the first step was the genotypic characterization of a certain number of collected samples, in order to identify the most common Mediterranean β thalassemia mutations, aimed both to design genotyping assays specific for those mutations, and to use samples different genotype for each mutation to optimize and validate the detection approaches.

4.2.2.3.1. Detection of mutations in the β globin gene of healthy carriers and β thalassemia patients by DNA sequencing

According to the experimental strategy (**Figure 4.18**), we decided to characterize the β globin genotype of several DNA samples extracted from both blood and buccal swabs of subjects of different clinical conditions. In particular, we selected 25 total samples from healthy subjects, 33 total samples from healthy carriers, and 19 samples from β thalassemia patients.

Genomic DNA was used as a template for PCR reactions, in order to amplify the β globin gene before DNA sequencing. For this purpose we used three pairs of primers to amplify the whole nucleotide sequence. The first pair (BG1F-BG2R) amplifies a region of 641 bp, that includes the first two exons and the first intron of the β globin gene; the product of the two primers BG3F-BG4R, instead, contains the second intron and part of the second and third exons, with a length of 954 bp; finally, the third pair of primers (BG5F-BG6R) covers the third exon, and the 5' UTR region until the polyadenylation site. The three different

amplification reactions were carried out from different amounts of template genomic DNA, 100 ng or 30 ng according to extraction from blood or buccal swab, respectively, because of the different yields obtained with purification, as already discussed.

The PCR products were then purified in order to remove excess of primers that could interfere with the subsequent sequencing reactions, and for each sample six sequencing reactions were finally set-up, three with the forward primers BG1F, BG3F, BG5F and three with the reverse primers BG2R, BG4R, BG6R, based on Sanger's DNA sequencing method [Sanger et al., 1977]. The electrophoretic analysis and detection of fragments generated by sequencing reactions were performed by *BMR Genomics* company at the University of Padua.

In the case of heathy donors, DNA sequencing confirmed the absence of mutations within the β globin gene (data not shown), whereas the **Table 4.7** shows the resulting β globin alleles genotypes of the selected healthy carriers and β thalassemia patients.

For all subjects, the results were consistent with what expected: healthy carriers displayed a heterozygous genotype with a normal allele and a mutated allele, instead mutations in both the β globin alleles were found for β thalassemia patients, resulting in a homozygous genotype or a double heterozygous genotype for two different alterations. The identified molecular defects consisted of the most common mutations that cause β thalassemia in the Mediterranean area: $\beta^0$39 C→T, β^+IVSI-110 G→A, β^+IVSI-6 T→C and β^0IVSI-1 G→A [Cao & Galanello, 2010].

In **Figure 4.20**, representative examples of electropherograms obtained by samples of different genotype are reported for each mutation: $\beta^0$39 (**Figure 4.20.A**), β^+IVSI-110 (**Figure 4.20.B**), β^+IVSI-6 (**Figure 4.20.C**), β^0IVSI-1 (**Figure 4.20.D**), respectively. For every alteration, it is clear the difference between the homozygous genotype, lacking the mutation (N/N) or carrying the mutation on both alleles (M/M), where only one peak is present, and the heterozygous condition of the healthy carriers or the double heterozygous patients, who carry both the normal and the mutated alleles (N/M). In the last case, two peaks are overlapping in the electropherograms.

4.2.2.3.2. Analysis of genomic DNA carrying different genotype for each mutation by genotyping assay

Once known the genotypes of the selected samples, we designed four genotyping assays to be employed in qRT-PCR, with the aim to distinguish the different genotypic conditions for the four point mutations identified in the β globin gene. In order to detect a point mutation, it is necessary to distinguish the normal allele from that carrying the mutated nucleotide. For

Samples	*Genotype*	*Blood*	*Buccal swabs*
Healthy carriers	*$\beta^0$39/N*	8	3
	β^+IVSI-110/N	8	3
	β^+IVSI-6/N	5	2
	β^0IVSI-1/N	1	3
β thalassemia patients	*$\beta^0$39/$\beta^0$39*	5	0
	β^+IVSI-110/β^+IVSI-110	5	0
	β^+IVSI-6/β^+IVSI-6	2	0
	β^0IVSI-1/β^0IVSI-1	1	0
	β^+IVSI-6/β^+IVSI-110	1	0
	β^+IVSI-110/$\beta^0$39	1	0
	β^0IVSI-1/$\beta^0$39	1	0
	β^+IVSI-6/$\beta^0$39	1	0
	β^+IVSI-110/β^0IVSI-1	1	0
	β^+IVSI-6/β^0IVSI-1	1	0

Table 4.7. List of β globin allele genotypes resulting from DNA sequencing of the selected healthy carriers and β thalassemia patients. For each genotype, the number of specimens deriving from blood or buccal swabs is indicated.

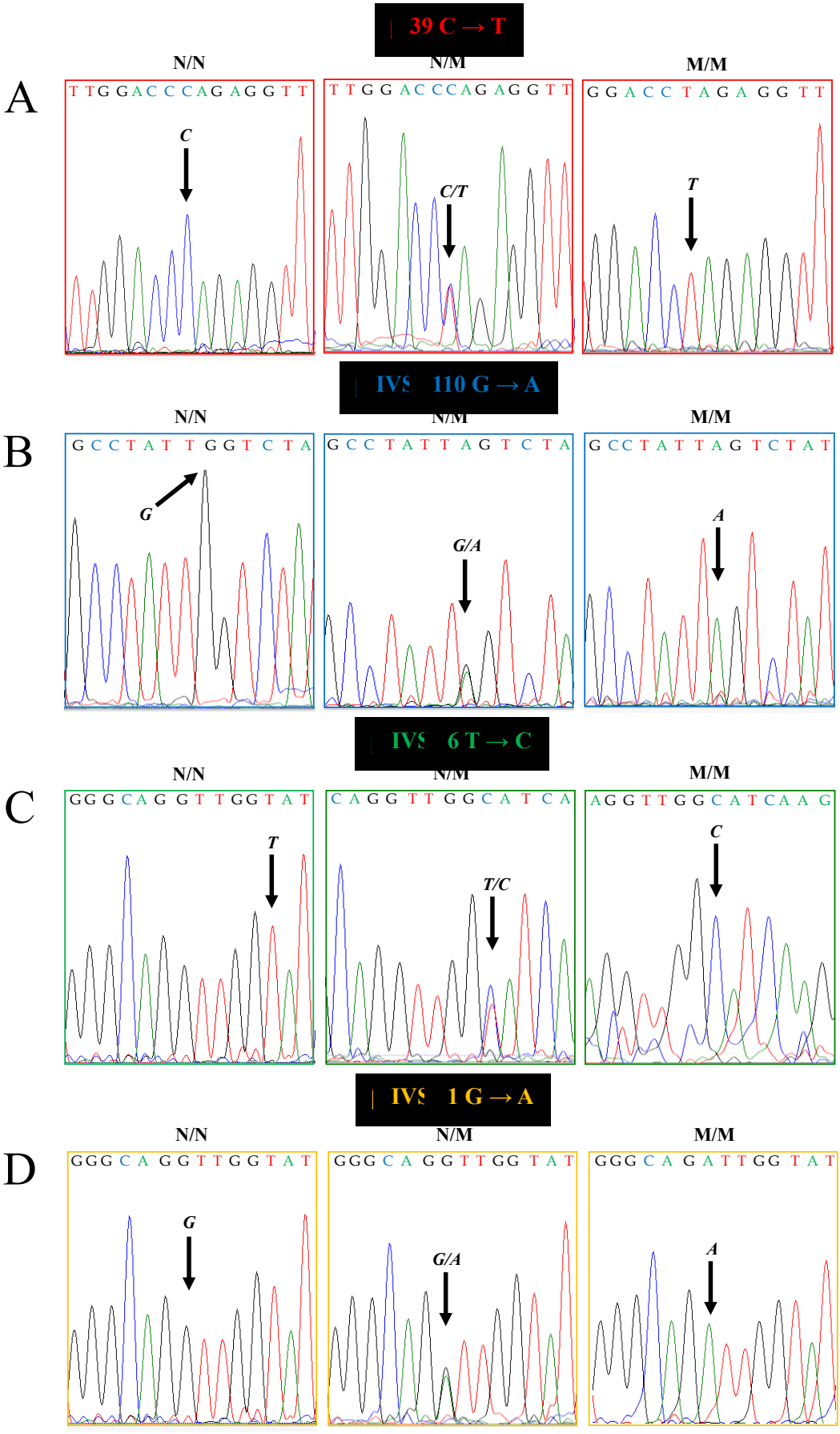


Figure 4.20. Portions of representative electropherograms obtained by genomic DNA sequencing. Examples of electropherograms obtained by samples normal homozygous (N/N), heterozygous (N/M) or mutated homozygous (M/M) are reported for each β globin gene alterations: $\beta^0$39 C→T (A), β^+IVSI-110 G→A (B), β^+IVSI-6 T→C (C) and β^0IVSI-1 G→A (D). Only one peak is present for normal and mutated homozygous genotype, instead two peaks are present in the case of heterozygous genotype.

this reason, each assay was designed by Life Technologies and contains two primers, able to recognize both the normal and the mutated sequence and so to amplify them, and two differently labeled TaqMan® probes: a probe specific for the normal sequence, labeled at the 5' end with VIC®, and FAM™-conjugated probe able to hybridize with the mutated sequence [Twyman, 2005]. At the 3' end, both the probes are coupled with the quencher NFQ, a non-fluorescent quencher acting as energy transfer acceptor [Afonina et al., 1997].

One of the most critical issues when dealing with genotyping assays is the specificity, which is the ability to efficiently discriminate the normal sequence from the mutated one, as they differ only for a single nucleotide. Therefore, the challenge has been to determine the best experimental conditions in order to achieve the most specific interactions between probes and complementary sequences; in this view, an optimization and a validation are required before assaying the approach for the molecular diagnosis of unknown samples.

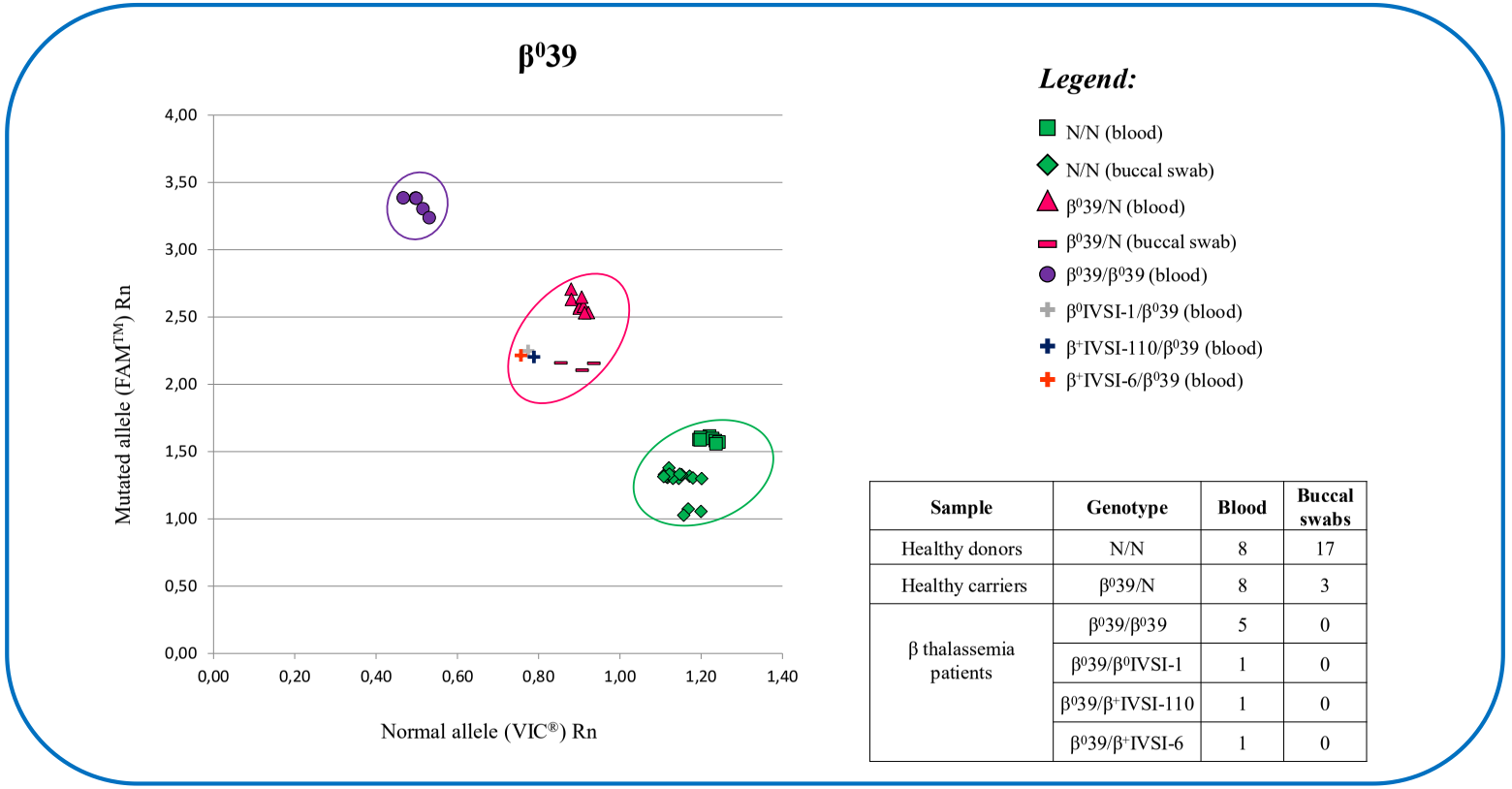
To this aim, for each mutation under investigation we chose, as templates, DNA samples having different genotypes among those previously analyzed: healthy donors (N/N), homozygous patients for the specific mutation (M/M), healthy carriers (N/M) and double heterozygous patients.

We then performed the optimization for all the four genotyping assays by amplifying the template DNAs in different experimental conditions, by evaluating in particular the number of amplification cycles and the annealing and elongation temperatures, in order to obtain an efficient discrimination between the normal and the mutated sequences.

To achieve such a specificity $\beta^0$39, β^+IVSI-110 and β^+IVSI-6 assays required 50 amplification cycles with annealing temperature of 62°C. As regards the β^0IVSI-1 assay, it required 60 amplification cycles with annealing temperature of 60°C.

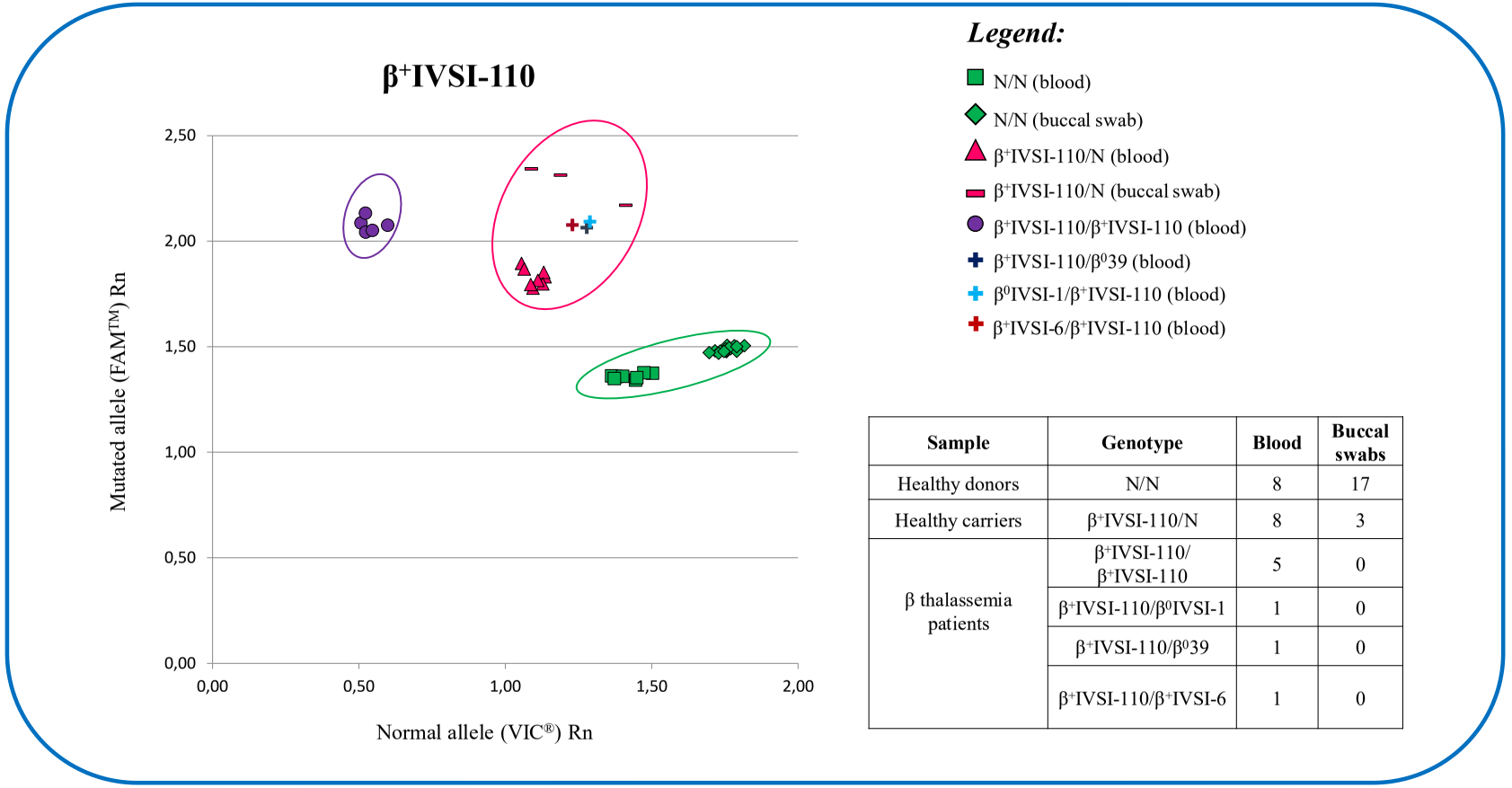
After the determination of the optimal amplification conditions for each assay, the four genotyping assays were finally validated with the samples of known genotype previously identified. Each sample DNA was undergone to the specific genotyping assay with the StepOne™ Real-Time PCR System (Applied Biosystem, Life Technologies), while the StepOne Software (Applied Biosystem, Life Technologies) was employed for the data analysis.

At the end of the reaction, the end-point fluorescence values for VIC®and FAM™ probes were extrapolated and normalized with the internal reference dye ROX™, obtaining an Rn value that is finally displayed in an allelic discrimination plot (**Figures 4.21-4.24**). In particular, the Rn values generated by the VIC® labeled normal probe and the FAM™ labeled mutated probe are reported on the x-axis and the y-axis, respectively.



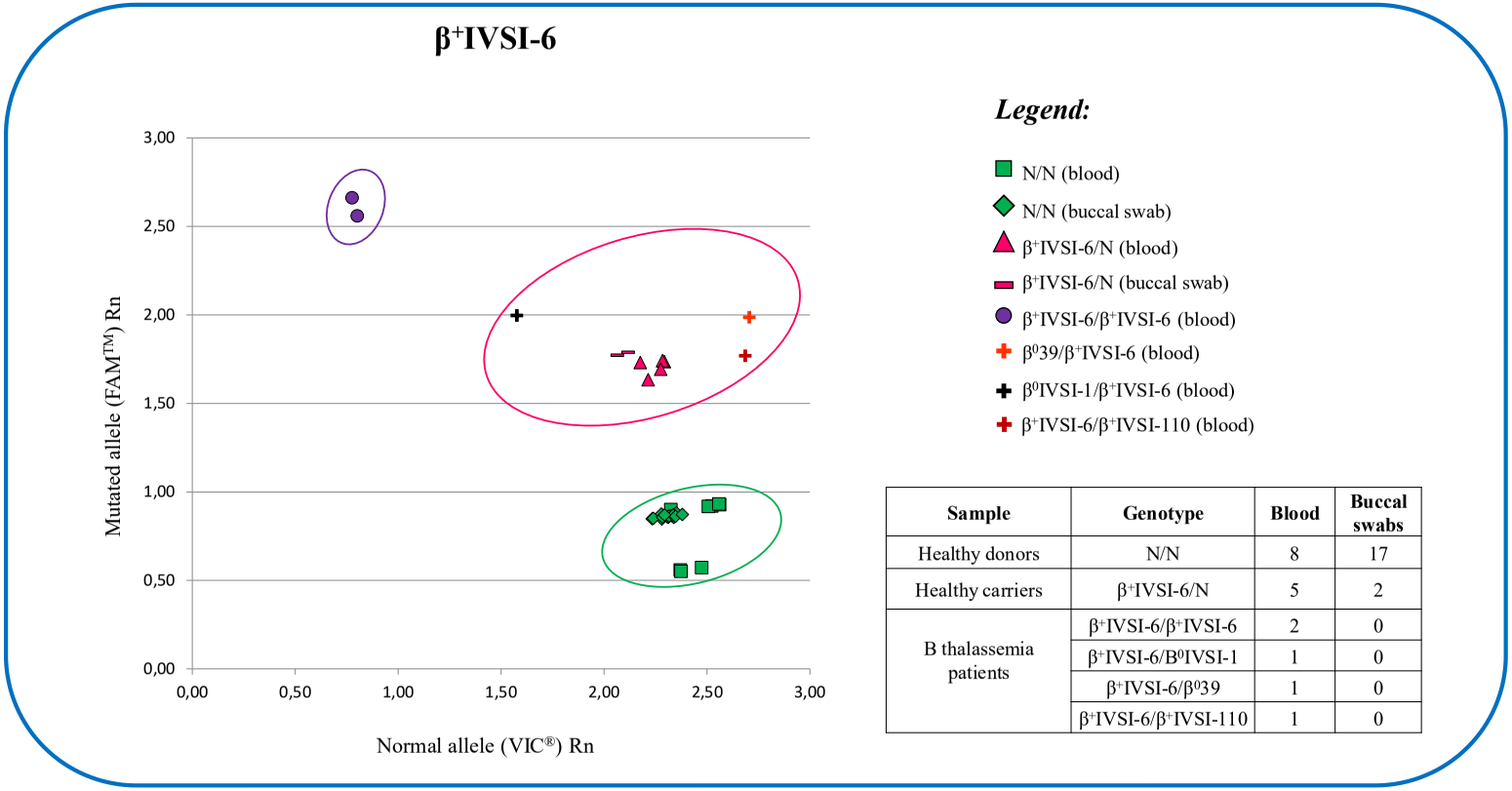
Sample	Genotype	Blood	Buccal swabs
Healthy donors	N/N	8	17
Healthy carriers	β⁰39/N	8	3
β thalassemia patients	β⁰39/β⁰39	5	0
	β⁰39/β⁰IVSI-1	1	0
	β⁰39/β⁺IVSI-110	1	0
	β⁰39/β⁺IVSI-6	1	0

Figure 4.21. Allelic discrimination plot resulting from validation analyses for β⁰39 genotyping assay. The genomic DNAs extracted from blood and buccal swabs belonging to healthy donors, healthy carriers and β thalassemia patients, double heterozygous and homozygous, for the β⁰39 mutation, were analyzed with the specific genotyping assay. The normalized end-point fluorescence (Rn) values generated by the VIC® labeled probe for the normal allele and by the FAM™ labeled probe for the mutated allele, are reported along the x-axis and the y-axis, respectively. Three distinct samples'groups are identified: healthy subjects (green circle), having a high value of VIC® and a low value of FAM™ fluorescence; homozygous patients (violet circle), characterized by an high FAM™ and a low VIC® fluorescence values; healthy carriers and double heterozygous patients (pink circle), having similar values of FAM™ and VIC® fluorescences. The table shows the number of samples, deriving from blood or buccal swabs, analyzed for each genotype.



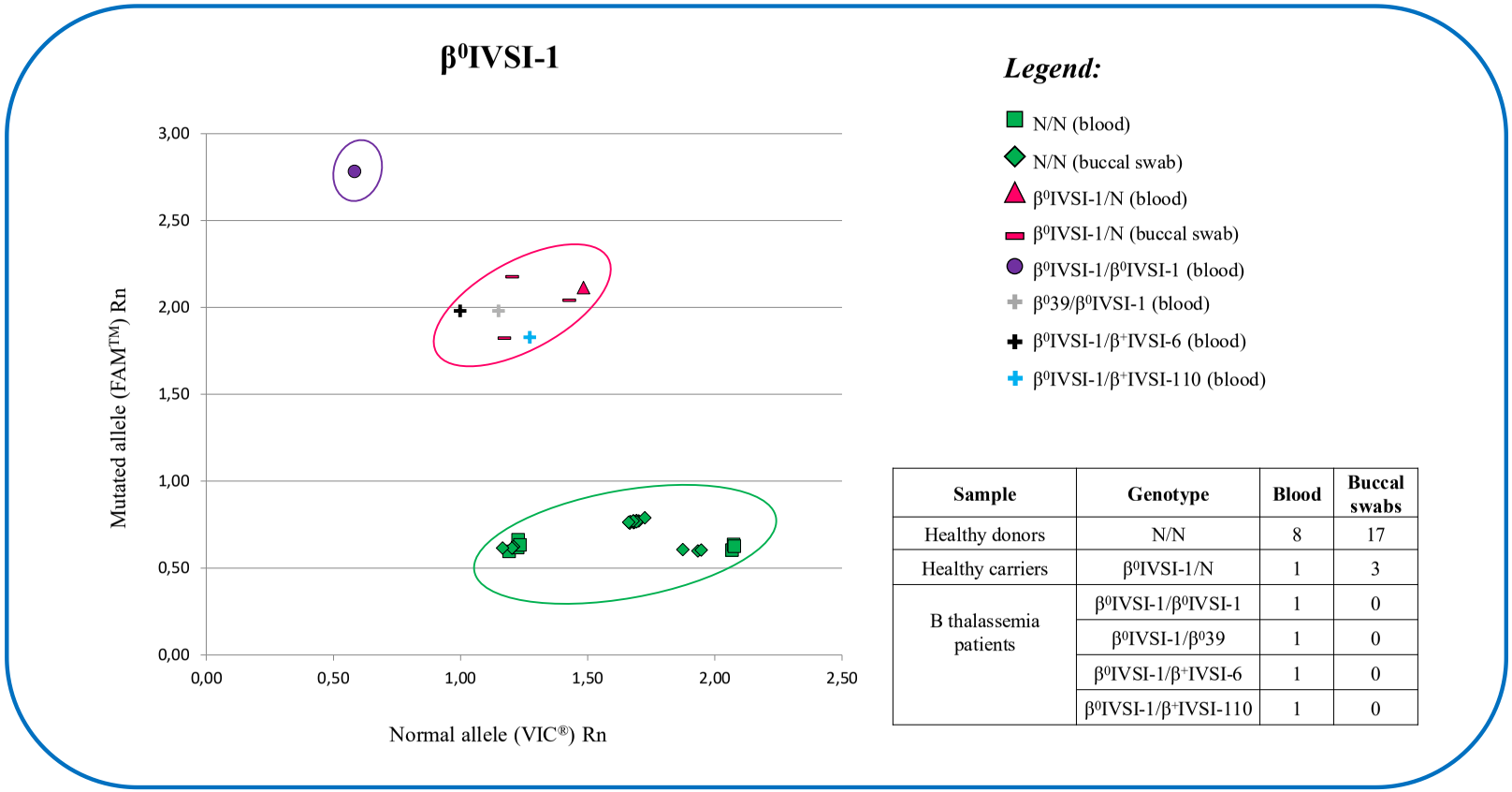
Sample	Genotype	Blood	Buccal swabs
Healthy donors	N/N	8	17
Healthy carriers	β+IVSI-110/N	8	3
β thalassemia patients	β+IVSI-110/ β+IVSI-110	5	0
	β+IVSI-110/β⁰IVSI-1	1	0
	β+IVSI-110/β⁰39	1	0
	β+IVSI-110/β+IVSI-6	1	0

Figure 4.22. Allelic discrimination plot resulting from validation analyses for β+IVSI-110 genotyping assay. The genomic DNAs extracted from blood and buccal swabs belonging to healthy donors, healthy carriers and β thalassemia patients, double heterozygous and homozygous, for the β+IVSI-110 mutation, were analyzed with the specific genotyping assay. The normalized end-point fluorescence (Rn) values generated by the VIC® labeled probe for the normal allele and by the FAM™ labeled probe for the mutated allele, are reported along the x-axis and the y-axis, respectively. Three distinct samples'groups are identified: healthy subjects (green circle), having a high value of VIC® and a low value of FAM™ fluorescence; homozygous patients (violet circle), characterized by an high FAM™ and a low VIC® fluorescence values; healthy carriers and double heterozygous patients (pink circle), having similar values of FAM™ and VIC® fluorescences. The table shows the number of samples, deriving from blood or buccal swabs, analyzed for each genotype.



Sample	Genotype	Blood	Buccal swabs
Healthy donors	N/N	8	17
Healthy carriers	β+IVSI-6/N	5	2
B thalassemia patients	β+IVSI-6/β+IVSI-6	2	0
	β+IVSI-6/B0IVSI-1	1	0
	β+IVSI-6/β039	1	0
	β+IVSI-6/β+IVSI-110	1	0

Figure 4.23. Allelic discrimination plot resulting from validation analyses for β+IVSI-6 genotyping assay. The genomic DNAs extracted from blood and buccal swabs belonging to healthy donors, healthy carriers and β thalassemia patients, double heterozygous and homozygous, for the β+IVSI-6 mutation, were analyzed with the specific genotyping assay. The normalized end-point fluorescence (Rn) values generated by the VIC® labeled probe for the normal allele and by the FAM™ labeled probe for the mutated allele, are reported along the x-axis and the y-axis, respectively. Three distinct samples'groups are identified: healthy subjects (green circle), having a high value of VIC® and a low value of FAM™ fluorescence; homozygous patients (violet circle), characterized by an high FAM™ and a low VIC® fluorescence values; healthy carriers and double heterozygous patients (pink circle), having similar values of FAM™ and VIC® fluorescences. The table shows the number of samples, deriving from blood or buccal swabs, analyzed for each genotype.



Sample	Genotype	Blood	Buccal swabs
Healthy donors	N/N	8	17
Healthy carriers	β⁰IVSI-1/N	1	3
B thalassemia patients	β⁰IVSI-1/β⁰IVSI-1	1	0
	β⁰IVSI-1/β⁰39	1	0
	β⁰IVSI-1/β⁺IVSI-6	1	0
	β⁰IVSI-1/β⁺IVSI-110	1	0

Figure 4.24. Allelic discrimination plot resulting from validation analyses for β⁰IVSI-1 genotyping assay. The genomic DNAs extracted from blood and buccal swabs belonging to healthy donors, healthy carriers and β thalassemia patients, double heterozygous and homozygous, for the β⁰IVSI-1 mutation, were analyzed with the specific genotyping assay. The normalized end-point fluorescence (Rn) values generated by the VIC® labeled probe for the normal allele and by the FAM™ labeled probe for the mutated allele, are reported along the x-axis and the y-axis, respectively. Three distinct samples'groups are identified: healthy subjects (green circle), having a high value of VIC® and a low value of FAM™ fluorescence; homozygous patients (violet circle), characterized by an high FAM™ and a low VIC® fluorescence values; healthy carriers and double heterozygous patients (pink circle), having similar values of FAM™ and VIC® fluorescences. The table shows the number of samples, deriving from blood or buccal swabs, analyzed for each genotype.

Figures 4.21-4.24 display the allelic discrimination plots generated by the genotyping assays specific for the $\beta^0$39 (**Figure 4.21**), β^+IVSI-110 (**Figure 4.22**), β^+IVSI-6 (**Figure 4.23**) and β^0IVSI-1 (**Figure 4.24**). All the diagrams show that, in according to their genotype, the samples are arranged into three major and distinct groups in the allelic discrimination plot. Samples from healthy subjects (N/N), having a high value of VIC® fluorescence and a low value of FAM™ fluorescence, are arranged in the lower right position (green circle). In contrast, homozygous samples for a specific mutation (M/M), are arranged in the upper left position (violet circle) characterized by an high FAM™ and a low VIC® fluorescence values, respectively. Samples from healthy carriers (N/M) and double heterozygous patients, giving similar values of FAM™ and VIC® fluorescences, are placed in the middle (pink circle).

The expected distribution of values was obtained for all the analyzed samples, despite the low number of mutated homozygous specimens available for every mutation, and some experimental variability in the results within single genotype groups.

In conclusion, once set the experimental conditions, the four genotyping assays were completely able to distinguish the different possible genotypes for each analyzed mutation, demonstrating the suitability of each genotyping assay for molecular discrimination.

In addition, in all cases no differences were found among results obtained by DNA samples purified by blood and by buccal swabs, indicating that the latter may be considered a very interesting simple and non-invasive alternative to blood sampling as a source of genomic DNA for molecular diagnosis, improving the subject's compliance. In fact, even if a lower amount of DNA is recovered, the good DNA quality useful for easy amplification, together with the high sensitivity of the technique, allow a correct identification of possible point mutations.

4.2.2.4. Diagnosis of β thalassemia mutations

Various techniques based on PCR or genomic sequencing are currently used for molecular post-natal diagnostic screening of β thalassemia, but all of them are time-consuming and labor-intensive, and require technical expertise, suggesting the need for new easier and inexpensive technologies.

In order to evaluate whether the genotyping assays could be efficiently employed as a simple and fast method in molecular diagnostic for β thalassemia, after validation all the four genotyping assays were used to analyze unknown samples (**Figure 4.18**).

Twenty-five specimens from blood and buccal swabs with unknown genotype were chosen among the collected DNAs from patients affected and were undergone to four different genotyping reactions, each with one of the optimized genotyping assay for the specific β

thalassemia mutation, with the aim to identify the pathogenic alterations (**Supplementary table 1**).

The **Figure 4.25** shows the allelic discrimination plots obtained from every assay, specific for the $\beta^0$39 (**Figure 4.25.A**), β^+IVSI-110 (**Figure 4.25.B**), β^+IVSI-6 (**Figure 4.25.C**) and β^0IVSI-1 (**Figure 4.25.D**) mutations, respectively.

Each unknown sample was identified with a black dot. In addition, to verify the adequacy of the analysis and to identify the positions of the expected fluorescence values from each genotype in the plot, for every assay some references of different genotypes were chosen among those used to validate the assay, represented by red indicators N/N (triangle), N/M (diamond) and M/M (square).

As expected, in all the plots the values obtained by the unknown samples arranged in three groups defined and verified through the reference samples, and identified by coloured circles including the values: the N/N group at the bottom right (green circle), the M/M group at the top left (violet circle), the N/M in the middle (pink circle).

So, according to the position of the unknown sample in every plot, it has been possible to deduce its genotype for each analyzed mutation.

The samples in the green circles appear not to be mutated for the specific considered mutation, but they will probably carry therefore another alteration on both the alleles. The samples in the pink circles seemed to be heterozygous for the specific considered mutation, so only one allele carries this alteration, and probably another mutation was expected on the other allele.

By comparing the discrimination plots obtained from each mutation (**Figure 4.25**) it has been formulated a hypothesis about the genotypes of the 25 analyzed specimens: the samples in the pink circles present in two discrimination plots for two specific mutations, seemed to be heterozygous for those alterations.

For all the samples, the resulting hypothesized genotypes are shown in **Table 4.8**.

In order to confirm the diagnostic outcomes, the 25 unknown samples where then subjected to Sanger's DNA sequencing of the β globin gene, by using the same experimental protocol previously described. The genotype confirmation is reported in **Table 4.8** as well.

All the results were confirmed, demonstrating that genotyping assays might be efficiently employed for post-natal diagnosis of point mutations, also after non-invasive DNA sampling method as a buccal swab.

Overall, in conclusion, a simple inexpensive and versatile method, for the post-natal diagnosis of β thalassemia mutations was developed.

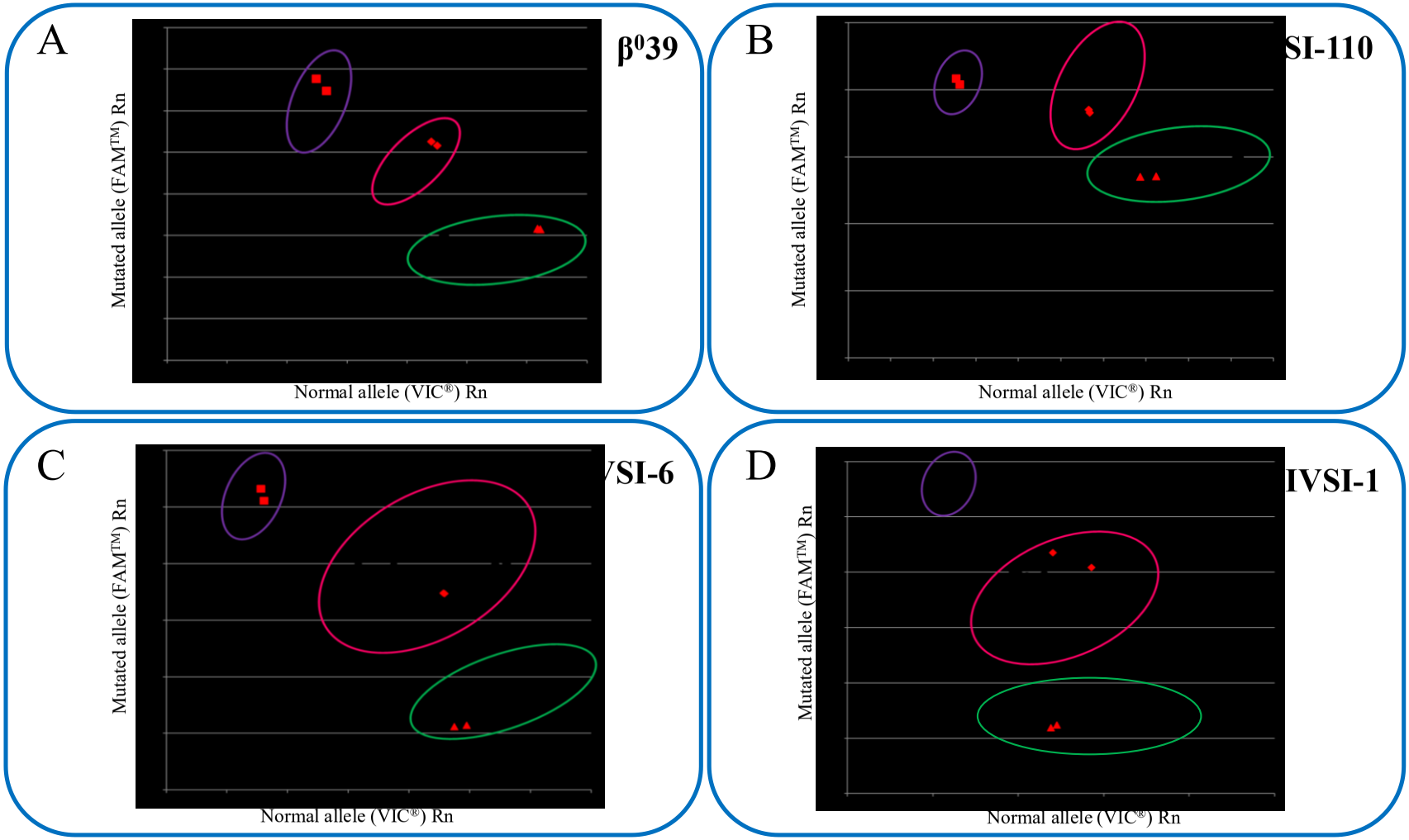


Figure 4.25. Allelic discrimination plots obtained from 25 total samples of genomic DNA with unknown genotype. The genomic DNAs, with unknown genotype (black dots), extracted from β thalassemia patients were analyzed with $\beta^0$39 (A), β^+IVSI-110 (B), β^+IVSI-6 (C), β^0IVSI-1 (D) genotyping assays. For each plot, the normalized end-point fluorescence (Rn) values generated by the VIC® labeled probe for the normal allele and the FAM™ labeled probe for the mutated allele are reported along the x-axis and the y-axis, respectively. Some reference samples of different known genotype are also displayed as red indicators [Modified from: Breveglieri et al., 2017].

# Sample	Genotype resulting from genotyping assays	Genotype confirmation by DNA sequencing
1	β^{+}IVSI-6/β^{+}IVSI-110	√
2	β^{+}IVSI-110/β^{0}39	√
3	β^{+}IVSI-1/β^{0}39	√
4	β^{+}IVSI-1/β^{0}39	√
5	β^{+}IVSI-6/β^{0}39	√
6	β^{+}IVSI-110/β^{0}39	√
7	β^{+}IVSI-6/β^{0}39	√
8	β^{+}IVSI-1/β^{0}39	√
9	β^{+}IVSI-110/β^{+}IVSI-110	√
10	β^{0}39/β^{0}39	√
11	β^{+}IVSI-110/β^{0}39	√
12	β^{0}IVSI-1/β^{+}IVSI-110	√
13	β^{+}IVSI-110/β^{+}IVSI-110	√
14	β^{0}39/β^{0}39	√
15	β^{0}39/β^{0}39	√
16	β^{+}IVSI-6/β^{+}IVSI-110	√
17	β^{+}IVSI-110/β^{+}IVSI-110	√
18	β^{0}IVSI-1/β^{+}IVSI-6	√
19	β^{+}IVSI-6/β^{+}IVSI-6	√
20	β^{+}IVSI-6/β^{+}IVSI-6	√
21	β^{0}IVSI-1/β^{+}IVSI-6	√
22	β^{+}IVSI-6/β^{+}IVSI-6	√
23	β^{0}IVSI-1/β^{+}IVSI-6	√
24	β^{0}IVSI-1/β^{+}IVSI-6	√
25	β^{0}IVSI-1/ β^{0}IVSI-1	√

Table 4.8. List of β globin allele genotypes resulting by genotyping assays. The analysis was performed for 25 samples genomic DNA from β thalassemia patients. For each sample the genotype resulting from genotyping assays and the genotype confirmation by DNA sequencing are indicated. √, confirmed.

Also, this method could be applied to any genetic disease caused by a point mutation.

In addition, correct genotypes have been also efficiently determined by DNA extracted from buccal swabs, thus demonstrating that this non-invasive sampling method can be efficiently used.

4.2.3. Non-invasive pre-natal diagnosis of β thalassemia mutations by using genotyping assays

Invasive techniques (for instance amniocentesis or chorionic villus sampling) are the currently employed sampling methods for pre-natal diagnosis and the only ones to be legally recognized for dealing with complex decisions about pregnancy. However, they carry a risk of miscarriage, which cannot be underestimated [Reece, 1999].

For this reason, in recent years, non-invasive pre-natal diagnosis has become increasingly importance, because, although it retains only a predictive/probabilistic value, it allows to investigate the state of fetal health without any risk for the fetus or the mother [Webb et al., 2012]. It is based on the discovery of ccffDNA in maternal circulation [Lo et al., 1997]. Even though a huge number of studies has been performed with the aim to develop experimental approaches for detecting, at an early gestational age, the possible presence of fetal abnormalities, no commercial assays are available to recognize point mutations. The non-invasive screening tests offered to pregnant women can detect only aneuploidies, fetal sex, small deletions or insertions [Norbury & Norbury, 2008]. In addition, the techniques employed in commercially-available screening tests are laborious and expensive [Sekizawa & Saito, 2001; Wright & Burton, 2009; Bianchi, 2012].

Therefore, after demonstrating the suitability of the simple and inexpensive genotyping assays for molecular post-natal diagnosis, the attention was focused on their possible application to non-invasive pre-natal screening, in order to detect possible fetal point mutations, responsible for β thalassemia, inherited from the father.

The identification of paternally inherited sequences has been one of the first issues addressed after the discovery of ccffDNA in maternal plasma, because of the relative easiness of detection as they are not present in maternal contaminating DNA [Tang et al., 1999; Sekizawa & Saito, 2001; Wright & Burton, 2009; Bianchi, 2012].

Moreover, there is a great interest in β thalassemia because it is a widely studied monogenic disease with a relatively high incidence in the Mediterranean area [Cao & Galanello, 2010], where it is not so difficult to find couples of healthy carrier parents needing pre-natal diagnosis. In particular, the benefit of finding pathogenic mutations would be not only to

take appropriate decision about pregnancy, but especially to plan suitable and early therapeutic approach for the newborn.

4.2.3.1. Samples collection

In order to apply genotyping assays for the non-invasive pre-natal diagnosis, almost 300 samples, of whole peripheral blood from pregnant women, were collected, after informed consent. This was done in collaboration with the *Laboratory of Chemical and Clinical Analysis and Microbiology* of *S. Anna* University Hospital (Cona, Ferrara), directed by Dr. Giovanni Guerra. In particular, Dr. Patrizia Pellegatti has been responsible for addressing pregnant women and for promptly carrying collected blood samples to the laboratory. A progressive number was assigned to each specimen, to keep the donors anonymous.

Within a few hours from blood sampling, plasma was prepared, and stored in single-use aliquots at -80°C. In fact many studies describe that plasma is the election source for the best recovery of ccffDNA, that can be found in maternal circulation together with maternal cfDNA and a few fetal cells [Lo et al., 1997]. Lo et al. demonstrated that fetal DNA concentration was very similar in maternal plasma and serum but in serum there is a larger maternal DNA contribution causing a less robust fetal DNA detection [Lo et al., 1998].

So, a biobank was established including all plasma samples obtained by pregnant women, showing a wide variability in terms of weeks of gestation. Indeed the interest was not only in late gestation to optimize and develop diagnostic methods, but especially in early gestation to which pre-natal diagnosis should be applied. In the samples collection a careful attention has been made to select specimens from future parents carrier of β thalassemia, whose DNA carries a pathogenic point mutation possibly inherited by children: only the mother, only the father of both the parents.

In particular, in this part of the study, the attention was focused on the detection of alterations inherited from the father, easier to be identified in circulating DNA because absent in the maternal DNA. So, in those cases where the future father was a healthy carrier, if possible, a biological sample (blood or buccal swab) from him was obtained, in order to be able to extract his genomic DNA and to characterize the β thalassemia mutation. The most difficult aspect in this case was to get the paternal biological material, as the future father often was not present at the time of the blood sampling of the pregnant woman: as a consequence, unfortunately several paternal genotypes are still unknown.

4.2.3.2. Experimental strategy

The experimental strategy in order to apply genotyping assays for non-invasive pre-natal diagnosis is shown in **Figure 4.26**. After selecting samples deriving from healthy mother and carrier father, the first goal was to determine the mutation of the father, by sequencing the β globin gene after DNA purification from blood or buccal swab (**Figure 4.26.A**). Then, the total circulating cfDNA from maternal plasma was extracted in order to determine if the fetus had inherited the paternal mutation by analysis with the genotyping assay specific for that mutation (**Figure 4.26.B**). Finally, the diagnostic outcome was confirmed extracting the newborn genomic DNA from buccal swab and sequencing the β globin gene, after the birth (**Figure 4.26.C**).

4.2.3.3. Detection of β thalassemia mutations in carrier father by DNA sequencing

A total of 26 samples of ccffDNA were analyzed with a carrier father, differing for the gestational age of the pregnant woman (**Figure 4.27**). The samples are grouped in four gestational ranges according to the pregnant woman gestational week at the time of blood collection: 39-30 weeks (in pink), 29-20 weeks (in yellow), 19-10 weeks (in green) and 9-4 weeks (in light blue). The samples belonging to 39-30 weeks' range represent the 19% (n=5) of the total samples; instead, the samples belonging to 29-20 weeks' range constitute the 38% (n=10), percentage follows by 31% (n=8) and 12% (3) corresponding to the samples belonging to 19-10 and 9-4 weeks' range, respectively.

While the majority of specimens were collected at late gestation, some earlier samples were available as well, to possibly evaluate the assay sensitivity, as it is known that the concentration of ccffDNA is increasing in maternal circulation with increasing gestational age [Lun et al., 2008].

In order to identify the β thalassemia mutations of the 26 carrier fathers, a buccal swab or a blood sample was collected from the future father and genomic DNA was extracted by the use of QIAamp® DNA Blood Mini Kit (Quiagen). Then, it was employed as a template for PCRs, in order to amplify the β globin gene before performing Sanger's DNA sequencing, according to what previously described for mutation detection in carriers or patients affected by β thalassemia for post-natal diagnosis.

The tables on the right part of **Figure 4.27** report the resulting paternal genotype and are divided according to the gestational range.

The identified mutations, in the β globin gene were the most frequent alterations causing β thalassemia in the Mediterranean area [Cao & Galanello, 2010], and also within this small

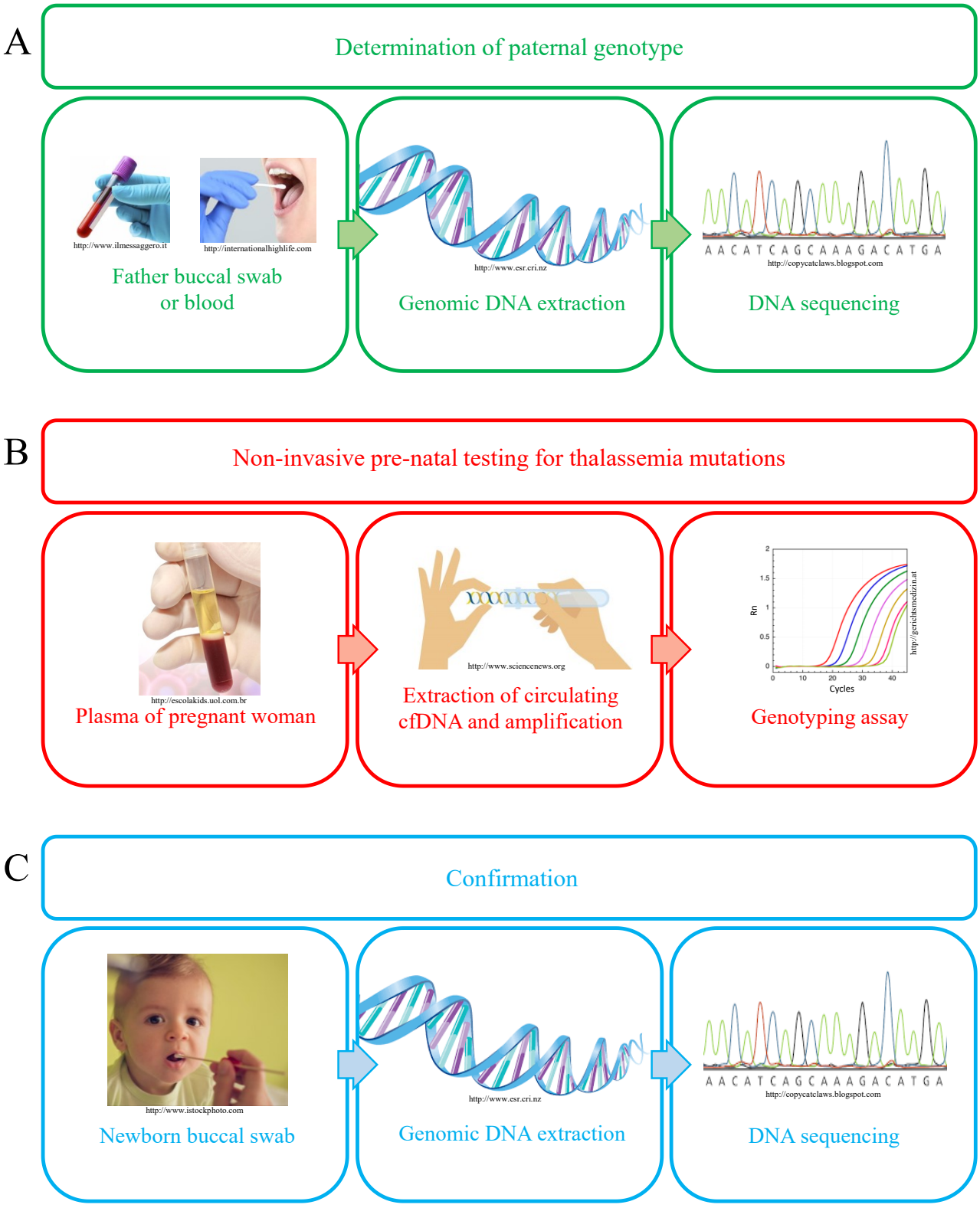
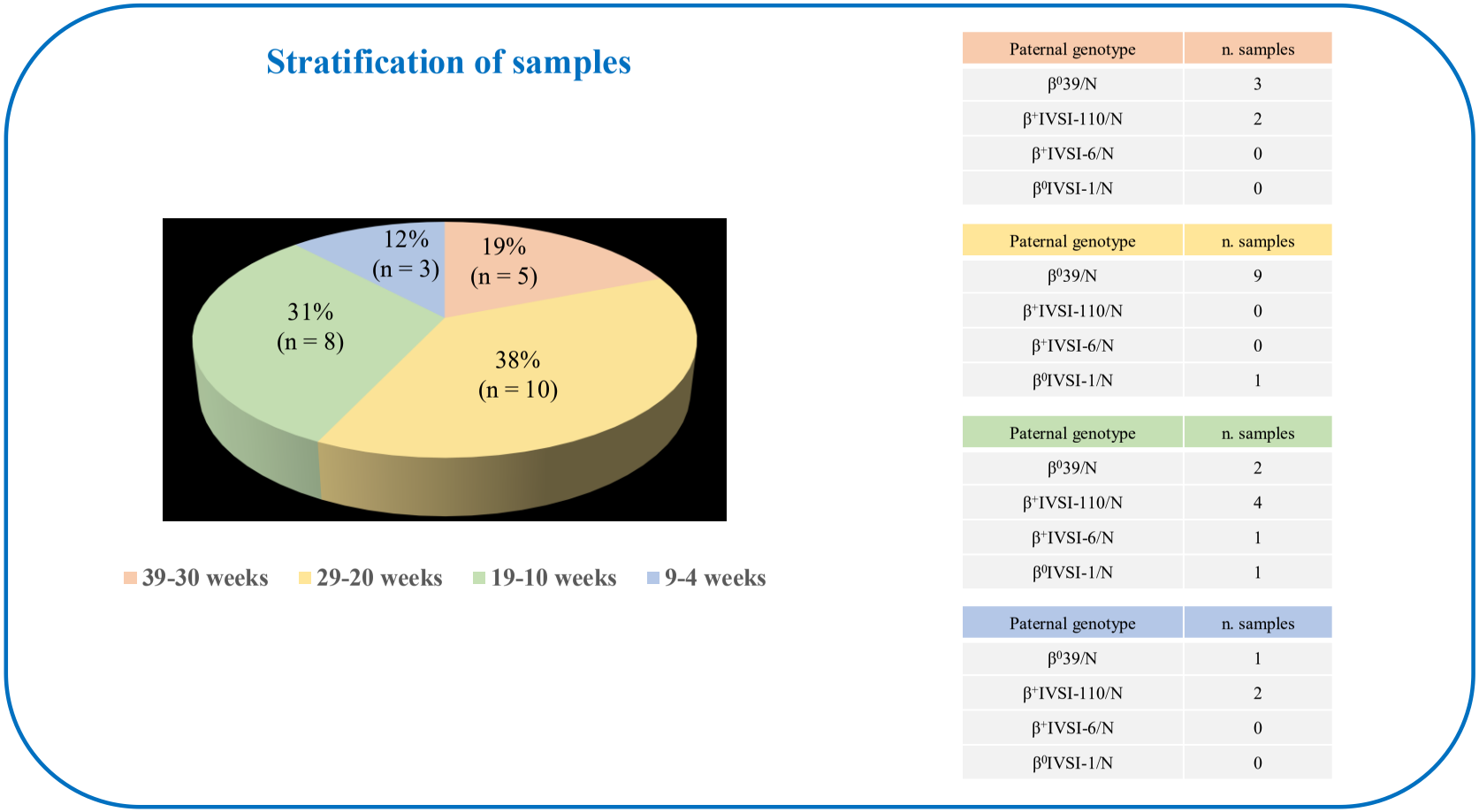


Figure 4.26. Experimental strategy for the development of a non-invasive pre-natal testing technique based on genotyping assays. After determination of the paternal mutation by DNA sequencing of genomic DNA extracted from blood or buccal swab (A), the total circulating cfDNA was extracted from maternal plasma and characterized by genotyping assays (B). After the recovery of the newborn buccal swab, the diagnosis was confirmed by DNA sequencing (C).



Paternal genotype	n. samples
$\beta^0$39/N	3
β^+IVSI-110/N	2
β^+IVSI-6/N	0
β^0IVSI-1/N	0

Paternal genotype	n. samples
$\beta^0$39/N	9
β^+IVSI-110/N	0
β^+IVSI-6/N	0
β^0IVSI-1/N	1

Paternal genotype	n. samples
$\beta^0$39/N	2
β^+IVSI-110/N	4
β^+IVSI-6/N	1
β^0IVSI-1/N	1

Paternal genotype	n. samples
$\beta^0$39/N	1
β^+IVSI-110/N	2
β^+IVSI-6/N	0
β^0IVSI-1/N	0

Figure 4.27. Stratification of the 26 samples with a father carrier of a β thalassemia mutation, chosen for the genotyping analysis and paternal genotype obtained from DNA sequencing. The 26 plasma samples from pregnant women are divided according to the gestational age in four ranges: 39-30 (in pink), 29-20 (in yellow), 19-10 (in green) and 9-4 (in light blue) gestational weeks, respectively. For each range, the percentage and the number of samples are indicated. The tables in the right part of the figure list the β globin allele genotypes resulting from sequencing of carrier paternal genomic DNA divided according to the gestational range. For each genotype of each group, the number of specimens is indicated.

group of samples the relative abundance was the same: the majority of fathers carried the $\beta^0$39 mutation, a lower number the β^+IVSI-110 mutation, and only a few the β^+IVSI-6 and β^0IVSI-1 mutations.

The next step was to assay the genotyping assays specific for each mutation to look for the alteration in circulating DNA extracted from maternal plasma, containing fetal DNA.

4.2.3.4. Purification and amplification of circulating DNA from maternal plasma

In order to obtain the fetal DNA for genotyping analyses, the circulating DNA was extracted from 2 mL of plasma derived from those 26 pregnant women whose partner had been characterized for the β globin gene genotype (**Figure 4.27**). The extraction was carried out by using the commercial QIAamp® DSP Virus Spin Kit (Qiagen), one of the best purification method in recovery and purity as reported in literature [Clausen et al., 2007; Repisaka et al., 2013]. The purified circulating cfDNA contains both maternal and fetal sequences.

An important issue to consider when dealing with ccffDNA extracted from maternal plasma, is its low amount, especially when compared to maternal background. To overcome this problem and avoid false negative results, a pre-amplification approach was assayed aimed to increase the amount of target fetal sequences before the genotyping detection.

To this aim, a pair of primers for each β thalassemia mutation, to be detected, was designed. As circulating cfDNA is expected to be highly fragmented, with fragments less than 300 bp in length, particular attention was payed in looking for primers amplifying short sequences, as well as in designing them upstream and downstream the region amplified by each genotyping assay.

Then, every pre-amplification reaction needed to be optimized, in order to find the best conditions to obtain both an efficient amplification of the template DNA, and a subsequent properly working genotyping assay.

So, 1 µL of circulating DNA, extracted from maternal plasma was employed for each PCR reaction: this low volume was sufficient to ensure a detectable amplification and was found to prevent a possible PCR inhibition in case of presence of residual contaminants, reducing the sample consumption. Also the number of amplification cycles was tested: for each PCR 30 cycles were finally performed, because a too high amount of amplicon was found to inhibit the genotyping assay. Finally, the annealing temperature, in order to achieve specific hybridization between primers and the DNA sequence of interest, was set for each PCR, so that the two primers specifically bind to the template DNA: 61°C for $\beta^0$39 primers, and 55°C for all the others (β^+IVSI-110, β^+IVSI-6 and β^0IVSI-1).

While the amplification conditions were the same set-up for post-natal diagnosis, the expected results were obtained when 1 μL of template PCR was pre-amplified before genotyping assay.

4.2.3.5. Non-invasive prenatal testing of β thalassemia mutations inherited from the father by genotyping assays

As reported in literature [Wright & Burton, 2009; Vermeulen et al., 2017], the detection of fetal mutations inherited from the father should be quite feasible because they are not present in maternal DNA, that is the main component of circulating DNA extracted from maternal plasma.

In the case of genotyping assays, when the mutation is transmitted by the father, maternal circulating DNA does not carry the mutated sequence and so will be amplified only by the VIC® labeled probe complementary to the normal sequence. Therefore, if the fetus is N/N like the mother, only an amplification signal from the VIC® labeled probe is expected; while if the fetus has inherited the father mutation, his circulating DNA, in addition to be amplified by the normal VIC® labeled probe, will be recognized also by the FAM™ labeled probe carrying the mutated sequence. In conclusion, the generation of a FAM™ fluorescence signal, related to the presence of a mutated allele, should occur only in the case that the fetus has inherited the father mutation.

Each circulating DNA among the 26 samples previously chosen, after pre-amplification, underwent the genotyping assay specific for the relative paternal mutation.

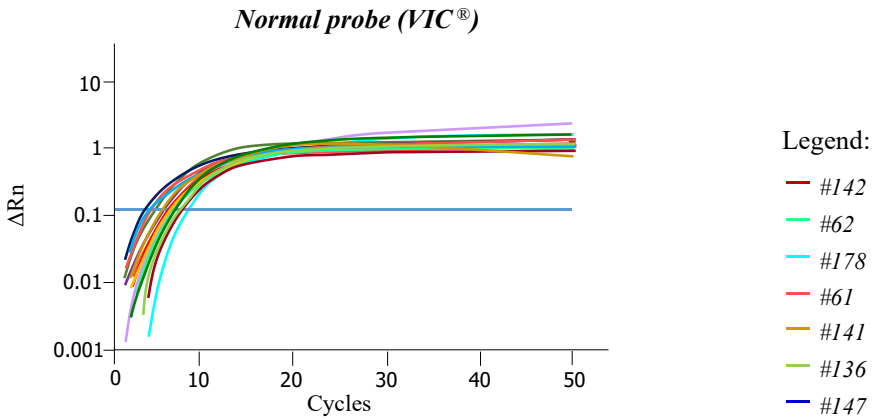
The results are reported in **Figures 4.28-4.31**, one for the genotypic analysis of every mutation: $\beta^0$39 (**Figure 4.28**), β^+IVSI-110 (**Figure 4.29**), β^+IVSI-6 (**Figure 4.29**), and β^0IVSI-1 (**Figure 4.29**).

All the samples generated amplification curves when the normal VIC® labeled probe was considered (**Figures 4.28.A**, **4.29.A**, **4.30.A**, **4.31.A**). This was expected as the fetus should carry almost a normal allele; in addition, both the maternal normal alleles would be detected. The FAM™ labeled mutated probe could instead amplify only the mutated allele possibly inherited form the father.

As regards the father carrier of $\beta^0$39 mutation (**Figure 4.28**), 15 samples were collected. The FAM™ labeled mutated probe (**Figure 4.28.B**) allowed to identify mutated alleles only for samples #62, #178, #61, #287, #132, #31, #36, #186, #193, #217 and #265, suggesting a heterozygous $\beta^0$39/N genotype. On the contrary, for the other four samples (#142, #141, #136, #1479) no mutated curves were produced, in accordance with a N/N genotype.

β⁰39

A



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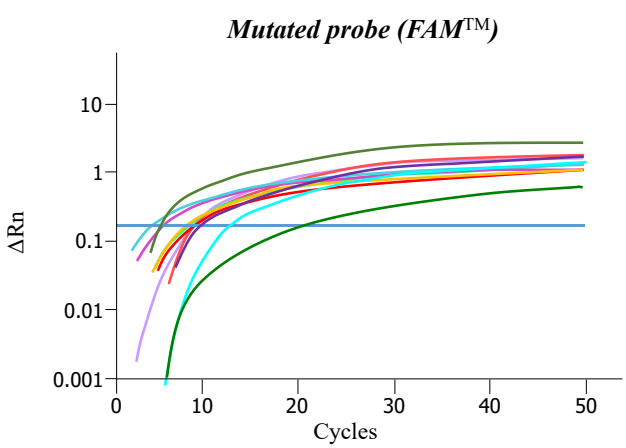
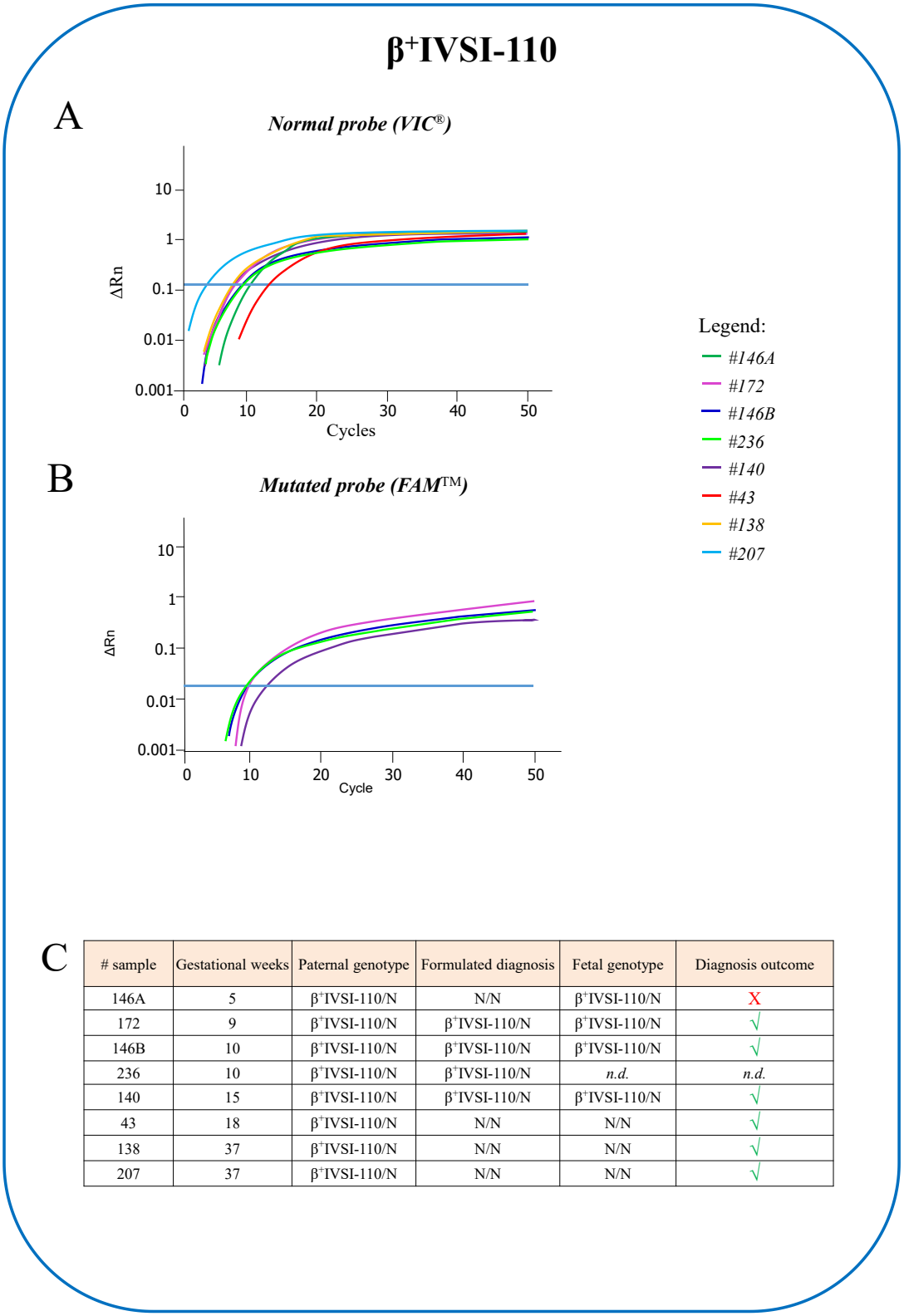


C

# sample	Gestational weeks	Paternal genotype	Formulated diagnosis	Fetal genotype	Diagnosis outcome
142	5	β⁰39/N	N/N	β⁰39/N	X
62	13	β⁰39/N	β⁰39/N	β⁰39/N	√
178	14	β⁰39/N	β⁰39/N	β⁰39/N	√
61	21	β⁰39/N	β⁰39/N	β⁰39/N	√
141	22	β⁰39/N	N/N	N/N	√
136	23	β⁰39/N	N/N	N/N	√
147	24	β⁰39/N	N/N	N/N	√
287	24	β⁰39/N	β⁰39/N	β⁰39/N	√
132	25	β⁰39/N	β⁰39/N	β⁰39/N	√
31	26	β⁰39/N	β⁰39/N	β⁰39/N	√
36	28	β⁰39/N	β⁰39/N	β⁰39/N	√
186	29	β⁰39/N	β⁰39/N	β⁰39/N	√
193	33	β⁰39/N	β⁰39/N	β⁰39/N	√
217	35	β⁰39/N	β⁰39/N	β⁰39/N	√
265	39	β⁰39/N	β⁰39/N	β⁰39/N	√

Figure 4.28. Amplification curves obtained and list of samples analyzed by β⁰39 genotyping assay from circulating DNA extracted from maternal plasma with father carrier for β⁰39 point mutation. qRT-PCR with β⁰39 genotyping assay, containing a normal probe (VIC®) (A) and a mutated probe (FAM™) (B) were performed for circulating DNA extracted from maternal plasma where the father was a carrier of β⁰39 thalassemia mutation. The plots show the ΔRn (the normalization of the Rn obtaining by subtracting the baseline) values as a function of the number of amplification cycles, while the threshold line is drawn in blue. Samples are listed according to increasing gestational ages and are marked with different colours. The table (C) reports for each sample, assayed with the β⁰39 genotyping assay, the gestational week, the paternal genotype, the formulated diagnosis by genotyping assay, the fetal genotype and the diagnosis outcome. The amplification was performed by using the StepOne™ Real-Time PCR System. √, confirmed. X, wrong [Modified from: Breveglieri et al., 2017].



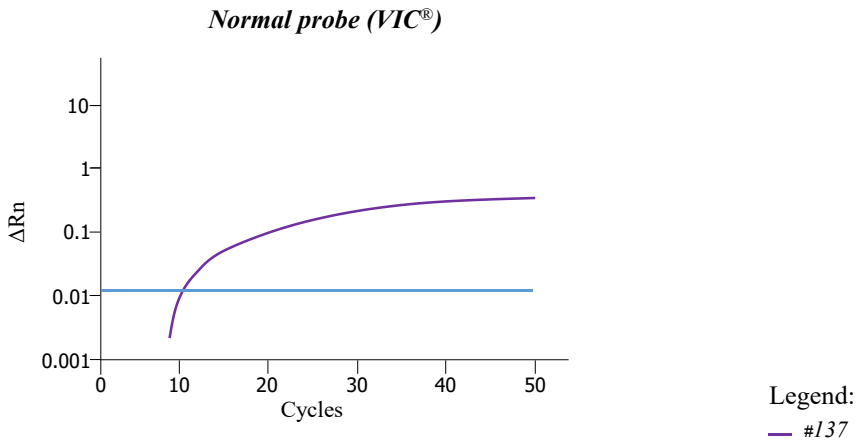
C

# sample	Gestational weeks	Paternal genotype	Formulated diagnosis	Fetal genotype	Diagnosis outcome
146A	5	β+IVSI-110/N	N/N	β+IVSI-110/N	X
172	9	β+IVSI-110/N	β+IVSI-110/N	β+IVSI-110/N	√
146B	10	β+IVSI-110/N	β+IVSI-110/N	β+IVSI-110/N	√
236	10	β+IVSI-110/N	β+IVSI-110/N	*n.d.*	*n.d.*
140	15	β+IVSI-110/N	β+IVSI-110/N	β+IVSI-110/N	√
43	18	β+IVSI-110/N	N/N	N/N	√
138	37	β+IVSI-110/N	N/N	N/N	√
207	37	β+IVSI-110/N	N/N	N/N	√

Figure 4.29. Amplification curves obtained and list of samples analyzed by β+IVSI-110 genotyping assay from circulating DNA extracted from maternal plasma with father carrier for β+IVSI-110 point mutation. qRT-PCR with β+IVSI-110 genotyping assay, containing a normal probe (VIC®) (A) and a mutated probe (FAM™) (B) were performed for circulating DNA extracted from maternal plasma where the father was a carrier of β+IVSI-110 thalassemia mutation. The plots show the ΔRn (the normalization of the Rn obtaining by subtracting the baseline) values as a function of the number of amplification cycles, while the threshold line is drawn in blue. Samples are listed according to increasing gestational ages and are marked with different colours. #146A and #146B refer to samples collected from the same pregnant woman at different gestational ages: 5 weeks and 10 weeks, respectively. The table (C) reports for each sample, assayed with the β+IVSI-110 genotyping assay, the gestational week, the paternal genotype, the formulated diagnosis by genotyping assay, the fetal genotype and the diagnosis outcome. The amplification was performed by using the StepOne™ Real-Time PCR System. √, confirmed. X, wrong. *n.d.*, not determined [Modified from: Breveglieri et al., 2017].

β⁺IVSI-6

A



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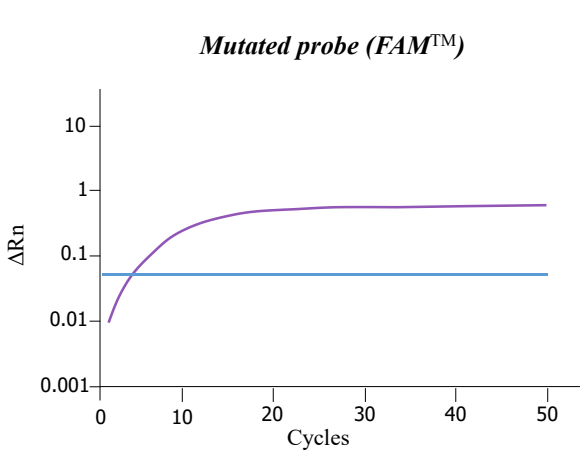


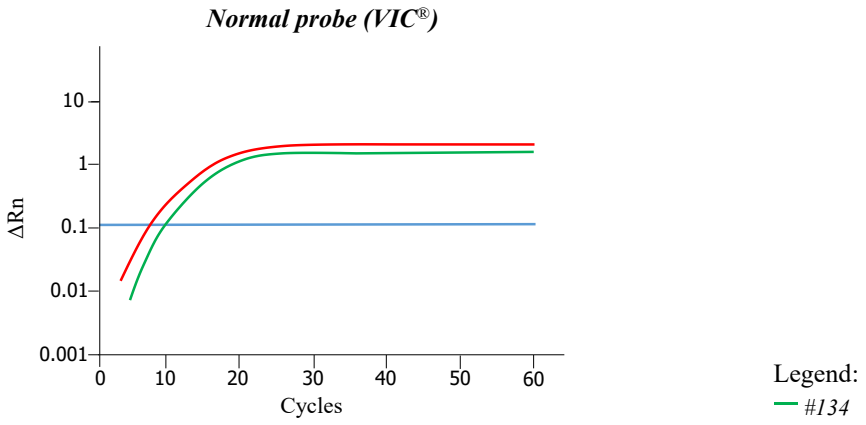
C

# sample	Gestational weeks	Paternal genotype	Formulated diagnosis	Fetal genotype	Diagnosis outcome
137	11	β⁺IVSI-6/N	β⁺IVSI-6/N	*n.d.*	*n.d.*

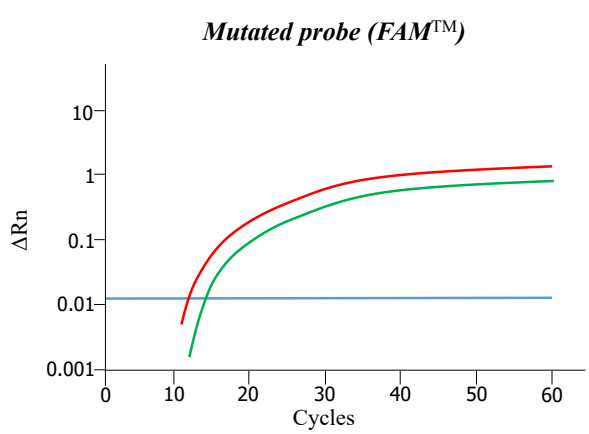
Figure 4.30. Amplification curves obtained and list of samples analyzed by β⁺IVSI-6 genotyping assay from circulating DNA extracted from maternal plasma with father carrier for β⁺IVSI-6 point mutation. qRT-PCR with β⁺IVSI-6 genotyping assay, containing a normal probe (VIC®) (A) and a mutated probe (FAM™) (B) were performed for circulating DNA extracted from maternal plasma where the father was a carrier of β⁺IVSI-6 thalassemia mutation. The plots show the ΔRn (the normalization of the Rn obtaining by subtracting the baseline) values as a function of the number of amplification cycles, while the threshold line is drawn in blue. The table (C) reports for each sample, assayed with the β⁺IVSI-6 genotyping assay, the gestational week, the paternal genotype, the formulated diagnosis by genotyping assay, the fetal genotype and the diagnosis outcome. The amplification was performed by using the StepOne™ Real-Time PCR System. *n.d.*, not determined [Modified from: Breveglieri et al., 2017].

β⁰IVSI-1

A



B



C

# sample	Gestational weeks	Paternal genotype	Formulated diagnosis	Fetal genotype	Diagnosis outcome
134	17	β⁰IVSI-1/N	β⁰IVSI-1/N	β⁰IVSI-1/N	√
151	28	β⁰IVSI-1/N	β⁰IVSI-1/N	β⁰IVSI-1/N	√

Figure 4.31. Amplification curves obtained and list of samples analyzed by β⁰IVSI-1 genotyping assay from circulating DNA extracted from maternal plasma with father carrier for β⁰IVSI-1 point mutation. qRT-PCR with β⁰IVSI-1 genotyping assay, containing a normal probe (VIC®) (A) and a mutated probe (FAM™) (B) were performed for circulating DNA extracted from maternal plasma where the father was a carrier of β⁰IVSI-1 thalassemia mutation. The plots show the ΔRn (the normalization of the Rn obtaining by subtracting the baseline) values as a function of the number of amplification cycles, while the threshold line is drawn in blue. Samples are listed according to increasing gestational ages and are marked with different colours. The table (C) reports for each sample, assayed with the β⁰IVSI-1 genotyping assay, the gestational week, the paternal genotype, the formulated diagnosis by genotyping assay, the fetal genotype and the diagnosis outcome. The amplification was performed by using the StepOne™ Real-Time PCR System. √, confirmed [Modified from: Breveglieri et al., 2017].

For the confirmation of the diagnostic outcome, it was necessary to wait until the newborn birth, sequencing the β globin gene of DNAs extracted from buccal swabs, all the genotypes were confirmed (**Figure 4.28.C**), except sample #142: in this case, the actual baby's genotype was $\beta^0$39/N, but no mutated curves was obtained (**Figure 4.28.B**). Perhaps this depends on the too low amount of fetal DNA, probably due to the early period of pregnancy where the sample was collected: 5 gestational weeks.

The same problem has arisen in the β^+IVSI-110 diagnosis (**Figure 4.29**), where no mutated curve was produced by sample #146A (**Figure 4.29.B**), in contrast with his β^+IVSI-110/N genotype, as found after postnatal baby's DNA sequencing: also this sample had been collected after only 5 weeks of gestation. To corroborate the hypothesis that the wrong diagnosis is due to the early period of pregnancy, the sample #146B collected from the same pregnant woman at a different gestational age (10 weeks), produced a mutated curve in accordance with a β^+IVSI-110/N genotype.

Regarding the other samples, the FAM^{TM} labeled mutated probe allowed to identify mutated alleles only for the samples #172, #236 and #140, suggesting heterozygous β^+IVSI-110/N genotype, that were then confirmed, for the samples #172 and #140, after extraction of the newborn genomic DNA from buccal swabs. It was not possible to confirm the #236 actual genotype because of the absence of the relative buccal swab (*n.d.*, not determined).

Normal genotype was deduced from the other three samples (#43, #138, #207) (**Figure 4.29.C**).

For β^+IVSI-6 mutation (**Figure 4.30**) the only sample #137 was analyzed. The pre-amplified circulating DNA generated amplification curves with both normal (**Figure 4.30.A**) and mutated (**Figure 4.30.B**) probes, so we could propose the heterozygous β^+IVSI-6/N genotype: unfortunately the diagnosis is still waiting for confirmation because we have not yet received the buccal swab of the newborn (*n.d.*, not determined) (**Figure 4.30.C**).

Finally, only two samples were collected deriving from fathers carrier of β^0IVSI-1 mutation (**Figure 4.31**), #134 and #151. For both samples, the FAM^{TM} labeled mutated probe (**Figure 4.31.B**) identified mutated alleles, showing a heterozygous β^0IVSI-1/N genotype that was then confirmed by sequencing the newborns' genomic DNAs obtained from buccal swabs (**Figure 4.31.C**).

In conclusion, these data show that the developed genotyping assays could be efficiently employed for non-invasive pre-natal diagnosis of paternally inherited β thalassemia mutations, at least until the 9th gestational week. At the 5th week of gestation the ccffDNA amount is very low and not detectable with this approach.

Anyway, the simplicity and reproducibility of TaqMan® genotyping assays permit its application in laboratories as a rapid and cost-effective diagnostic tool for the identification of common β thalassemia mutations in the Mediterranean area.
The discussed results have been recently published in *PLOS ONE* [Breveglieri et al., 2017].

In order to extend the non-invasive detection of possible fetal point mutations also to early pregnancy, as it has been reported that ccffDNA is detectable in maternal plasma from 4-5 weeks of gestation [Illanes et al., 2007; D'Aversa et al., 2018], a new approach could be proposed.
The approach is based on the new technology called ddPCR, that allows to detect mutations present only in a small fraction of template DNA, also without need of pre-amplification. The sensitivity of the technology is based on the division of the sample into a very large number of separate small volume reactions, such that to obtain no more than one target molecule in any individual reaction where the analytical technique is applied [Pinheiro et al., 2012], as previously underlined.
Therefore, this technique may also be very useful to address the problem of determining fetal mutations inherited from the mother, and both the parents, through the quantification of normal and mutated alleles in maternal plasma evaluating whether they are balanced or unbalanced. Allelic balance is expected when the fetal genotype is identical to the mother's one, whereas allelic imbalance occurs if there is an under-representation (the fetus is homozygous for the normal allele) or over-representation (the fetus is homozygous for the mutated allele) of the mutant allele with respect to the normal one [Lun et al., 2008].

4.2.4. Non-invasive pre-natal diagnosis of β thalassemia mutations by using ddPCR

The objective of the last part of the study was to apply the ddPCR advanced technology for pre-natal non-invasive diagnosis of point mutations causing β thalassemia.
As previously mentioned, the only validated techniques for pre-natal diagnosis of fetal DNA mutations require the collection of fetal material using invasive techniques that may constitute a risk of miscarriage [Reece, 1999]. But recently, thanks to the discovery of ccffDNA in maternal plasma [Lo et al., 1997], an increasing number of studies have focused on the development of more sensitive techniques suitable for the non-invasive detection of hereditary disorders in fetal DNA.

Those molecular techniques are very expensive, laborious and time-consuming, so it is necessary to develop less expensive, rapid, simple and sensitive techniques for the non-invasive pre-natal diagnosis of point mutations from ccffDNA extracted from maternal plasma.

In addition, no commercial kits for non-invasive pre-natal diagnosis are available to recognize single point mutations, as currently the non-invasive screening tests offered to pregnant women can detect only aneuploidies, fetal sex, small deletions or insertions [Sekizawa & Saito, 2001; Wright & Burton, 2009; Bianchi, 2012].

In the previously chapter, the genotyping assay method was proposed for this purpose. Even if the method was find to be able to correctly determine the paternally inherited alterations, some limitations were detected. First, it was possible to correctly determine the mutation at least until the 9th gestational week. Indeed, one of the most critical issue is the very low amount of fetal DNA in maternal plasma, in particular at early gestational weeks, that makes its detection very difficult. In fact, at the 5th week of gestation the ccffDNA amount is very low to be detected with this approach, requiring the use of a more sensitive and precise technique able to detect the mutation also at very early gestational ages. Second, in order to obtain an amplification curve from ccffDNA, a previously PCR amplification is required, so, also in this case is necessary a new more sensitive approach pre-amplification free, to avoid possible contamination that can produce false positive, and reduce the time of the analysis. Third, with the genotyping assay approach it is possible to detect only mutations paternally inherited, because it does not provide an accurate quantification, because of the low amount of the fetal DNA in maternal circulation, for the allelic dosage, considering the maternal allelic contribution that causes fetal DNA contamination. So, with the aim to detect also the maternally inherited mutations a technology able to perform an accurate quantification is required.

For this reason, in order to detect paternally inherited β thalassemia mutations also at early gestational weeks, and determine the fetal genotype in case of maternally or both maternally and paternally inherited β thalassemia mutations, the ddPCR technology was employed.

This new advanced technology, as previously mentioned, is based on the partition of nucleic acid samples in thousands of nanoliter-sized water-oil emulsion droplets, permitting the precise quantification of rare target nucleic acids in the sample [Pinheiro et al., 2012]. The massive partitioning is a key aspect of the ddPCR technique, whose, therefore, present several advantages: (A) absolute quantification, (B) high precision and accuracy, (C) reduction in cost due to the nanoliter-range, (D) precious sample preserving, (E) high tolerance to inhibitors [Hindson et al., 2011].

4.2.4.1. Experimental strategy

The experimental strategy, in order to apply ddPCR technology for non-invasive pre-natal diagnosis of β thalassemia mutations, is shown in **Figure 4.32** and consists of three main stages: method set-up and validation, and samples diagnosis. After genomic DNA extraction from blood (**Figure 4.32.A**) of healthy donors (N/N), healthy carriers (N/M) and β thalassemia patients (M/M), it was employed to set-up the experimental conditions (**Figure 4.32.B**) and validate the assays by the use of mixtures of digested genomic DNA of different genotypes (**Figure 4.32.C**) simulating fetal and maternal circulating cfDNA. Finally, after determination of father and mother genotypes by sequencing of the β globin gene after DNA purification from blood and buccal swab (**Figure 4.32.D**), the total circulating cfDNA was extracted from maternal plasma, as previously described, and analyzed by ddPCR assays (**Figure 4.32.E**), in order to determinate the fetal genotype. After the recovery of the newborn buccal swab and the extraction of genomic DNA, the diagnostic outcome was confirmed by DNA sequencing (**Figure 4.32.F**).

Since the samples available of the plasma biobank were related only to the $\beta^0$39 and the β^+IVSI-110 mutations, the analysis was carried out for these β thalassemia mutations.

4.2.4.2. Method set-up by genomic DNA analysis

According to the experimental strategy, the first stage was the experimental conditions set-up of the amplification assays, in order to: (1) correctly discriminate the normal and the mutated alleles, evaluating the effects of annealing temperature change with the aim to prevent cross-hybridization events. Indeed, one of the most critical issues when dealing with genotyping assays is the specificity, as they differ only for a single nucleotide. Therefore, the challenge has been to determine the best experimental conditions in order to achieve the most specific interactions between probes and complementary sequences, even more so when the amount of the target sequences is low, as in this case; (2) quantify the two alleles by optimizing the amplification efficiency. The latter aspect is fundamental in ddPCR because the absolute quantification of the technique is based on the evaluation of the positive events relative to the negative ones. So, optimal amplification conditions are require in order to distinguish the droplets with inside the target from the empty ones.

4.2.4.2.1. Set-up for β^+IVSI-110 thalassemia mutation

With the aim to discriminate and quantify the normal and mutated alleles for the β^+IVSI-110 mutation, an amplification assay was used in ddPCR, consisting of two primers (forward and

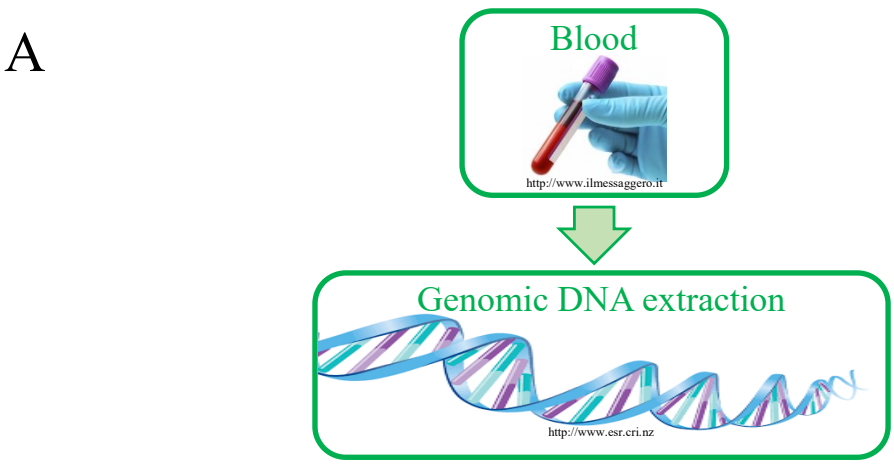


Figure 4.32. Experimental strategy for the development of a non-invasive pre-natal method based on ddPCR assay, to detect β thalassemia point mutations. After genomic DNA extraction from blood (A) of healthy donors (N/N), healthy carriers (N/M) and β thalassemia patients (M/M), it was employed to set-up the experimental conditions (B) and validate the assays by the use of mixtures of digested genomic DNA (C) simulating fetal and maternal circulating cfDNA. Finally, after determination of the two parental genotypes by DNA sequencing of genomic DNA extracted from blood or buccal swab (D), the total circulating cfDNA was extracted from maternal plasma and analyzed by ddPCR assays (E). After the recovery of the newborn buccal swab and the extraction of genomic DNA, the diagnosis was confirmed by DNA sequencing (F).

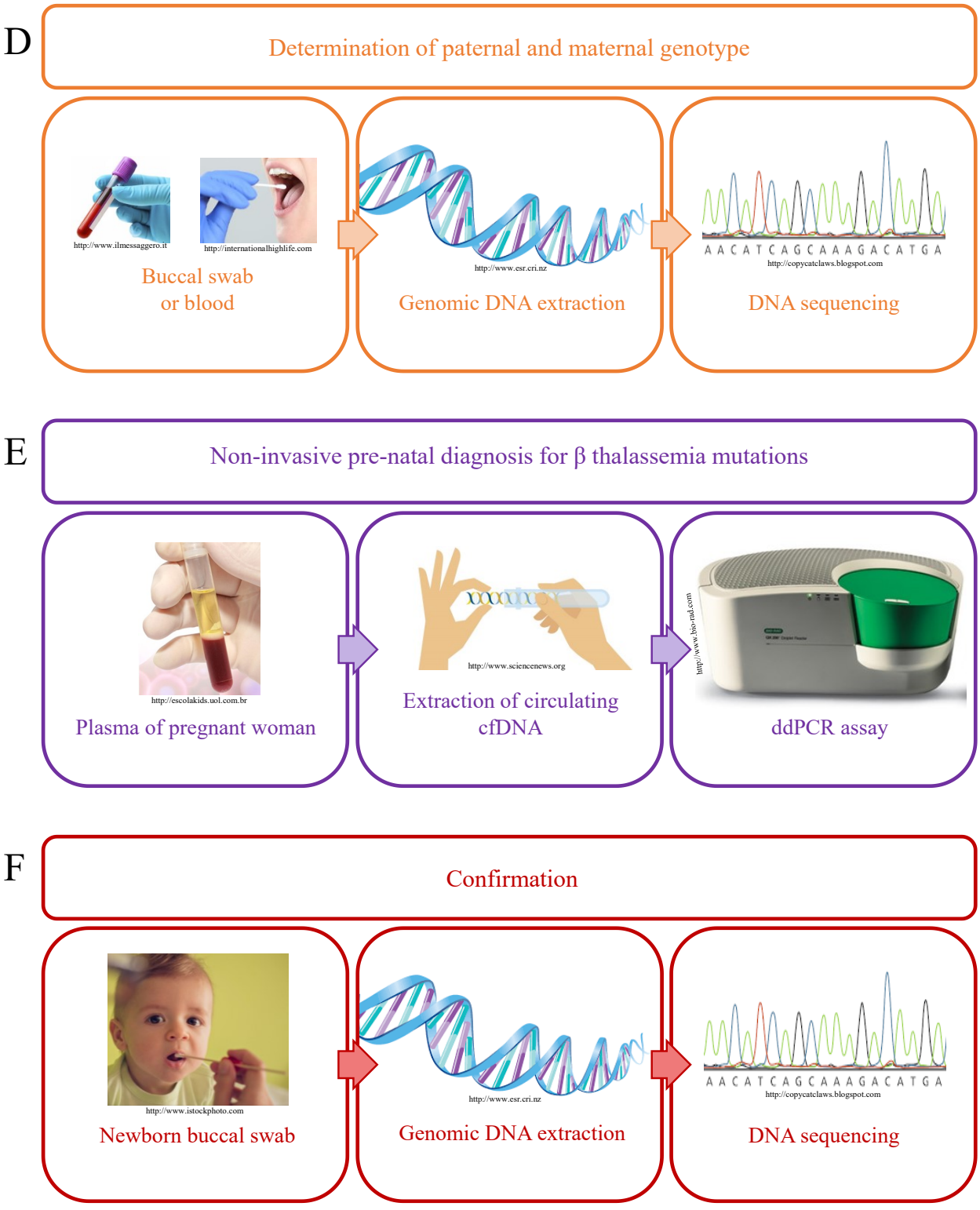


Figure 4.32. Experimental strategy for the development of a non-invasive pre-natal method based on ddPCR assay, to detect β thalassemia point mutations. After genomic DNA extraction from blood (A) of healthy donors (N/N), healthy carriers (N/M) and β thalassemia patients (M/M), it was employed to set-up the experimental conditions (B) and validate the assays by the use of mixtures of digested genomic DNA (C) simulating fetal and maternal circulating cfDNA. Finally, after determination of the two parental genotypes by DNA sequencing of genomic DNA extracted from blood or buccal swab (D), the total circulating cfDNA was extracted from maternal plasma and analyzed by ddPCR assays (E). After the recovery of the newborn buccal swab and the extraction of genomic DNA, the diagnosis was confirmed by DNA sequencing (F).

reverse) and two hydrolysis probes: one FAM™ labeled, recognizing the mutated allele, and the second VIC® labeled, that binds the normal allele. This genotyping assay is exactly the same one previously used in qRT-PCR [Breveglieri et al., 2017], but applied to ddPCR.

One of the most relevant topic when optimizing the experimental conditions is the assay annealing temperature: usually, a decrease in temperature corresponds to a better and efficient amplification; on the contrary, an increase in annealing temperature is related to a greater hybridization specificity of primers and probes with the target sequence [Breveglieri et al., 2017].

For this purpose, after the genomic DNA extraction from blood of a healthy donor (N/N), a healthy carrier (N/M) and a β thalassemia patient (M/M) for the β^+IVSI-110 mutation, four set-up experiments were performed employed different annealing temperatures.

The **Figure 4.33** shows the ddPCR graphs related to the different annealing temperatures: 62°C [**Figure 4.33.A**], 61°C [**Figure 4.33.B**], 60°C [**Figure 4.33.C**] and 58°C [**Figure 4.33.D**]. The graphs on the left side correlate the FAM™ fluorescence intensity, corresponding to the mutated allele (blue dots), to the events number; the graphs on the right side report the HEX™ fluorescence intensity, relative to the normal allele (green dots), to the number of events. As is well known, the black dots are the negative droplets in which no amplification events occur.

The threshold line (in fuchsia) was set by the operator to discriminate the positive from the negative events; a clear separation between the two populations is the goal to be achieved for an efficient and specific amplification.

One ng of genomic DNA of normal homozygous (N/N), heterozygous (N/M) and mutated homozygous (M/M) for the β^+IVSI-110 mutation was analyed in ddPCR, for mutated (in blue) and normal (in green) allele targets.

The first amplification reaction was performed at 62°C, the same temperature previously used in qRT-PCR for the same genotyping assay [Breveglieri et al., 2017], but as shown in the graphs in **Figure 4.33.A**, there was no a clear separation between the positive and the negative events, having both a low fluorescence intensity: 5000 and 2500 respectively.

Therefore, with the aim to increase the fluorescence intensity of the target sequences, the same analysis was performed at the three lower temperatures (**Figures 4.33.B-4.33.D**).

By observing the graphs is clear that when the annealing temperature decreases the fluorescence intensity of both the fluorophores increases, allowing a clear separation between the positive and the negative events and, consequently, improving the method reliability. On this basis, the annealing temperature of 58°C was find to be the optimal one for the targets amplification using this specific assay.

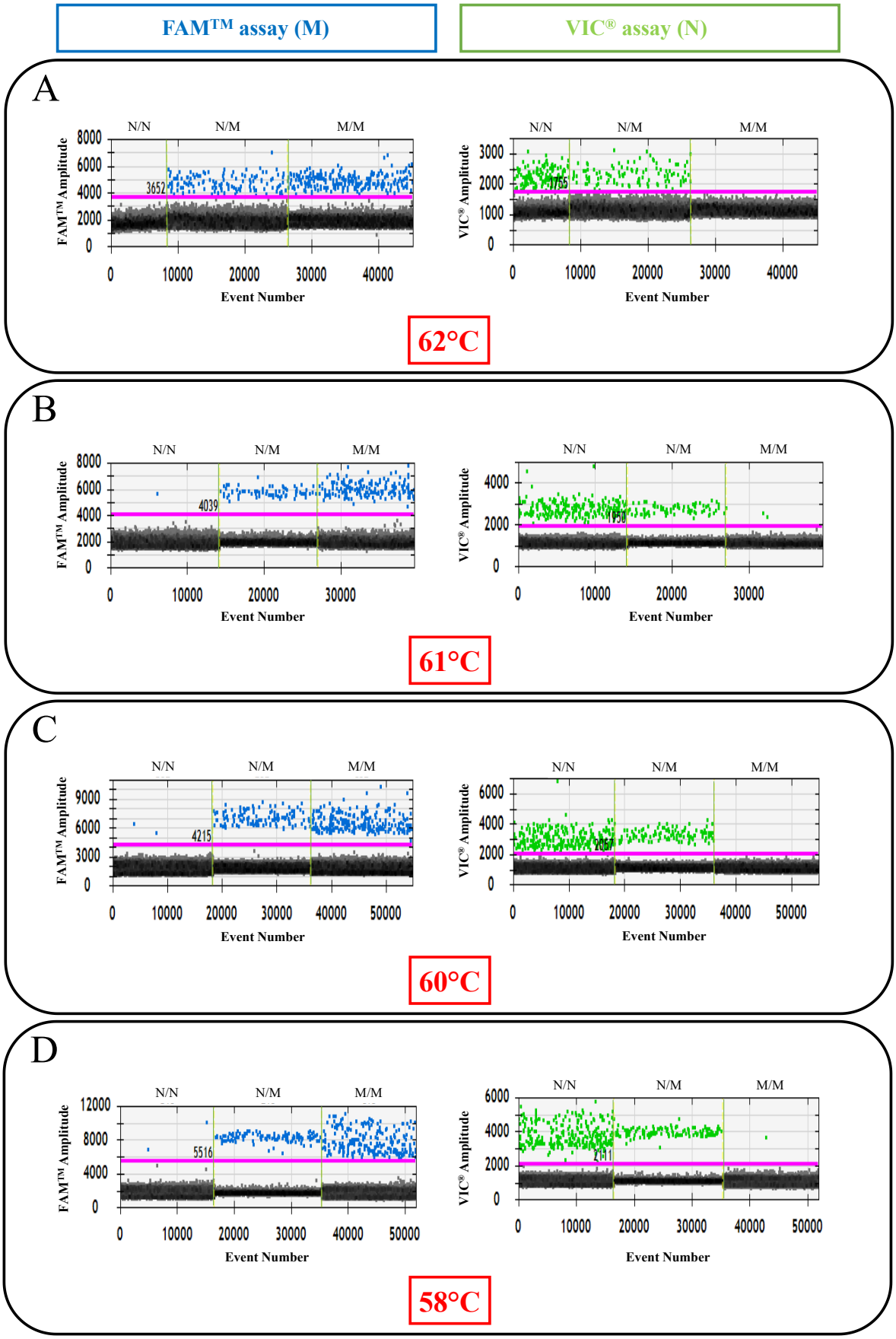


Figure 4.33. ddPCR graphs for not digested genomic DNA samples of different genotypes, for β⁺IVSI-110 mutation, in order to set-up the assay annealing temperature. With the aim to set-up the ddPCR experimental conditions, normal homozygous (N/N), heterozygous (N/M) and mutated homozygous (M/M) for the β⁺IVSI-110 mutation samples were analyed in ddPCR, for mutated (in blue) and normal (in green) allele targets, at different annealing temperatures: 62°C (A), 61°C (B), 60°C (C) and 58°C (D). The graphs on the left side correlate the FAM™ fluorescence intensity, corresponding to the mutated allele (blue dots), to the number of events; the graphs on the right side report the HEX™ fluorescence intensity, relative to the normal allele (green dots), to the number of events. Black dots indicate negative droplets (no amplification events). The threshold lines are coloured in fuchsia.

As regard the probes specificity, the obtained results for all the four temperatures were the expected ones: the homozygous normal DNA (N/N) was amplified only by the VIC® labeled probe; on the contrary, the homozygous mutated DNA (M/M) was exclusively amplified by the FAM™ labeled probe; about the heterozygous DNA (N/M), it was recognized and amplified both by the normal and the mutated probe, as expected. The presence of one or two non-specific events, recovered from both the probes, is not sufficient for the Poisson's statistic application, which required at least three or more positive events to consider a sequence detectable and, consequently, quantifiable (manufactures guidelines). The obtained results have confirmed the assay specificity in targets recognition.

In addition, since the genomic DNA amount was the same for all the three samples (1 ng), the densities of the positive events were greater for the homozygous samples and lower for the heterozygous sample, as expected, because, for the latter sample, the copies number of each target sequence was half.

Circulating cfDNA in maternal circulation is highly fragmented [Bischoff et al., 2005]. The 99% of fetus-derived DNA is shorter than 312 bp, whereas maternal cfDNA had a medium length of about 400-500 bp [Chan et al., 2004; Li et al., 2004].
For this reason, we decided to repeat the same four set-up experiments using as amplification target the digested genomic DNA, in order to simulate the fragmentation of circulating cfDNA: final target of the analysis.
SspI is the restriction enzyme employed for the digestion reaction: it was chosen because is able to recognize and cut two regions of the β globin gene. The recognized sequences are located upstream of the promoter and inside the second intron, respectively, remaining the region around the mutation intact to avoid interference in primers and probes hybridization.
The obtained findings for the digested genomic DNA of N/M and M/M genotypes were the same achieved for the not digested DNA, in term of annealing temperature, efficiency and specificity (data not shown).
Based on the number and the distribution of negative and positive events, the ddPCR applies the Poisson's statistic and determines an absolute quantification of the samples analyzed, expressed in copies/µL of reaction.
The **Figure 4.34** shows the absolute quantification obtained from the five samples analyzed (3 not digested genomic DNA and 2 digested genomic DNA) at the chosen temperature of 58°C. For each sample, the absolute concentration, expressed in copies/µL of reaction, of the β^{+}IVSI-110 mutated allele (FAM™, blue dots) and the normal allele (VIC®, in green) is

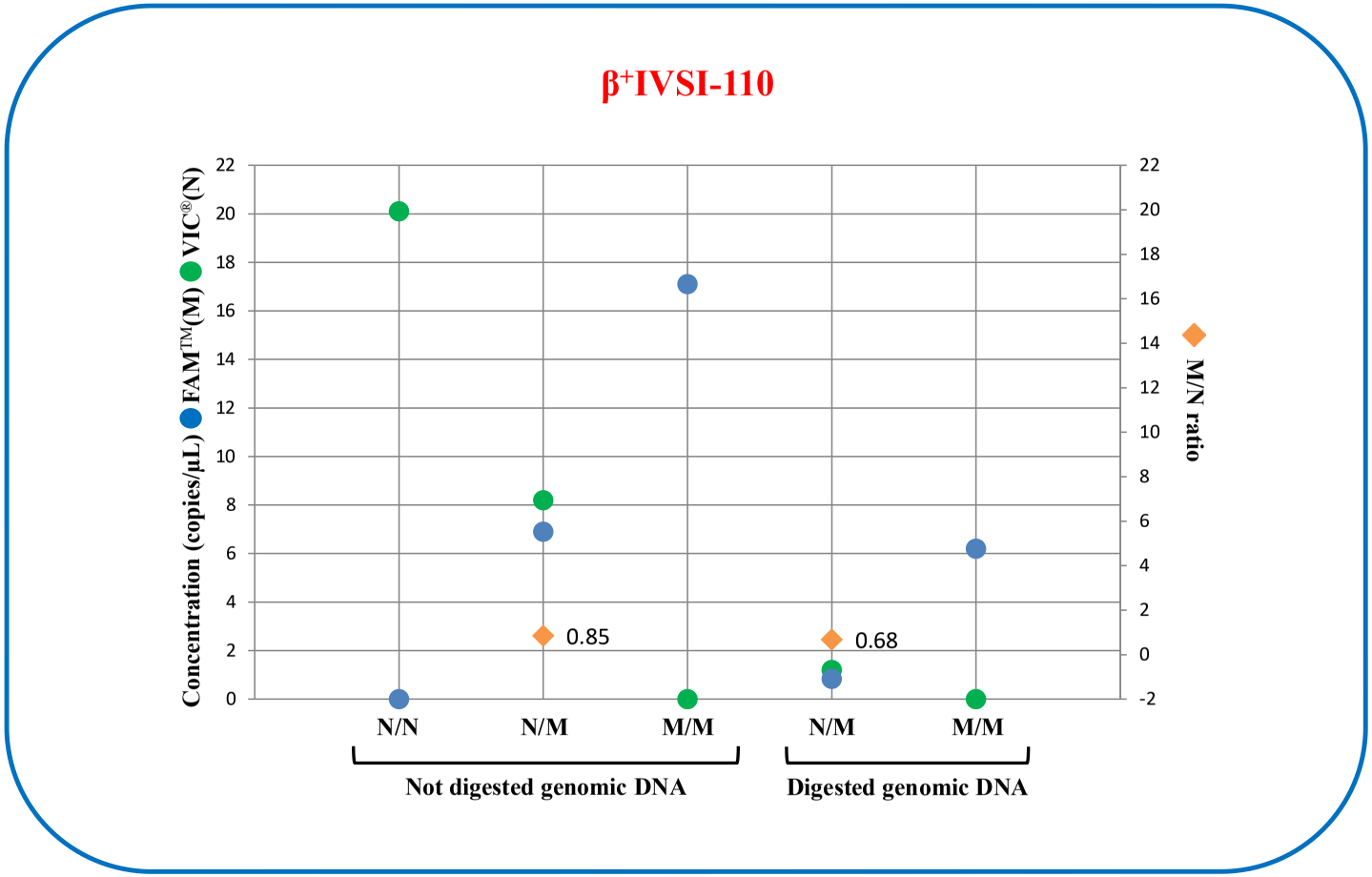


Figure 4.34. Graphical representation of ddPCR analysis on not digested and digested genomic DNA samples in order to set-up β+IVSI-110 assay. After ddPCR analysis at 58°C, the absolute concentrations of not digested genomic DNA samples normal homozygous (N/N), heterozygous (N/M) and mutated homozygous (M/M), and digested N/M and M/M genomic DNA samples, were obtained. The concentration of β+IVSI-110 mutated (FAM™, blue dots) and normal (VIC®, green dots) alleles was expressed in copies/µL of reaction, obtained through QuantaSoft, based on Poisson's statistics; the indicators with diamond shape represent the M/N ratio.

indicated. Only the normal sequences were quantified for the normal homozygous sample, as well only the mutated allele was quantified for the mutated homozygous samples (digested and not digested), instead the concentration of the other allele is equal to zero confirming the absence, as expected. On the contrary, for the heterozygous samples (digested and not digested) both the alleles were detected and quantified: the amount was comparable considering that each allele was present in a single copy. Indeed, for both the digested and not digested DNA, the absolute quantification of the homozygous samples was nearly twice the quantification of the heterozygous samples, for the same reason explained before.

In addition, the graphs show the ratio between the mutated and normal alleles (M/N ratio, orange indicators): since for the homozygous samples each allele is present in a single copy, the M/N ratio should be around 1. The obtained values are consistent with the expected results: mild deviations from 1 are probably due to the nature and the quality of samples.

As can be seen, unlike the expectations, the absolute quantification of the digested samples was lower than the one for the non digested samples of the same genotype: the variability could be caused by a not equal distribution of the sample inside the water-oil droplets, due to the fragmented nature of the digested samples, leading to a different amplification that affects the final statistical calculation. However, such differences could be also caused by mild different amount of genetic DNA, since an housekeeping gene was not employed in the analysis. Nevertheless, the normalization was not necessary for our purpose, because we were not interested in the quantification of the single alleles but in allelic imbalance, in order to detect the β^{+}IVSI-110 in fetal DNA.

In conclusion, the annealing temperature of 58°C was chosen for the next experiments because it has given the best results, in term of amplification efficiency and hybridization specificity, for the ddPCR β^{+}IVSI-110 assay in the set-up reactions.

4.2.4.2.2. Set-up for β^{0}39 thalassemia mutation

After the optimization of the ddPCR β^{+}IVSI-110 assay, the experimental conditions of the ddPCR β^{0}39 assay were tested.

With the aim to discriminate and quantify the normal and mutated alleles for the β^{0}39 mutation, at first, the same genotyping assay used in qRT-PCR [Breveglieri et al., 2017], was applied in ddPCR, consisting of two primers (forward and reverse) and two hydrolysis probes: one FAM™ labeled, recognizing the mutated allele, and the second VIC® labeled, that binds the normal allele.

Unfortunately, the genotyping assay was not applicable in ddPCR because a correct discrimination between the two alleles was not reached: the normal VIC® labeled probe recognized also the mutated one, resulting in an assay not specific (data not shown).

For this reason, we designed another genotyping assay for the $\beta^0$39 mutation specific for ddPCR amplification.

Also in this case, 1 ng of five samples was employed for the set-up analysis: a not digested normal homozygous sample (N/N), a digested and not digested heterozygous sample (N/M) and a digested and not digested mutated homozygous sample (M/M) for the $\beta^0$39 mutation.

The first annealing temperature employed was 60°C as suggested in the assay datasheet. Subsequently, other two annealing temperatures were tested, in order to increase the separation between the positive and the negative events: 59 and 58°C.

The chosen annealing temperature was 59°C, in fact, the temperature was enough low to allow an efficient amplification but not too much to impair the hybridization specificity of primers and probes with the target sequences (data not shown).

The same conclusions for the β^+IVSI-110 ddPCR assay can be apply to the $\beta^0$39 assay.

In this regard, the **Figure 4.35** shows the absolute quantification, of the two alleles, obtained from the five samples analyzed (3 not digested genomic DNA and 2 digested genomic DNA) at the chosen temperature of 59°C. In this case too, only the normal sequences were quantified for the normal homozygous sample, as well only the mutated allele was quantified for the mutated homozygous samples (digested and not digested), instead the concentration of the other allele is equal to zero confirming the absence, as expected. On the contrary, for the heterozygous samples (digested and not digested) both the alleles can be detected and quantified: the amount is comparable considering that each allele is present in a single copy. The absolute quantification of the homozygous samples is nearly twice the quantification of the heterozygous samples, as expected.

The M/N ratio for the not digested and digested genomic heterozygous DNA was 0.9 and 1.5, respectively. Here again the M/N ratio was around 1 and mild deviations from 1 were probably due to the nature and the quality of samples.

In conclusion, the annealing temperature of 59°C was chosen for the next experiments because it has given the best results, in term of amplification efficiency and hybridization specificity, for the ddPCR $\beta^0$39 assay in the set-up reactions.

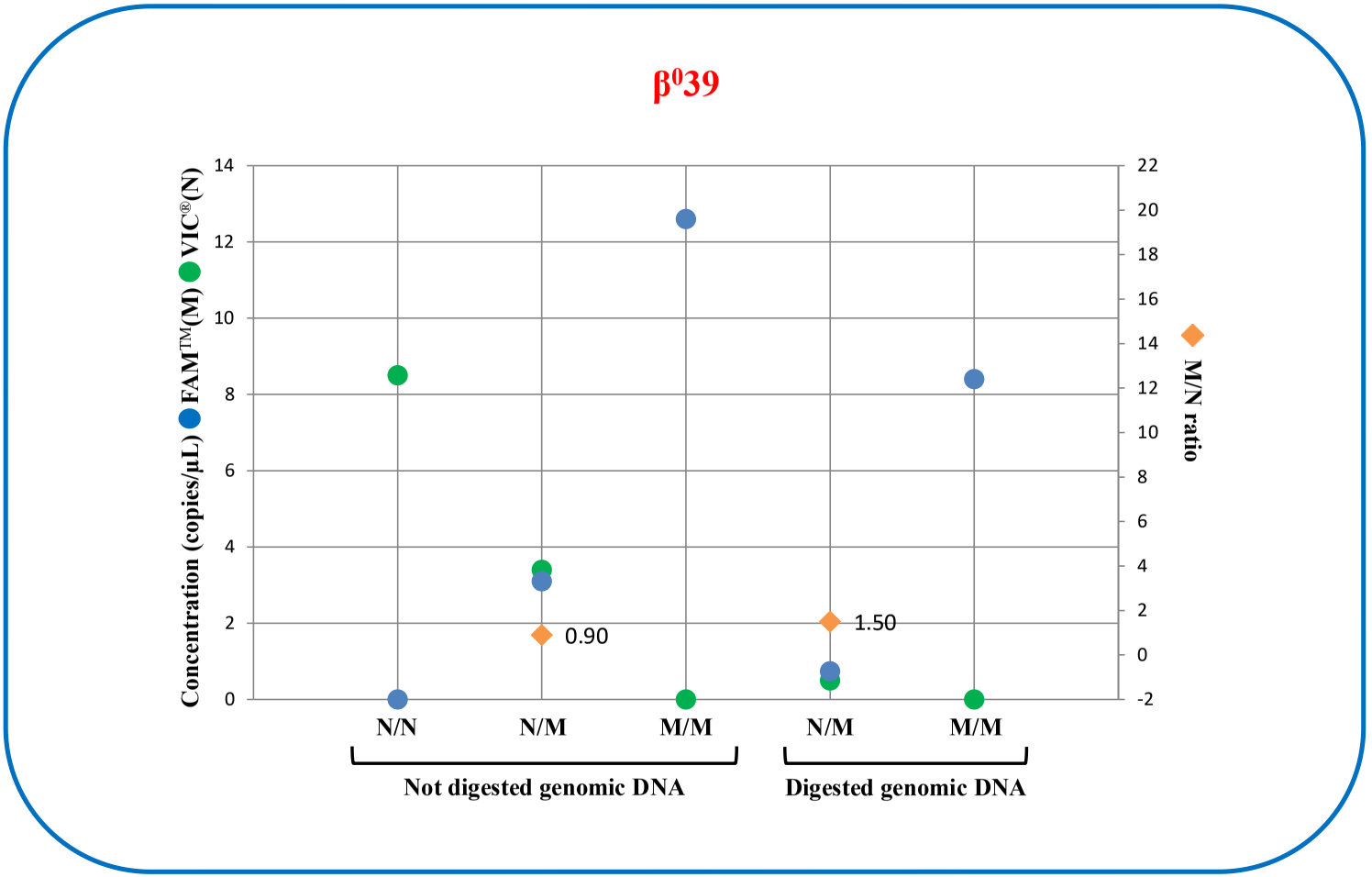


Figure 4.35. Graphical representation of ddPCR analysis on not digested and digested genomic DNA samples in order to set-up β⁰39 assay. After ddPCR analysis at 59°C, the absolute concentrations of not digested genomic DNA samples normal homozygous (N/N), heterozygous (N/M) and mutated homozygous (M/M), and digested N/M and M/M genomic DNA samples, were obtained. The concentration of β⁰39 mutated (FAM™, blue dots) and normal (VIC®, green dots) alleles was expressed in copies/µL of reaction, obtained through QuantaSoft, based on Poisson's statistics; the indicators with diamond shape represent the M/N ratio.

4.2.4.3. Method validation by genomic DNA mixtures analysis simulating fetal and maternal circulating cfDNA

According to the experimental strategy, after the optimization of the experimental conditions, the second step of this work was the assays validation using mixtures of digested genomic DNA of different genotypes (**Figure 4.32.C**), simulating fetal and maternal circulating cfDNA: the real target of this work for the development of a non-invasive pre-natal method of β thalassemia diagnosis.

The fetal DNA is present in maternal blood at very low concentrations increasing during the progression of the pregnancy. The amount of ccffDNA depends, besides the gestation period, on other factors, such as maternal diseases and body weight [Zhou et al., 2015]. Usually, in the plasma of pregnant women, the percentage of fetal DNA is between 3% and 10-20% of the total circulating cfDNA [Drury et al., 2016].

In order to simulate the circulating cfDNA real samples at different gestational ages, different percentages of two genomic DNA of different genotypes, previously enzymatically digested, were employed. As previously mentioned, circulating cfDNA in maternal circulation is highly fragmented [Bischoff et al., 2005], so the genomic DNA fragmentation allows a better simulation of the real analysis target.

0%, 1.5%, 3%, 6% and 18% are the percentages of the pseudo-fetal DNA chosen, since the ccffDNA in maternal plasma is between 3% and 10-20% of the total circulating DNA [Drury et al., 2016].

For each β thalassemia mutation, four experiments were performed simulating the most significant genotypes: mother normal homozygous (N/N) and fetus heterozygous (N/M) (1), mother heterozygous (N/M) and fetus normal homozygous (N/N) (2), heterozygous (N/M) (3) and mutated homozygous (M/M) (4). In the first case the paternally inherited mutation was simulated, in the other cases the maternally or paternally inherited mutation was mimicked; the most interesting case for a pre-natal diagnostic purpose of β thalassemia is the (4).

After the emulsion generation, the reactions were performed in the amplification conditions optimized for each ddPCR assay, respectively.

4.2.4.3.1. Validation for β^{+}IVSI-110 thalassemia mutation

In **Figure 4.36**, the four experiments for the β^{+}IVSI-110 mutation are reported, where different percentages of pseudo-fetal DNA were employed in order to simulate the four genotype cases: (1) mother N/N and fetus β^{+}IVSI-110/N (**Figure 4.36.A**), (2) mother

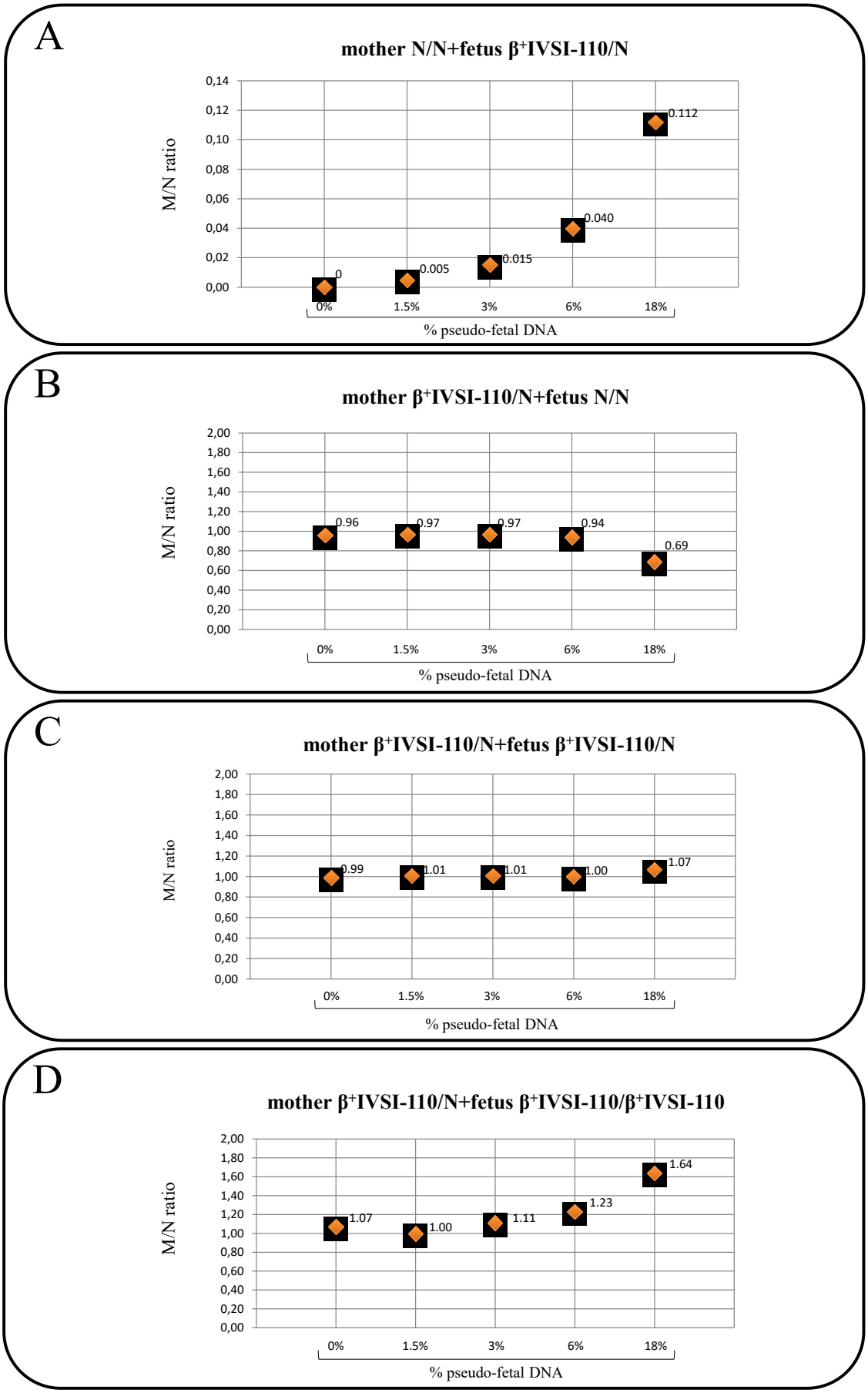


Figure 4.36. Graphical representation of ddPCR analysis on digested genomic DNA mixtures in order to validate β+IVSI-110 assay. After β+IVSI-110 ddPCR analysis at 58°C, the M/N allelic ratio (indicators with diamond shape) of digested genomic DNA mixtures, were obteined, in order to simulate the maternal and ccffDNA of different genotypes: normal homozygous mother (N/N) and heterozygous fetus (β+IVSI-110/N) (A); heterozygous mother (β+IVSI-110/N) and homozygous normal (N/N) (B), or heterozygous (β+IVSI-110/N) (C) or homozygous mutated (β+IVSI-110/β+IVSI-110) (D) fetus. For each graph the percentage of pseudo-fetal DNA analyzed is reported.

β^+IVSI-110/N and fetus N/N (**Figure 4.36.B**), (3) mother and fetus β^+IVSI-110/N (**Figure 4.36.C**), (4) mother β^+IVSI-110/N and fetus β^+IVSI-110/ β^+IVSI-110 (**Figure 4.36.D**).

In the graphs (**Figure 4.36**), the M/N ratio (orange indicators) is indicated for each pseudo-fetal percentage.

If the mother is normal for the considered mutation, only the normal sequences will be amplify, and the M/N ratio will be equal to 0, as obtained (**Figure 4.36.A**, 0% pseudo-fetal DNA). If the fetus is heterozygous for the β^+IVSI-110 mutation, also the mutated sequences of fetal origin will be amplify, and the M/N ratio will be different from 0 but less than 1 considering that the amount of fetal DNA is lower than the circulating cfDNA of maternal origin, as obtained (**Figure 4.36.A**).

Even if the allelic imbalance is an under-representation of the mutated allele with respect to the normal one, the M/N allelic ratio increases with increasing preudo-fetal DNA percentage. On the contrary, if the mother is heterozygous both the normal and the mutated sequences will be amplify, and the M/N ratio will be around 1, as obtained (**Figure 4.36.B-4.36.D**, 0% pseudo-fetal DNA). If the fetus is normal for the same mutation of the mother, a mild allelic imbalance is expected, due to the fetal contribution, resulting in a decrease of M/N ratio, as obtained with increasing the percentage of pseudo-fetal DNA (**Figure 4.36.B**). Also if the fetus is homozygous for the same mutation of the mother, a mild allelic imbalance is expected resulting, in this case, in an increase of M/N ratio, as obtained with increasing the percentage of pseudo-fetal DNA (**Figure 4.36.D**). If the fetus in heterozygous, as the mother, no imbalance occurs, resulting in an M/N ratio around 1, as obtained even if the percentage of pseudo-fetal DNA increases (**Figure 4.36.C**).

4.2.4.3.2. Validation for $\beta^0$39 thalassemia mutation

After validating the ddPCR β^+IVSI-110 assay, we proceed to validate the assay for the mutation. In **Figure 4.37** are displayed the four experiments for the $\beta^0$39 mutation in which different percentages of pseudo-fetal DNA were employed in order to simulate the four genotype cases: (1) mother N/N and fetus $\beta^0$39/N (**Figure 4.37.A**), (2) mother $\beta^0$39/N and fetus N/N (**Figure 4.37.B**), (3) mother and fetus $\beta^0$39/N (**Figure 4.37.C**), (4) mother $\beta^0$39/N and fetus $\beta^0$39/ $\beta^0$39 (**Figure 4.37.D**).

In the graphs (**Figure 4.37**), the M/N ratio (orange indicators) is indicated for each pseudo-fetal percentage.

The obtained results for the validation of $\beta^0$39 assay were in accordance with the results obtained for the β^+IVSI-110 assay.

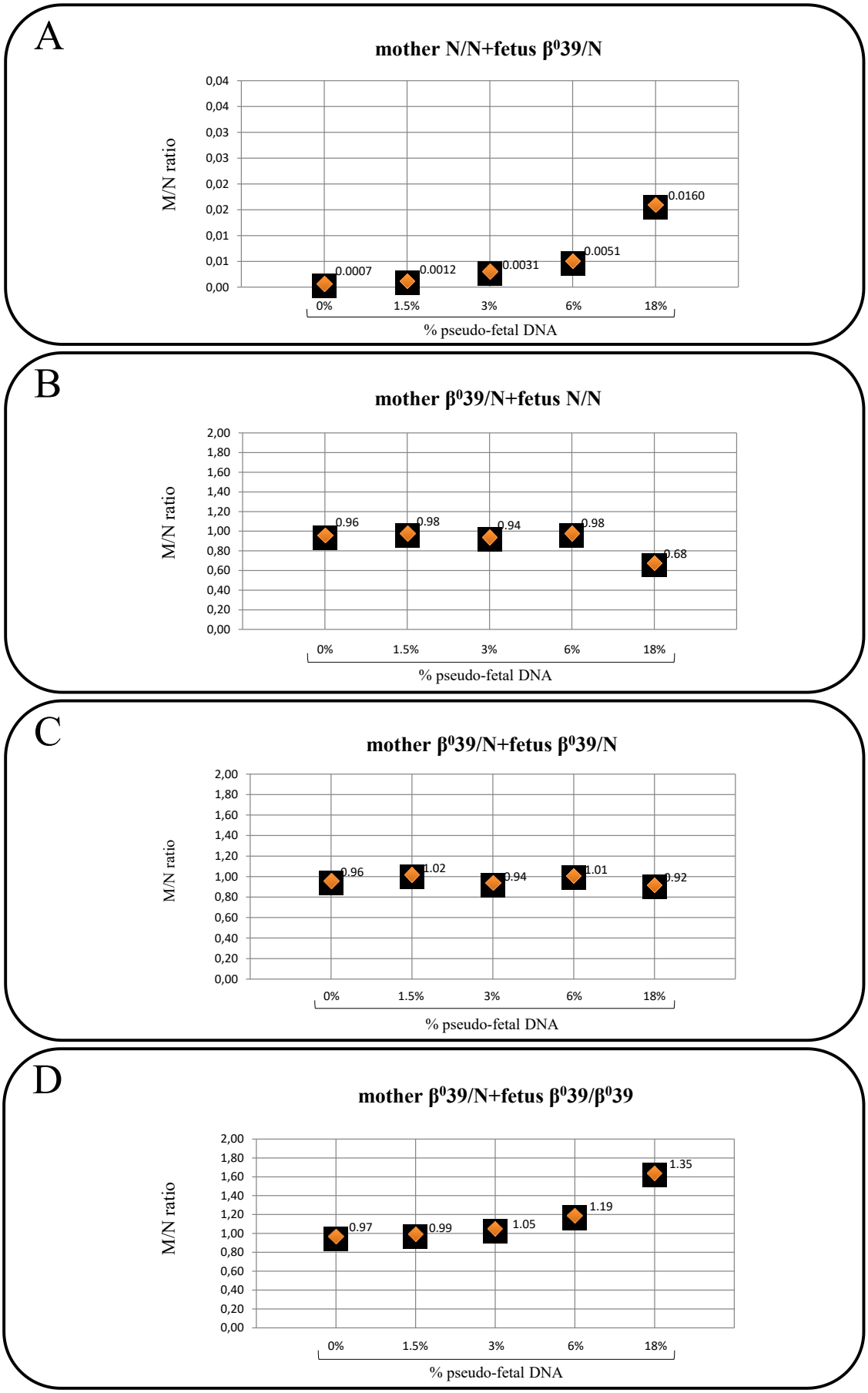


Figure 4.37. Graphical representation of ddPCR analysis on digested genomic DNA mixtures in order to validate β⁰39 assay. After β⁰39 ddPCR analysis at 59°C, the M/N allelic ratio (indicators with diamond shape) of digested genomic DNA mixtures of different genotypes, were obteined, in order to simulate the maternal and ccffDNA of different genotypes: normal homozygous mother (N/N) and heterozygous fetus (β⁰39/N) (A); heterozygous mother (β⁰39/N) and homozygous normal (N/N) (B), or heterozygous (β⁰39/N) (C) or homozygous mutated (β⁰39/β⁰39) (D) fetus. For each graph the percentage of pseudo-fetal DNA analyzed is reported.

In the **Figure 4.37.A**, which represents the case of mother N/N and fetus $\beta^0$39/N, the M/N ratio for 0% preudo-fetal percentage has a value of 0.007, instead to the expected value of 0. Indeed, the sample was shown only one positive event for the mutated allele, so, not sufficient for the Poisson's statistic application, which required at least three or more positive events to consider a sequence detectable and, consequently, quantifiable (manufactures guidelines).

In conclusion, in all the cases analyzed, with both the assays, the obtained results were the expected ones, and the M/N ratio was found to be a good value to efficiently determine the allelic imbalance due to the pseudo-fetal DNA contribution. Therefore, considering the promising results, the M/N ratio could be apply and employed in a pre-natal diagnostic view.

4.2.4.4. Non-invasive pre-natal diagnosis of β thalassemia mutations

After the successful set-up and validation of the two genotyping assays, they were employed for the analysis of the circulating cfDNA samples with the aim to detect the fetal genotype.

Thirty-six blood samples were collected, after informed consent, from pregnant women, and the plasma immediately prepared.
At first, the two parental genotypes were confirmed by sequencing of the β globin gene after genomic DNA purification from blood and buccal swab (**Figure 4.32.D**).
Then, the total circulating cfDNA was extracted from maternal plasma, and the samples analyzed by the specific ddPCR assay (**Figure 4.32.E**).
In particular, while for the β^+IVSI-110 assay no problems were found when the samples were analyzed at the optimized conditions, for the $\beta^0$39 assay it was not the same. At the annealing temperature of 59°C the real circulating cfDNA samples not showed a correctly separation between the positive and the negative events. So, using circulating cfDNA samples at late gestational weeks, the experimental conditions were again optimized, choosing 57°C as the new annealing temperature.

As previously underlined, the determination of fetal mutation paternally inherited is the easier case: if the mother is normal homozygous, the presence of positive events for the mutated allele is due to fetal contribution. The case of maternally or both parents inherited mutation is more complex: a precise quantification of normal and mutated alleles in maternal plasma is required to evaluate whether they are balanced or unbalanced.
For this reason, is better to distinguish the two different situations.

4.2.4.4.1. Analysis of β thalassemia mutations inherited from the father by ddPCR assays

As reported in the section **4.2.3**, the genotyping assay using qRT-PCR was proposed for non-invasive pre-natal diagnosis of β thalassemia mutations paternally inherited. Even if the method was find to be able to correctly determine the paternally inherited alteration, some limitations were detected. Primarily, it was possible to correctly determine the mutation at least until the 9th gestational week, due to the very low amount of fetal DNA in maternal plasma, in particular at early gestational weeks. Therefore, we employed the ddPCR technique as a sensitive approach for determining fetal mutations at early gestational weeks.

The **Table 4.9** lists the 19 samples collected from normal homozygous pregnant women with partner carrier of $\beta^0$39 (on the left side) or β^+IVSI-110 (on the right side) mutation. For each sample, the assigned number and the gestational age are indicated. The samples were heterogeneous in terms of gestational weeks (from 39 to 5).

Therefore, the circulating cfDNA samples were analyzed and the diagnostic outcomes are shown in **Table 4.10**: for each sample, the formulated diagnosis using ddPCR is indicated.

The sample was considered normal homozygous if no positive events for the mutated allele were detected, instead, is considered heterozygous for the specific mutation if more than two positive events for the mutated allele were detected.

After the recovery of the newborn buccal swab and the extraction of genomic DNA, the diagnostic outcome was confirmed by DNA sequencing (**Table 4.10**).

For all the samples analyzed the diagnostic outcome was confirmed, also at 5th gestational week, except for the sample #207 at 37th gestational week: it was resulted heterozygous for the β^+IVSI-110 mutation, but the genotype resulting from DNA sequencing is normal homozygous. Since the sample is the only one whose genotype was not confirmed and its gestational age is too late, probably there was a mistake in the DNA sequencing due to an exchange of samples. For this reason, the first future perspective is to check the real actual genotype of the fetus.

In conclusion, for the first time, we have demonstrated that ddPCR technology can be used for non-invasive pre-natal diagnosis of β^+IVSI-110 and $\beta^0$39 mutations paternally inherited at early gestation ages (prior to 9th week), confirming that the ddPCR is a robust, sensitive, efficient and reliable technology.

Pregnant women N/N with partner $\beta^0$39/N	
# sample	Gestational weeks
265	39
217	35
193	33
186	29
36	28
31	26
287	24
147	24
61	21
178	14
142	5

Pregnant women N/N with partner β^+IVSI-110/N	
# sample	Gestational weeks
207	37
138	37
43	18
276	16
140	15
146 B	10
172	9
146 A	5

Table 4.9. List of plasma samples collected from normal homozygous pregnant women with partner carrier of β thalassemia mutations. The table lists the plasma samples of pregnant women with normal genotype whose partners are heterozygous for $\beta^0$39 (on the left side) or β^+IVSI-110 (on the right side) mutations. For each sample the assigned number and the gestational age, in decreasing order, are indicated. The samples 146 A and 146 B were obtained from the same pregnant woman at two different gestational weeks.

Pregnant women N/N with partner $\beta^0$39/N				
# sample	**Gestational weeks**	**Formulated diagnosis**	**Fetal genotype**	**Diagnosis outcome**
265	39	$\beta^0$39/N	$\beta^0$39/N	√
217	35	$\beta^0$39/N	$\beta^0$39/N	√
193	33	$\beta^0$39/N	$\beta^0$39/N	√
186	29	$\beta^0$39/N	$\beta^0$39/N	√
36	28	$\beta^0$39/N	$\beta^0$39/N	√
31	26	$\beta^0$39/N	$\beta^0$39/N	√
287	24	$\beta^0$39/N	$\beta^0$39/N	√
147	24	N/N	N/N	√
61	21	$\beta^0$39/N	$\beta^0$39/N	√
178	14	$\beta^0$39/N	$\beta^0$39/N	√
142	5	$\beta^0$39/N	$\beta^0$39/N	√

Pregnant women N/N with partner β^+IVSI-110/N				
# sample	**Gestational weeks**	**Formulated diagnosis**	**Fetal genotype**	**Diagnosis outcome**
207	37	β^+IVSI-110/N	N/N	X
138	37	N/N	N/N	√
43	18	N/N	N/N	√
276	16	β^+IVSI-110/N	β^+IVSI-110/N	√
140	15	β^+IVSI-110/N	β^+IVSI-110/N	√
146 B	10	β^+IVSI-110/N	β^+IVSI-110/N	√
172	9	β^+IVSI-110/N	β^+IVSI-110/N	√
146 A	5	β^+IVSI-110/N	β^+IVSI-110/N	√

Table 4.10. Fetal genotype determination by ddPCR of circulating cfDNAs from plasma of normal homozygous pregnant women with partner carrier of β thalassemia mutations. The table shows, for each circulating cf sample of pregnant woman with normal genotype whose partner is heterozigous for $\beta^0$39 (on the left side) or β^+IVSI-110 (on the right side) mutations, the assigned number, the gestational age, the formulated diagnosis resulting by ddPCR assays, the actual fetal genotype, determinated by DNA sequencing, and the diagnosis outcome. The samples 146 A and 146 B were obtained from the same pregnant woman at two different gestational weeks. √, confirmed. X, wrong.

4.2.4.4.2. Analysis of β thalassemia mutations inherited from the mother or from both parents by ddPCR assays

With the genotyping assay using qRT-PCR approach it is possible to detect only mutations paternally inherited, because it does not provide an accurate quantification of the low amount of the fetal DNA in maternal circulation, necessary for the allelic dosage, considering the maternal allelic contribution causes fetal DNA contamination. So, with the aim to detect also the maternally and both parents inherited mutations the ddPCR was required.

The **Table 4.11** lists the 17 samples collected from heterozygous pregnant women for the $\beta^0$39 (on the left side) and β^+IVSI-110 (on the right side) mutations with partner normal homozygous (in the upper part) and carrier of the same maternal mutation.

For each sample, the assigned number and the gestational age are indicated. The samples, also in this case, were heterogeneous in terms of gestational weeks (from 39 to 10).

The two samples (#59; #133) for which both the parents are carrier of the same mutation are very informative and interesting form a diagnostic point of view, because they represent a real case in which the fetus could be a β thalassemia patient.

After the recovery of the newborn buccal swab and the extraction of genomic DNA, the genotype of each sample was determined by DNA sequencing.

Therefore, the circulating cfDNA samples were analyzed by ddPCR assays, amplifying both the normal and the mutated alleles, and the M/N allelic ratio was calculated for each sample. The distribution of the M/N ratio values, obtained in the performed experiments, is displayed in **Figure 4.38**, in which each dot is a sample. Despite some variability in the obtained values, two distinct value populations not overlapping each other were identified according to the different fetal genotype: samples in which the fetus is not carrier of the mutations (light blue dots) and samples in which the fetus is heterozygous for the mutations considered (red dots), together with the relevant statistical analyses, which confirm that the differences between the values obtained from different fetal genotypes were statistically significant, even if the number of samples is low.

As expected, the samples carrying the heterozygous fetus, in which the allelic balance is expected, showed an M/N ratio around 1. On the contrary, the samples carrying the normal fetus, in which the allelic imbalance is due to an under-representation of the mutant allele respect the normal one [Lun et al., 2008], showed a M/N ratio less than one and less than the M/N ratios of the samples carrying heterozygous samples. In this way, two diagnostic ranges based on the M/N ratio values were identify: the first one from 0.51 to 0.78 relating to normal fetuses, and the second from 0.83 to 1.11 relating to heterozygous foetuses.

Pregnant women $\beta^0$39/N with partner N/N	
# sample	Gestational weeks
60	39
131	36
83	29
74	26
94	20
139	18
75	14
Pregnant women $\beta^0$39/N with partner $\beta^0$39/N	
# sample	Gestational weeks
59	33

Pregnant women β^+IVSI-110/N with partner N/N	
# sample	Gestational weeks
128	38
214	33
144	30
109	30
78	14
63	11
64	10
120	9
Pregnant women β^+IVSI-110/N with partner β^+IVSI-110/N	
# sample	Gestational weeks
133	10

Table 4.11. List of plasma samples collected from heterozygous pregnant women for β thalassemia mutations with partner homozygous normal or carrier of the same mutation. The table lists the plasma samples of pregnant women with heterozygous genotype, for $\beta^0$39 (on the left side) or β^+IVSI-110 (on the right side), whose partners are normal homozygous (in the upper part) or heterozygous (in the lower part) for the same mutation. For each sample the assigned number and the gestational age, in decreasing oreder, are indicated.

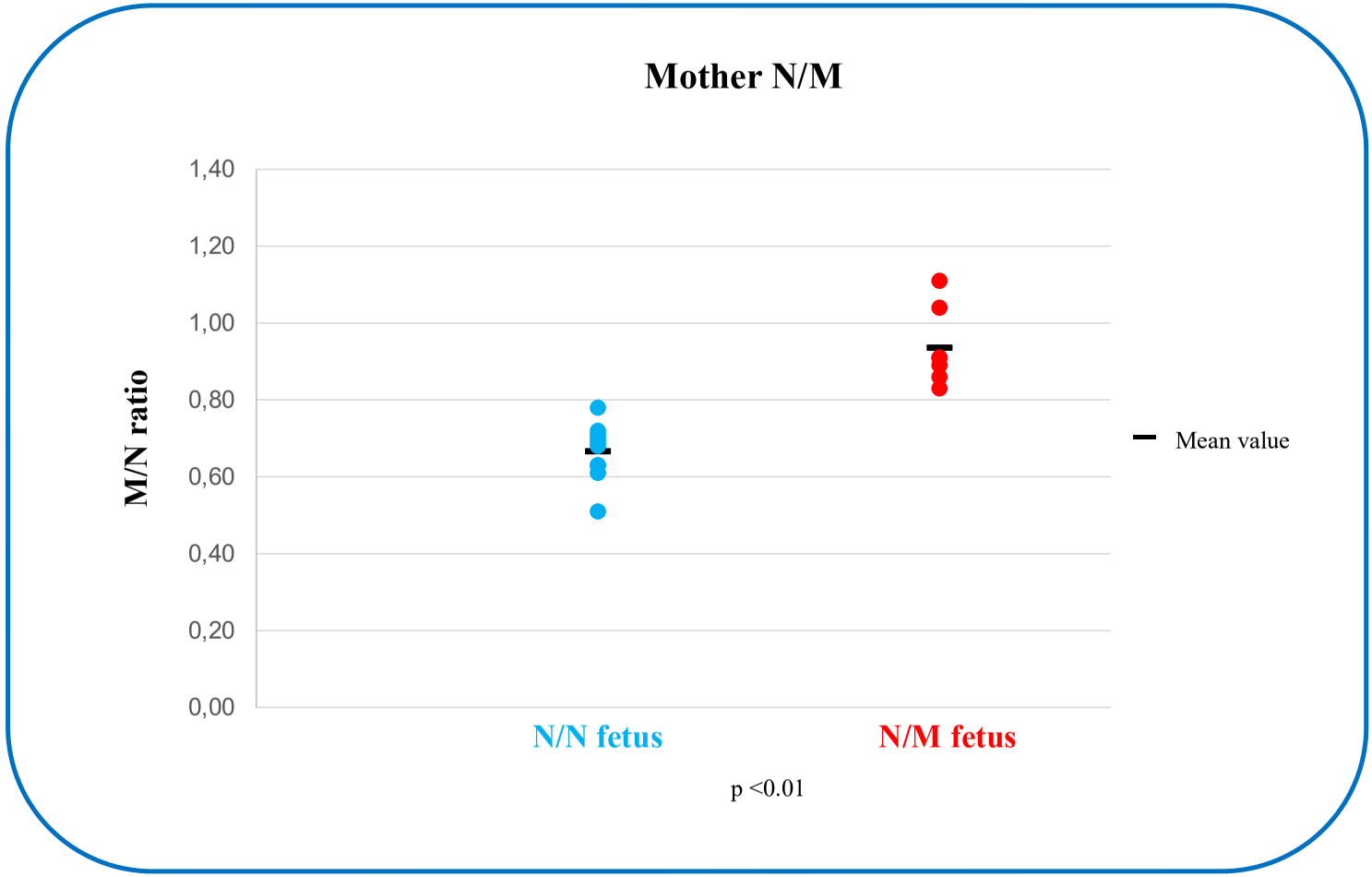


Figure 4.38. M/N allelic ratio distribution through ddPCR to detect β thalassemia fetal genotype from circulating cfDNAs of heterozygous pregnant women. All the 17 circulating cfDNAs samples analyzed, derived from pregnant women heterozygous for the $\beta^0$39 and β^+IVSI-110 mutations, are dysplayed. Two discrinct groups are identified: sampes in which the fetus is not carrier of the mutation (light blue dots) and samples in which the fetus is carrier of $\beta^0$39 or β^+IVSI-110 mutation (red dots). The mean value of each distribution and the statistical significance of the analysis are also indicated. Statistical differences between grups were compared by the Student's *t*-test, selecting a 99% confidence level.

The identified ranges are clearly distinct, demonstrating the suitability of the developed ddPCR-based approach to correctly discriminate the different allelic fetus conditions for the β thalassemic mutations taken into consideration.
Unfortunately, there were not samples with homozygous fetus for one of the two β thalassemia mutations considered.
The **Table 4.12** summarizes the obtained results: for each sample the single M/N ratio, the formulated diagnosis using ddPCR, the fetal genotype determined by DNA sequencing and the diagnosis outcome are indicated. For all the samples analyzed the diagnostic outcome was confirmed, also at early gestational weeks (until the 9^{th} week).

In conclusion, for the first time, we have demonstrated that ddPCR technology can be used for non-invasive pre-natal diagnosis of β^{+}IVSI-110 and β^{0}39 mutations maternally or both maternally and paternally inherited, also at early gestation ages (until 9^{th} week), confirming that the ddPCR is a robust, sensitive, efficient and reliable technology.
In this way, a simple, fast and sensitive non-invasive pre-natal method for the identification of β thalassemia mutations maternally and both maternally and paternally inherited was developed, and therefore, it can be apply to other genetic diseases. However, in order to confirm the effective applicability and efficiency of the technology will be necessary to amplify the number of samples and recruit samples at earlier gestational weeks.

Ultimately, the second part of the work aimed to detect β thalassemia point mutations, in pre-natal and post-natal ages, can be considered a key milestone for the development of protocols for patients and fetuses stratification in personalized therapy on the road of precision medicine.

Pregnant women $\beta^0$39/N with partner N/N					
# sample	Gestational weeks	M/N ratio	Formulated diagnosis	Fetal genotype	Diagnosis outcome
60	39	1.11	$\beta^0$39/N	$\beta^0$39/N	√
131	36	0.89	$\beta^0$39/N	$\beta^0$39/N	√
83	29	0.71	N/N	N/N	√
74	26	0.51	N/N	N/N	√
94	20	0.68	N/N	N/N	√
139	18	0.78	N/N	N/N	√
75	14	0.63	N/N	N/N	√

Pregnant women $\beta^0$39/N with partner $\beta^0$39/N					
# sample	Gestational weeks	M/N ratio	Formulated diagnosis	Fetal genotype	Diagnosis outcome
59	33	0.69	N/N	N/N	√

Pregnant women β^+IVSI-110/N with partner N/N					
# sample	Gestational weeks	M/N ratio	Formulated diagnosis	Fetal genotype	Diagnosis outcome
128	38	0.605	N/N	N/N	√
214	33	0.63	N/N	N/N	√
144	30	0.86	β^+IVSI-110/N	β^+IVSI-110/N	√
109	30	0.83	β^+IVSI-110/N	β^+IVSI-110/N	√
78	14	0.91	β^+IVSI-110/N	β^+IVSI-110/N	√
63	11	0.91	β^+IVSI-110/N	β^+IVSI-110/N	√
64	10	1.04	β^+IVSI-110/N	β^+IVSI-110/N	√
120	9	0.72	N/N	N/N	√

Pregnant women β^+IVSI-110/N with partner β^+IVSI-110/N					
# sample	Gestational weeks	M/N ratio	Formulated diagnosis	Fetal genotype	Diagnosis outcome
133	10	0.70	N/N	N/N	√

Table 4.12. Fetal genotype determination by ddPCR of circulating cfDNAs from plasma of heterozygous pregnant women for β thalassemia mutations with partner homozygous normal or carrier of the same mutation. The table shows, for each circulating cf sample of pregnant woman with heterozygous genotype, for $\beta^0$39 (on the left side) or β^+IVSI-110 (on the right side), whose partner is normal homozygous (in the upper part) or heterozygous (in the lower part) for the same mutation pregnant, the assigned number, the gestational age, the M/N allelic ratio, the formulated diagnosis resulting by ddPCR assays, the actual fetal genotype, determinated by DNA sequencing, and the diagnosis outcome. √, confirmed.

5. CONCLUSIONS AND FUTURE PERSPECTIVES

Commonly, the diagnosis in fetal age is obtained using invasive procedures such as amniocentesis and chorionic villus sampling, but these procedures hide a risk of miscarriage of 1% and cannot be performed until 11 weeks of gestation [Sillence et al., 2015]. So, recently non-invasive pre-natal diagnosis techniques are widely under investigation for detecting pre-natal disorders and pregnancy monitoring [Galbiati et al., 2005; Tounta et al., 2011; Edlow & Bianchi, 2012; Pescia et al., 2017].

Non-invasive pre-natal diagnosis started after the discovery by Lo et al. [Lo et al., 1997] of ccffDNA in maternal circulation, and is based on fetal DNA analysis starting from a simple maternal peripheral blood sampling, without impairing the unborn child and the pregnant woman health, avoiding the risks associated with conventional invasive techniques withdrawal [D'Aversa et al., 2018].

Nowadays more than 100 X-linked inherited human disorders have been identified, such as hemophilia and Duchenne muscular dystrophy [Germain, 2006]. Therefore, the fetal sex determination is crucial to predict the pathological phenotype for the unborn. Indeed, for recessive X-linked diseases, the possibility of pathological phenotype for female fetuses is excluded, while for males the risk persists [Mandieh et al., 2013].

In particular, the fetal sex determination is necessary for: (1) early conventional therapy employment, (2) timely personalized therapy employment for the newborn and (3) possible decision of pregnancy interruption from the parents.

In this context, the aim of the first part of the research proposed in this thesis, was the development of rapid, sensitive, and cost-effective non-invasive diagnostic methods for the determination of fetal sex, in particular at early gestational ages.

In this field, an already reported study was developed by our research group [Breveglieri et al., 2016]: after collecting 26 blood samples from pregnant women, plasma was prepared and circulating cfDNA was extracted and analyzed using, as diagnostic molecular techniques, the qRT-PCR and the SPR-based Biacore^{TM} technology, in order to determine the fetal sex detecting the SRY gene.

The obtained results demonstrated that the fetal sex diagnosis can correctly performed by qRT-PCR starting from the 9th gestational week, and from the 7th using Biacore^{TM} system. So, the latter technique has proved to be a higher sensitive and accurate method for molecular diagnosis of non-invasive detection of fetal gender, but prior to the 7th week we obtained wrong outcomes probably due to the low concentration of ccffDNA in maternal circulation increasing during the progression of pregnancy [Zhou et al., 2015].

Therefore, considering the low number of samples analyzed in this previous work, at first, we decided to extend the qRT-PCR analysis to 139 circulating cfDNA samples with a wide variability in term of gestational weeks. Even if the accuracy of the technique was again demonstrated, the gestational limit of the molecular technology was confirmed.
So, in order to allow a correct fetal diagnosis, at early weeks of gestation, the ddPCR was employed: in fact, the technology is known for its high sensitivity and specificity.
After optimizing the ddPCR experimental conditions using genomic and circulating DNAs belonging to male and female subjects, 29 ccffDNA samples at early gestational stages (12-4.5 weeks) were analyzed and for all of them the fetal gender was correctly determined even at earliest gestational age (4.5 weeks), achieving 100% accuracy.
In conclusion, for the first time, we have demonstrated that ddPCR technology can be used for non-invasive pre-natal diagnosis of Y chromosome at early gestation ages (prior to 7^{th} week), establishing that the ddPCR is a robust, sensitive, efficient and reliable technology for the earliest fetal sex determination from maternal plasma.
A future perspective could be the extension of the number of ccffDNA samples at early gestational ages with the aim to confirm the gestational limit of the technique and its great accuracy and efficiency.

β thalassemia is an autosomal recessive inherited disease, with high incidence in the Mediterranean area [Cao, 2010]. This genetic blood disorder, caused by nearly 300 mutations, is associated with the absence (β^0) or reduction (β^+) of adult hemoglobin causing a severe anemia [Silvestroni, 1998; Cao & Galanello, 2010]. β thalassemia major is the most severe form of the disease, and patients are unable to survive into adulthood without a therapeutic transfusion plan associated with iron chelation [Weatherall, 1980]. These therapies are just for maintenance, the only definitive treatment, for this condition, is the bone marrow transplantation, which hides transplant-related complications such as infections, rejection and GVHD [Silvestroni, 1998; Cai et al., 2018].
In this context, innovative therapeutic approaches have been investigated in order to employ a personalized therapy based on genetic, biomarkers and phenotypic characteristics of the patient [Jameson & Longo, 2015]. The antisense approach, targeting splicing mutations such as β^+IVSI-110, β^0IVSI-1 and β^+IVSI-6 [Suwanmanee et al., 2002], and the read-through approach, specifically studied for nonsense mutations [Doronina et al., 2006] such as $\beta^0$39 [Orkin & Goff, 1981], are some examples of β thalassemia personalized therapies.
For this reason, the detection of the pathogenic molecular alterations, causing the disease, could be of substantial importance for the employment of targeted and personalized therapy

for the patients. Clearly, an early diagnosis is also need to start a maintenance therapy as soon as possible, in order to improve the patients' quality of life. Even more, a pre-natal β thalassemia diagnosis is the kay for the timely personalized therapy application.

Several molecular techniques for the detection of point mutations, such as β thalassemia, have been developed over the years, and today genomic DNA sequencing is the approach routinely employed to identify molecular alterations [Mahdieh & Rabbani, 2013]. However, all these techniques are costly, labour intensive and technically demanding [Sekizawa & Saito, 2001; Wright & Burton, 2009; Bianchi, 2012; Gallo et al., 2017].

In this context, the aim of the second part of the research proposed in this thesis, was the development of rapid, sensitive and cost-effective diagnostic approaches for the identification of the most common mutations causing β thalassemia in the Mediterranean area ($\beta^0$39, β^+IVSI-110, β^0IVSI-1, β^+IVSI-6) [Cao & Galanello, 2010], from genomic DNA of patients (post-natal diagnosis) and circulating cfDNA extracted from plasma of pregnant women (pre-natal diagnosis).

Actually, no commercial assays are available to recognize point mutations; the non-invasive screening tests offered to pregnant women can detect only aneuploidies, fetal sex, small deletions or insertions [Norbury & Norbury, 2008].

The first technique employed for post-natal diagnosis of β thalassemic point mutations was Biacore™ system, chosen for its sensitivity and possibility of real-time analysis. After the immobilization of a normal and a mutated biotinylated oligonucleotide probes on the sensor chip, a pair for each mutation, they were validated evaluating the binding with normal and mutated complementary oligonucleotides. Finally the diagnosis was performed from single-stranded PCR products obtained from genomic DNA of 71 patients with different genotypes (NN, N/M and M/M), extracted from blood and buccal swab samples. For all the specimens analyzed it was possible to correctly discriminate the different allelic conditions for each mutation, demonstrating the suitability of the developed SPR-based approach to detect β thalassemia mutations, in a simple fast and reproducible way. In addition, no differences have been observed between the two DNA sources, demonstrating that the developed method may be efficiently applied to salivary swabs: much less invasive source that achieve patients' compliance.

Another technique employed for post-natal diagnosis of β thalassemia point mutations was the qRT-PCR based on genotyping assays.

After the extraction of genomic DNA from blood or buccal swab samples, the designed genotyping assays, one for each mutation, were optimized and validated selecting a limited pool of genomic DNA samples of different genotypes (N/N, N/M and M/M). Finally, the

molecular diagnosis was performed for 25 unknown samples belonging to patients affected by β thalassemia. After DNA sequencing, the diagnostic outcomes, for each sample, were confirmed, demonstrating that qRT-PCR based on genotyping assays developed method may be efficiently employed for post-natal diagnosis of β thalassemia point mutations, also after non-invasive DNA sampling method as a buccal swab.

After demonstrating the suitability of the simple and inexpensive genotyping assays for molecular post-natal diagnosis, the attention was focused on their possible application to non-invasive pre-natal screening, in order to detect fetal point mutations, responsible for β thalassemia inherited from the father: a quite feasible case because the mutation is not present in maternal DNA, that is the main component of circulating DNA extracted from maternal plasma.

After collecting 26 blood samples from pregnant women whose partner is carrier of the thalassemic mutation, the plasma was prepared and a biobank was established with a wide variability in terms of weeks of gestation. Then, the total circulating cfDNA from maternal plasma was extracted, pre-amplified and analyzed with the specific genotyping assay in order to determine if the fetus has inherited the father mutation.

The obtained data have shown that the developed genotyping assays could be efficiently employed for non-invasive pre-natal diagnosis of paternally inherited β thalassemia mutations, at least until the 9^{th} gestational week. At the 5^{th} week of gestation the ccffDNA amount is very low and not detectable with this approach.

In order to extend the non-invasive pre-natal diagnosis to paternally inherited thalassemic mutations at early pregnancy (prior to 9^{th}), and to maternally and both maternally and paternally inherited thalassemic mutations a new sensitive and precise approach has been proposed: the employment of the ddPCR technology.

After optimizing $\beta^0$39 and β^+IVSI-110 assays in term of amplification efficiency and hybridization specificity, using genomic not digested and digested DNAs of different genotype (N/N, N/M, and M/M), the two assays were validated using mixtures of two digested genomic DNAs of different genotype in order to simulate fetal and maternal circulating cfDNA. Finally, after collecting 36 blood samples from pregnant women, the circulating cfDNA was extracted from plasma and the samples analyzed with the specific ddPCR assay in order to determine the fetal genotype.

In the samples in which the mutation is paternally inherited, the only presence of a mutated allele in a normal background indicates the heterozygous genotype of the fetus. For all the samples analyzed the diagnostic outcome was confirmed by DNA sequencing, also at 5^{th} gestational week.

The case of maternally or both parents inherited mutation is more complex: a precise quantification of normal and mutated alleles in maternal plasma is required to evaluate whether they are balanced or unbalanced. Allelic balance is expected when the fetal genotype is identical to the mother's one, whereas allelic imbalance occurs if there is an under-representation (the fetus is homozygous for the normal allele) or over-representation (the fetus is homozygous for the mutated allele) of the mutant allele with respect to the normal one [Lun et al., 2008]. For this reason in order to identify the fetal genotype, the M/N ratio parameter was introduced. The obtained results have been identify two diagnostic ranges of M/N ratio values statistically distinct and not overlapping, allowing the correctly determination of fetal genotype in all the samples analyzed also at early gestational weeks (until the 9^{th}). Also for the interesting case in which both the parents were carrier of the same mutation, a real case in which the future baby could be a thalassemic patient, the outcomes were confirmed.

In conclusion we have demonstrated that ddPCR technology can be used for non-invasive pre-natal testing of β^{+}IVSI-110 and β^{0}39 mutations paternally inherited at early gestational ages (prior to 9^{th} week), and, for the first time, maternally and both parents inherited mutation, also at early gestational ages (until 9^{th} week), confirming the development of a simple, sensitive, fast, and pre-amplification free approach for the determination of β thalassemia mutations, using maternal plasma.

In addition, in all the cases considered the ddPCR was found to be a robust, sensitive, efficient and reliable technology, with a detection limit lower than the other technologies.

However, in order to confirm the effective efficiency and applicability of the technology to β thalassemia mutations identification, will be necessary to increase the number of ccffDNA samples and recruit ccffDNA samples at early gestational weeks (prior to 9) to identify the gestational limit of the approach.

Another future perspective could be extend the analysis to the other thalassemic point mutations.

In future, both the post-natal and pre-natal developed approaches could be apply to point mutations causing other genetic diseases, with the aim to designed diagnostic protocols useful for patients and fetuses stratification in personalized therapy on the road of precision medicine.

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www.osservatoriodellemalattierare.it

www.rarepartners.org

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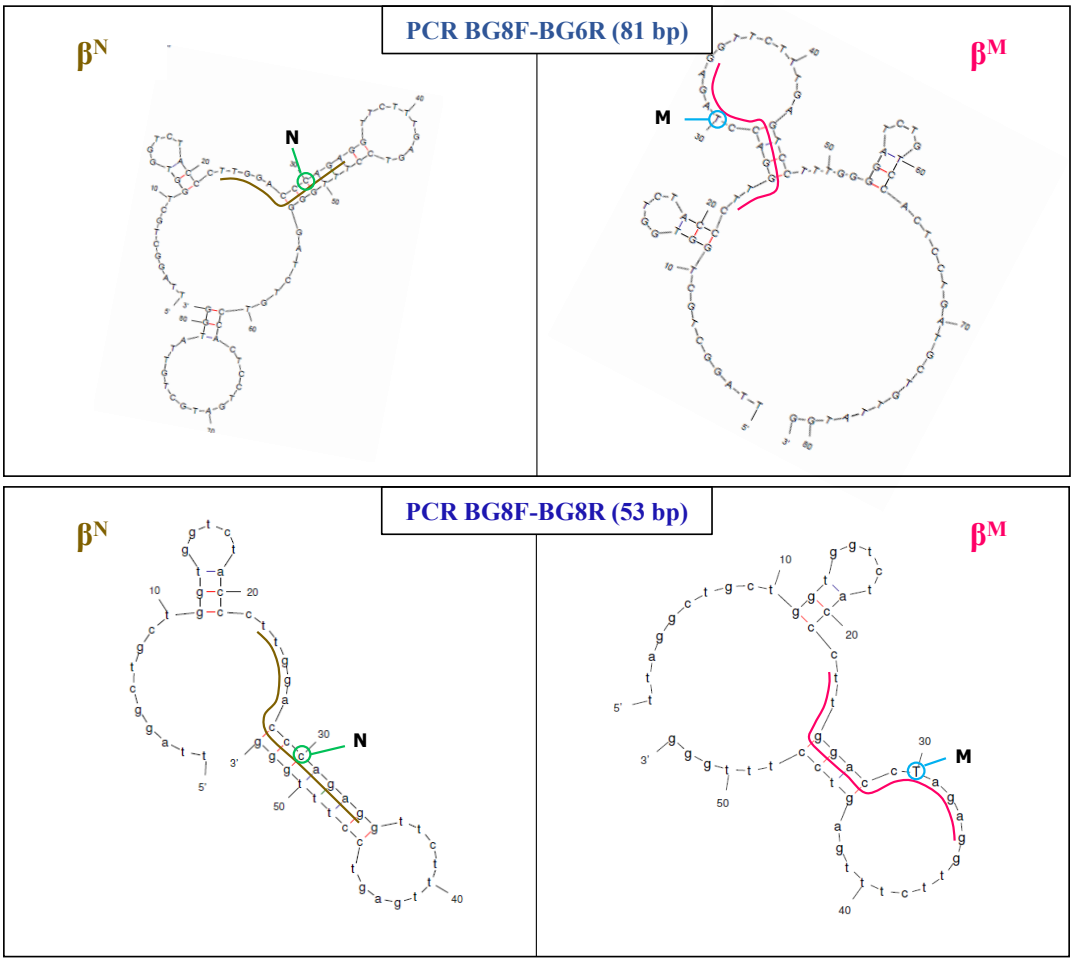
SUPPLEMENTARY MATERIALS

A

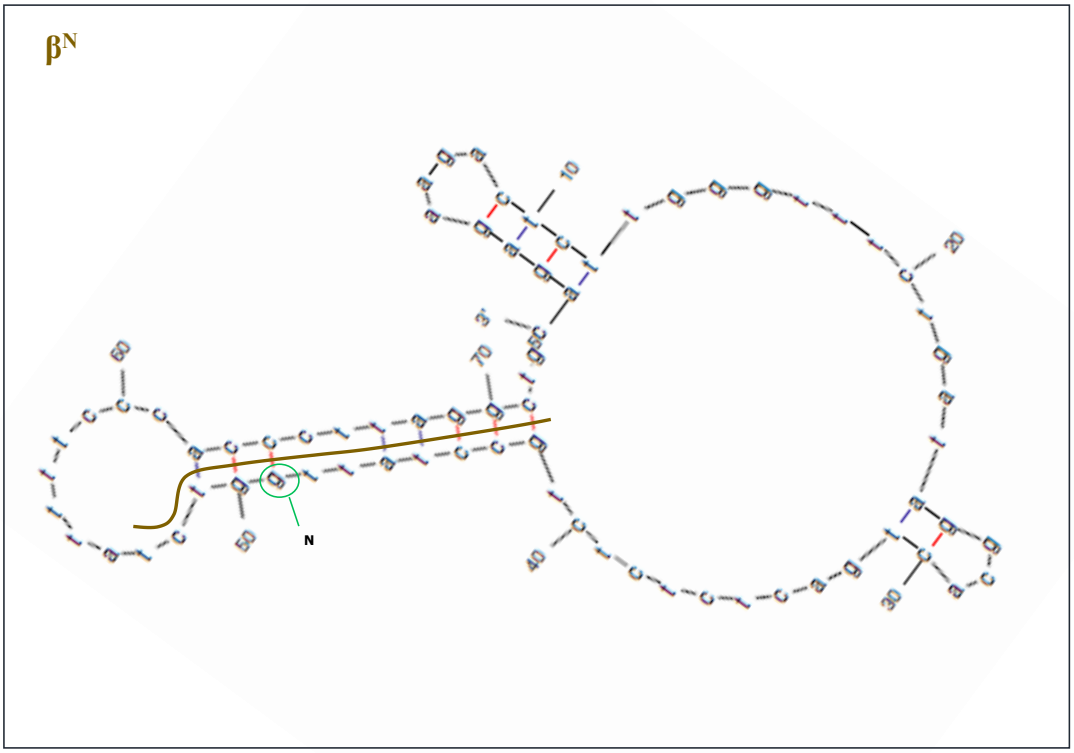
B



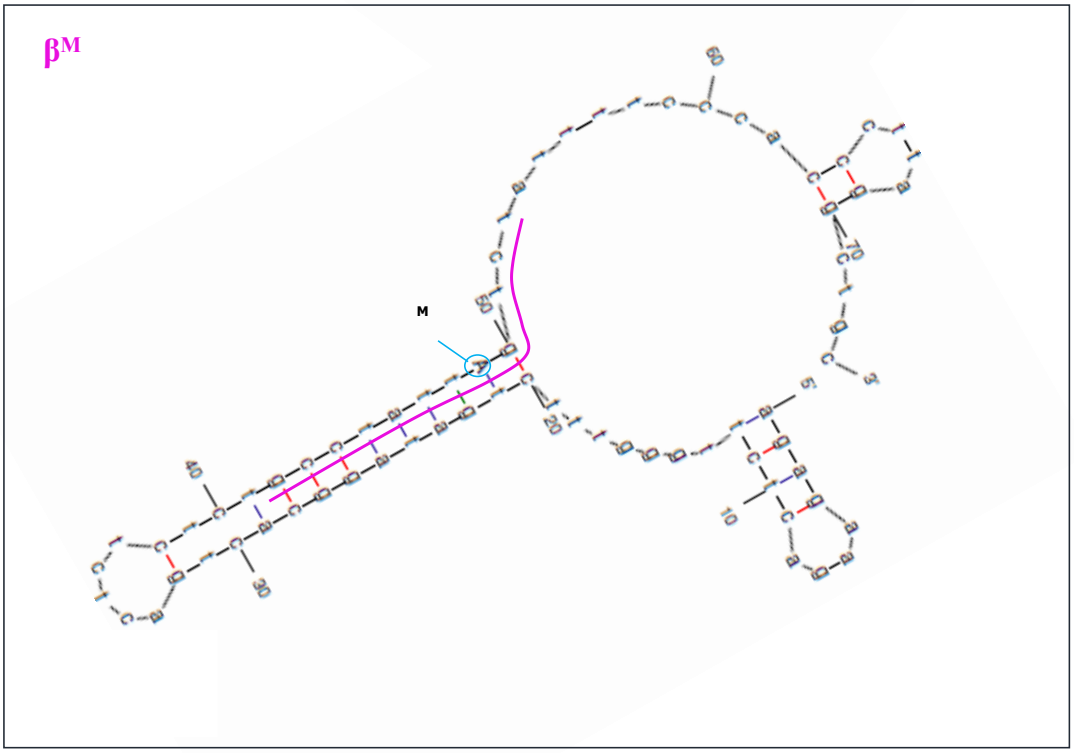
Supplementary figure 1. Secondary structures of the normal (β^N) and mutated (β^M) single-stranded 81 bp (A) and 53 bp (B) target sequences to be used as analytes in SPR-based diagnostic analyses. For each expected structure, the position of the $\beta^0$39 mutation and the region of base pairing with the immobilized complementary probe are indicated.

β⁺IVSI-110

A



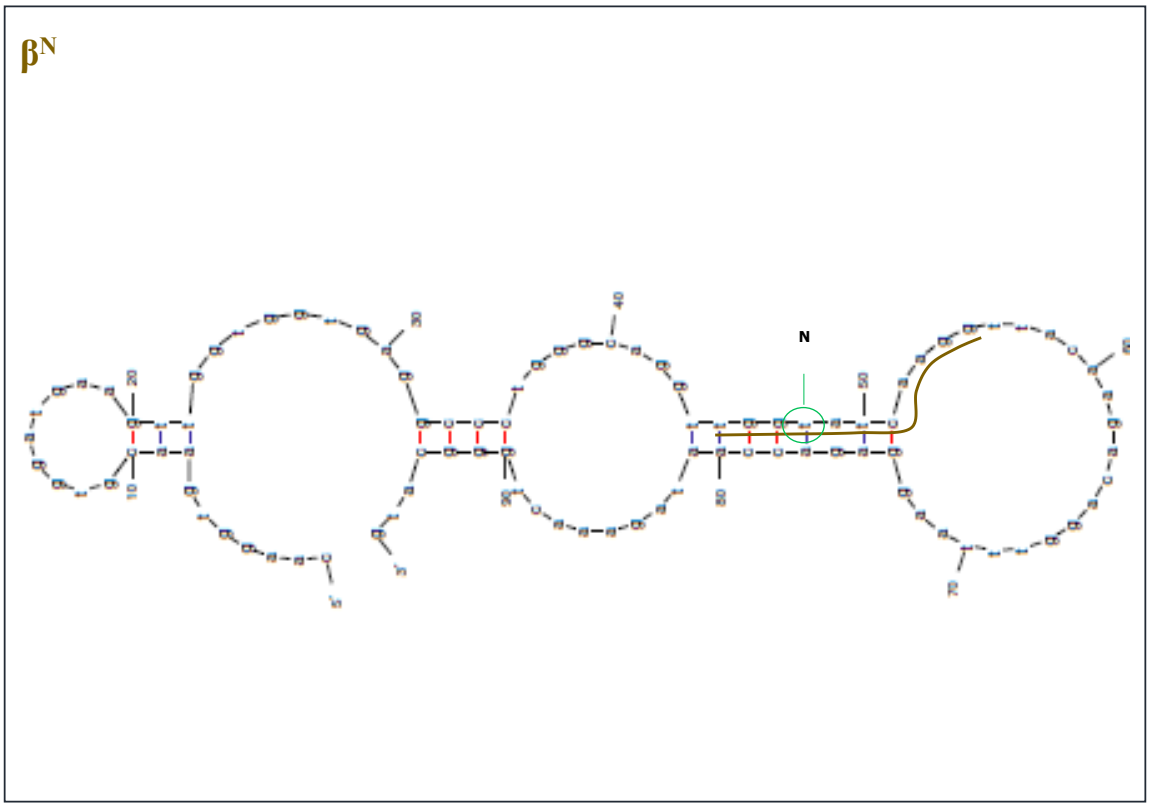
B



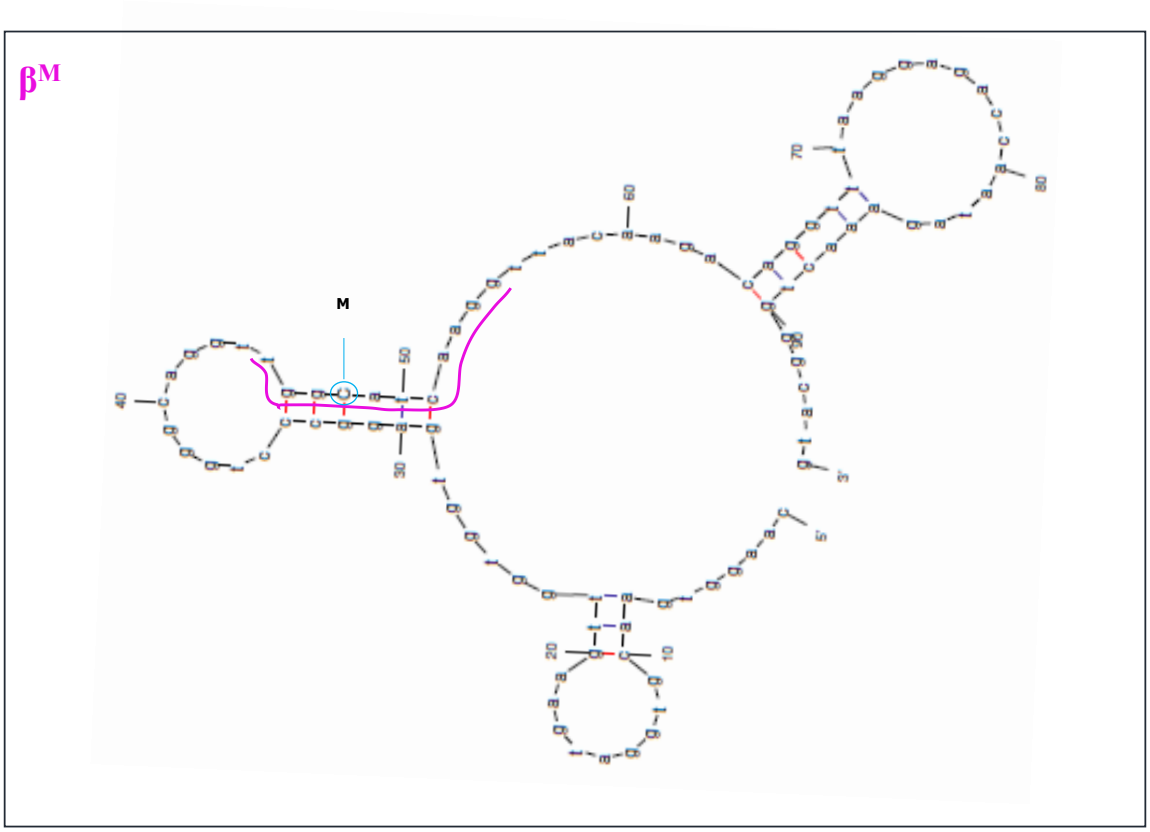
Supplementary figure 2. Secondary structures of the normal (β^N) (A) and mutated (β^M) (B) single-stranded β⁺IVSI-110 target sequences to be used as analytes in SPR-based diagnostic analyses. For each expected structure, the position of the mutation β⁺IVSI-110 and the region of base pairing with the immobilized complementary probe are indicated.

β+IVSI-6

A



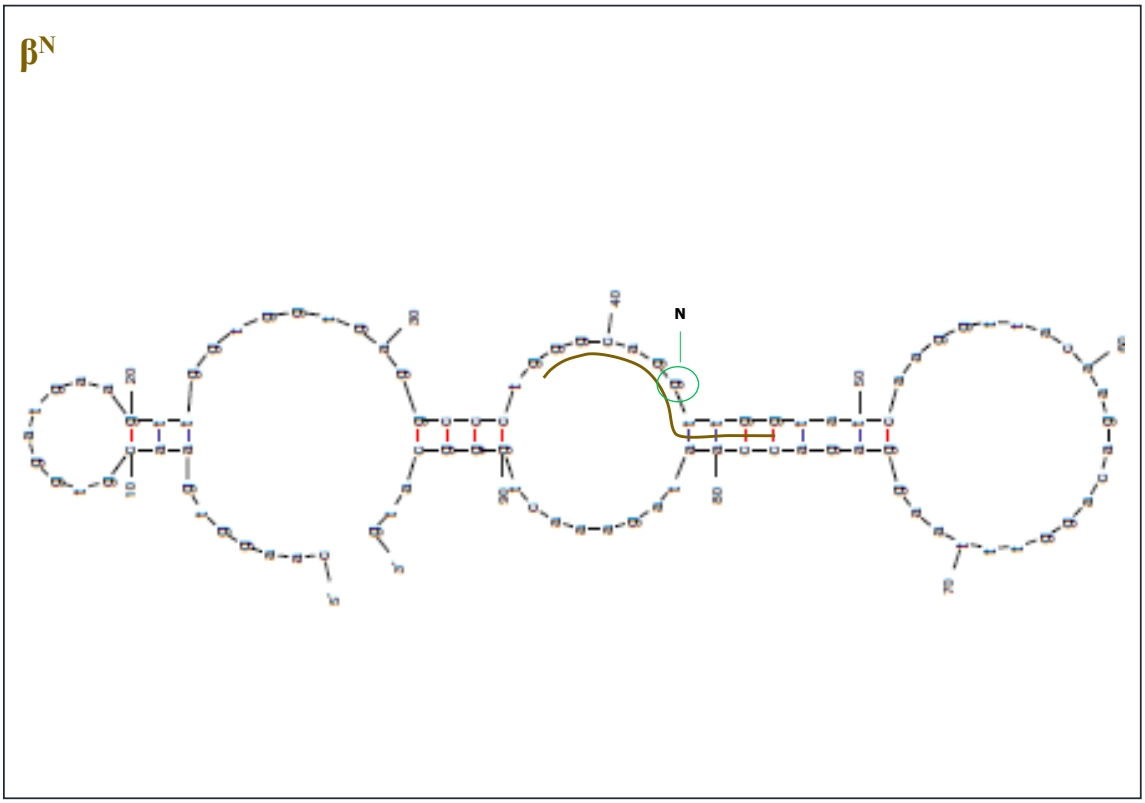
B



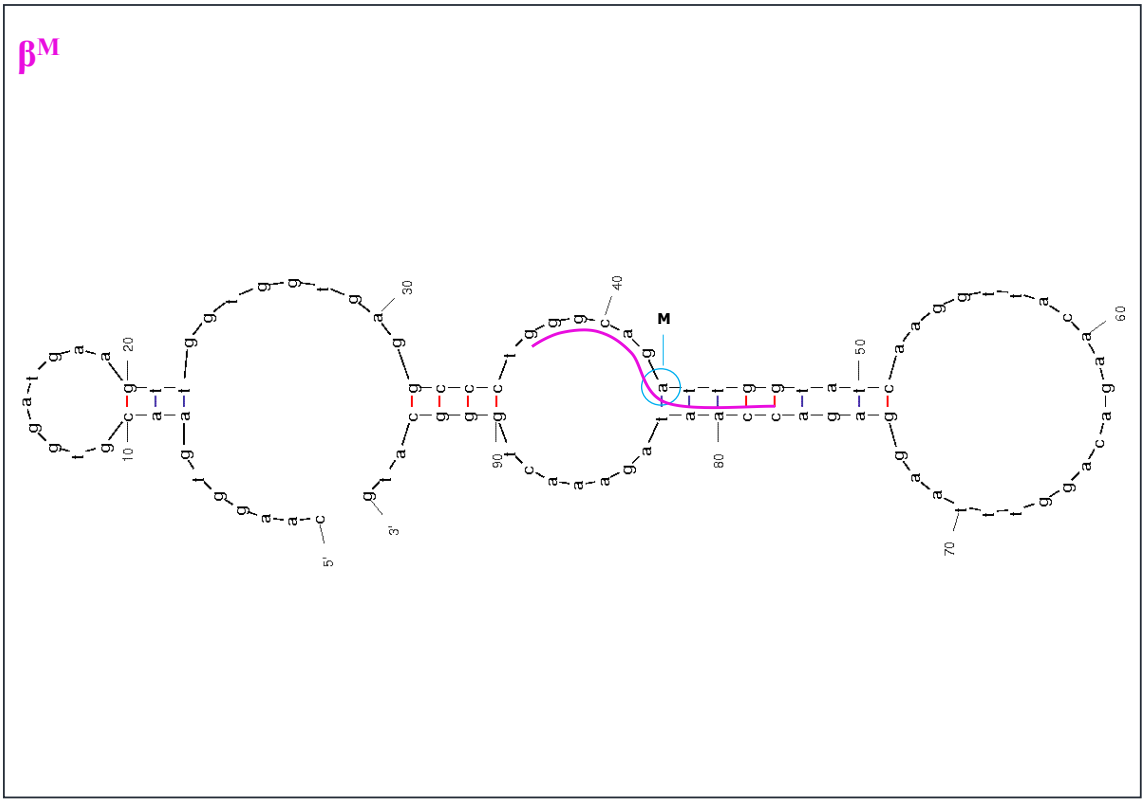
Supplementary figure 3. Secondary structures of the normal (β^N) (A) and mutated (β^M) (B) single-stranded β^+IVSI-6 target sequences to be used as analytes in SPR-based diagnostic analyses. For each expected structure, the position of the mutation β^+IVSI-6 and the region of base pairing with the immobilized complementary probe are indicated.

β^0IVSI-1

A



B



Supplementary figure 4. Secondary structures of the normal (β^N) (A) and mutated (β^M) (B) single-stranded β^0IVSI-1 target sequences to be used as analytes in SPR-based diagnostic analyses. For each expected structure, the position of the mutation β^0IVSI-1 and the region of base pairing with the immobilized complementary probe are indicated.

# Sample	Resulting genotype	$\beta^0$39		β^+IVSI-110		β^+IVSI-6		β^0IVSI-1	
		FAM™ Rn	VIC® Rn	FAM™ Rn	VIC® Rn	FAM™ Rn	VIC® Rn	FAM™ Rn	VIC® Rn
1	β^+IVSI-6/β^+IVSI-110	1.36	1.01	2.08	1.23	1.77	2.69	0.60	1.80
2	β^+IVSI-110/$\beta^0$39	2.20	0.79	2.06	1.28	1.09	3.09	0.61	1.82
3	β^+IVSI-1/$\beta^0$39	2.25	0.77	1.55	1.67	1.09	2.88	1.98	1.15
4	β^+IVSI-1/$\beta^0$39	2.15	0.80	1.57	1.67	1.09	2.90	2.01	0.97
5	β^+IVSI-6/$\beta^0$39	2.22	0.76	1.54	1.57	1.99	2.71	0.79	1.44
6	β^+IVSI-110/$\beta^0$39	2.14	0.80	2.05	1.27	1.09	3.04	0.61	1.92
7	β^+IVSI-6/$\beta^0$39	2.19	0.79	1.58	1.70	2.01	2.80	0.78	1.50
8	β^+IVSI-1/$\beta^0$39	2.26	0.75	1.59	1.67	1.12	2.99	1.85	1.18
9	β^+IVSI-110/β^+IVSI-110	1.37	0.99	2.09	0.54	1.10	3.16	0.78	1.67
10	$\beta^0$39/$\beta^0$39	3.43	0.51	1.57	1.61	1.11	3.17	0.60	1.81
11	β^+IVSI-110/$\beta^0$39	2.25	0.73	2.11	1.23	1.08	3.05	0.58	1.91
12	β^0IVSI-1/β^+IVSI-110	1.39	1.00	2.09	1.29	1.08	2.80	1.83	1.27
13	β^+IVSI-110/β^+IVSI-110	1.39	1.02	2.16	0.56	1.10	3.16	0.76	1.65
14	$\beta^0$39/$\beta^0$39	2.75	0.47	1.56	1.62	1.09	3.12	0.78	1.64
15	$\beta^0$39/$\beta^0$39	3.28	0.51	1.58	1.58	1.10	2.91	0.78	1.62
16	β^+IVSI-6/β^+IVSI-110	1.37	1.01	2.09	1.30	2.04	2.86	0.60	1.90
17	β^+IVSI-110/β^+IVSI-110	1.39	0.99	2.16	0.55	1.09	3.13	0.77	1.56
18	β^0IVSI-1/β^+IVSI-6	1.50	0.92	1.56	1.54	2.00	1.58	1.98	1.00
19	β^+IVSI-6/β^+IVSI-6	1.39	1.02	1.56	1.57	2.61	0.72	0.79	1.60
20	β^+IVSI-6/β^+IVSI-6	1.39	1.02	1.56	1.62	2.71	0.71	0.76	0.93
21	β^0IVSI-1/β^+IVSI-6	1.36	0.99	1.54	1.57	1.99	1.87	1.96	1.05
22	β^+IVSI-6/β^+IVSI-6	1.39	0.92	1.56	1.44	2.03	2.03	0.80	1.58
23	β^0IVSI-1/β^+IVSI-6	1.07	1.21	1.49	1.84	1.81	1.51	1.37	1.40
24	β^0IVSI-1/β^+IVSI-6	1.06	1.18	1.49	1.82	1.81	1.43	1.87	1.17
25	β^0IVSI-1/β^0IVSI-1	1.35	1.08	1.37	1.32	0.84	1.39	2.78	0.58

Supplementary table 1. List of the normalized end-point fluorescence (Rn) values generated by the VIC®-labeled probe and the FAM™-labeled probe, resulting from the analysis of 25 unknown samples by genotyping assays.

PUBLICATIONS

Mol Med. (2018); 24(1):14.

Non-invasive fetal sex diagnosis in plasma of early weeks pregnants using droplet digital PCR

D'Aversa E, Breveglieri G, Pellegatti P, Guerra G, Gambari R, Borgatti M.

Abstact

BACKGROUND:

Fetal sex determination is useful for families at risk of X-linked disorders, such as Duchenne muscular dystrophy, adrenal hypoplasia, hemophilia. At first, this could be obtained through invasive procedures such as amniocentesis and chorionic villus sampling, having a 1% risk of miscarriage. Since the discovery of cell-free fetal DNA (cffDNA) in maternal plasma, noninvasive prenatal testing permits the early diagnosis of fetal sex through analysis of cffDNA. However, the low amount of cffDNA relative to circulating maternal DNA requires highly sensitive molecular techniques in order to perform noninvasive prenatal diagnosis. In this context we employed droplet digital PCR (ddPCR) in order to evaluate the earliest possible fetal sex determination from circulating DNA extracted from plasma of pregnant women at different gestational ages.

METHODS:

We identified the fetal sex on cffDNA extracted from 29 maternal plasma samples at early gestational ages, several of them not suitable for qPCR determination, using ddPCR designed for SRY gene target.

RESULTS:

All maternal plasma samples were determined correctly for SRY gene target using ddPCR even at very early gestational age (prior to 7 weeks).

CONCLUSIONS:

The ddPCR is a robust, efficient and reliable technology for the earliest possible fetal sex determination from maternal plasma.

Chem Med Chem. (2018); 13(15):1549-1554.

Effect of Acylation on the Antimicrobial Activity of Temporin B Analogues

Avitabile C, D'Andrea LD, D'Aversa E, Milani R, Gambari R, Romanelli A.

Abstract

New peptides derived from the natural antimicrobial temporin B were obtained. The design, antimicrobial activity, and characterization of the secondary structure of peptides in the presence of bacterial cells is described herein. TB_KKG6K (KKLLPIVKNLLKSLL) has been identified as the most active analogue against Gram-positive and -negative bacteria, compared with natural temporin B (LLPIVGNLLKSLL) and TB_KKG6A (KKLLPIVANLLKSLL). Acylation with hydrophobic moieties generally led to reduced activity; however, acylation at the 6-position of TB_KKG6K led to retained sub-micromolar activity against Staphylococcus epidermidis.

BMC Biotechnol. (2018); 18(1):28.

UPF1 silenced cellular model systems for screening of read-through agents active on $\beta^0$39 thalassemia point mutation

Salvatori F, Pappadà M, Breveglieri G, D'Aversa E, Finotti A, Lampronti I, Gambari R, Borgatti M.

Abstract

BACKGROUND:

Nonsense mutations promote premature translational termination, introducing stop codons within the coding region of mRNAs and causing inherited diseases, including thalassemia. For instance, in $\beta^0$39 thalassemia the CAG (glutamine) codon is mutated to the UAG stop codon, leading to premature translation termination and to mRNA destabilization through the well described NMD (nonsense-mediated mRNA decay). In order to develop an approach facilitating translation and, therefore, protection from NMD, ribosomal read-through molecules, such as aminoglycoside antibiotics, have been tested on mRNAs carrying premature stop codons. These findings have introduced new hopes for the development of a pharmacological approach to the $\beta^0$39 thalassemia therapy. While several strategies, designed to enhance translational read-through, have been reported to inhibit NMD efficiency concomitantly, experimental tools for systematic analysis of mammalian NMD inhibition by translational read-through are lacking.

RESULTS:

We developed a human cellular model of the $\beta^0$39 thalassemia mutation with UPF-1 suppressed and showing a partial NMD suppression.

CONCLUSIONS:

This novel cellular model could be used for the screening of molecules exhibiting preferential read-through activity allowing a great rescue of the mutated transcripts.

Eur J Med Chem. (2018); 151:285-293.

Design, synthesis and biological evaluation of novel trimethylangelicin analogues targeting nuclear factor kB (NF-kB)

Marzaro G, Lampronti I, D'Aversa E, Sacchetti G, Miolo G, Vaccarin C, Cabrini G, Dechecchi MC, Gambari R, Chilin A.

Abstract

A series of trimethylangelicin (TMA) derivatives were designed and synthesized to overcome the unwanted effects of TMA, promising agent for treatment of inflammation-related diseases and other pathologies, such as cystic fibrosis. The new generation TMA analogues bore hindered substituents at the 4 position in order to minimize or avoid the photoreactions with DNA. Among them, the 4-isopropyl-6-ethyl derivative 23 exhibited TMA-like inhibitory activity on NF-κB/DNA interactions but it proved unable to photoreact with pyrimidine bases of DNA, nor to induce any other DNA damage. The isopropyl analogue 23 was proven to lack mutagenicity when assayed through Ames test and exhibited no anti-proliferative activity on cystic fibrosis IB3-1 cells, displaying at the same time inhibition of the TNF-α induced release of the NF-κB regulated PDGF-B chain, IL-10, IL-15, IL-17 and IFN-γ. Therefore compound 23 deserves further assay to determine its anti-inflammatory properties, since it lacks photoreaction properties and mutagenicity-related side effects.

Sensors and Actuators B 260 (2018) 710-718

A novel and efficient protocol for Surface Plasmon Resonance based detection of four β-thalassemia point mutations in blood samples and salivary swabs

Breveglieri G, D'Aversa E, Gallo TE, Pellegatti P, Guerra G, Cosenza LC, Finotti A, Gambari R, Borgatti M.

Abstract

Optical biosensors based on Surface Plasmon Resonance (SPR), such as the Biacore™ X100, are widely used to study in real-time and in label-free mode bio-molecular interactions, including those allowing the identification of single point mutations responsible of genetic diseases, such as thalassemia and cystic fibrosis. The aim of this study was to verify whether the Biacore™ X100 can be proposed for the real-time detection of four mutations of the human β-globin gene causing β-thalassemia, a genetic blood disorder associated with absence (β^0) or reduction (β^+) of adult hemoglobin and severe anemia. In particular we analyzed the most frequent thalassemia point mutations present in the Mediterranean area ($\beta^0$39, β^0IVSI-1, β^+IVSI-110 and β^+IVSI-6) using a novel SPR-based interaction format where two oligonucleotide probes (one complementary to the normal sequence and the other to the mutated one) were immobilized on sensor chips and asymmetric PCR targets obtained from genomic DNA of analyzed subjects were injected. For the development of the diagnostic approach, genomic DNAs of different genotypes for each mutation were obtained from blood samples or salivary swabs of 71 subjects, including healthy individuals, heterozygous β-thalassemia carriers and homozygous β-thalassemia patients. The results obtained allow proposing a new non-invasive diagnostic SPR-based protocol for thalassemia single point mutations using blood samples and salivary swabs as a source of genomic DNA.

Front Pharmacol. (2017); 8:236.

β-Sitosterol Reduces the Expression of Chemotactic Cytokine Genes in Cystic Fibrosis Bronchial Epithelial Cells

Lampronti I, Dechecchi MC, Rimessi A, Bezzerri V, Nicolis E, Guerrini A, Tacchini M, Tamanini A, Munari S, D'Aversa E, Santangelo A, Lippi G, Sacchetti G, Pinton P, Gambari R, Agostini M, Cabrini G.

Abstract

Extracts from Nigella arvensis L. seeds, which are widely used as anti-inflammatory remedies in traditional medicine of Northern Africa, were able to inhibit the expression of the pro-inflammatory neutrophil chemokine Interleukin (IL)-8 in Cystic Fibrosis (CF) bronchial epithelial IB3-1 cells exposed to the Gram-negative bacterium Pseudomonas aeruginosa. The chemical composition of the extracts led to the identification of three major components, β-sitosterol, stigmasterol, and campesterol, which are the most abundant phytosterols, cholesterol-like molecules, usually found in plants. β-sitosterol (BSS) was the only compound that significantly reproduced the inhibition of the P. aeruginosa-dependent expression of IL-8 at nanomolar concentrations. BSS was tested in CF airway epithelial CuFi-1 cells infected with P. aeruginosa. BSS (100 nM), showed a significant and consistent inhibitory activity on expression of the P. aeruginosa-stimulated expression chemokines IL-8, GRO-α GRO-β, which play a pivotal role in the recruitment of neutrophils in CF inflamed lungs. Preliminary mechanistic analysis showed that BSS partially inhibits the P. aeruginosa-dependent activation of Protein Kinase C isoform alpha, which is known to be involved in the transmembrane signaling activating IL-8 gene expression in bronchial epithelial cells. These data indicate BSS as a promising molecule to control excessive lung inflammation in CF patients.

PLoS One. (2017); 12(2):e0172756.

Postnatal and non-invasive prenatal detection of β-thalassemia mutations based on Taqman genotyping assays

Breveglieri G, Travan A, D'Aversa E, Cosenza LC, Pellegatti P, Guerra G, Gambari R, Borgatti M.

Abstract

The β-thalassemias are genetic disorder caused by more than 200 mutations in the β-globin gene, resulting in a total (β0) or partial (β+) deficit of the globin chain synthesis. The most frequent Mediterranean mutations for β-thalassemia are: β039, β+IVSI-110, β+IVSI-6 and β0IVSI-1. Several molecular techniques for the detection of point mutations have been developed based on the amplification of the DNA target by polymerase chain reaction (PCR), but they could be labor-intensive and technically demanding. On the contrary, TaqMan® genotyping assays are a simple, sensitive and versatile method suitable for the single nucleotide polymorphism (SNP) genotyping affecting the human β-globin gene. Four TaqMan® genotyping assays for the most common β-thalassemia mutations present in the Mediterranean area were designed and validated for the genotype characterization of genomic DNA extracted from 94 subjects comprising 25 healthy donors, 33 healthy carriers and 36 β-thalassemia patients. In addition, 15 specimens at late gestation (21-39 gestational weeks) and 11 at early gestation (5-18 gestational weeks) were collected from pregnant women, and circulating cell-free fetal DNAs were extracted and analyzed with these four genotyping assays. We developed four simple, inexpensive and versatile genotyping assays for the postnatal and prenatal identification of the thalassemia mutations β039, β+IVSI-110, β+IVSI-6, β0IVSI-1. These genotyping assays are able to detect paternally inherited point mutations in the fetus and could be efficiently employed for non-invasive prenatal diagnosis of β-globin gene mutations, starting from the 9th gestational week.

PUBLISHED ABSTRACT

21st World Congress on Advances in Oncology and 19th International Symposium on Molecular Medicine, Athens (Greece), 6-8 October 2016.

International Journal of Molecular Medicine, 38 (Supp1): S69, 2016.

SPR-based biosensor for non-invasive prenatal diagnosis of Y-chromosome

Breveglieri G, Cosenza LC, Pellegatti P, Guerra G, Salvatori F, D'Aversa E, Finotti A, Gambari R, Borgatti M.

Abstract

Since the discovery of cell-free fetal DNA (cffDNA) in maternal plasma, diagnostic non-invasive prenatal methods have been developed. As far as Y-chromosome identification, this might be important for therapeutic intervention on sex-associated pathologies such as Duchenne muscular dystrophy, hemophilia and congenital adrenal hyperplasia. Surface plasmon resonance (SPR)-based biosensors might be useful for these studies, because they allow to monitor the molecular interactions in real-time providing qualitative and quantitative information, through kinetics, affinity and concentration analyses.

In this study Biacore^{TM} X100 has been applied to identify the Y-chromosome in cffDNA obtained from plasma samples of 26 pregnant women at different gestational ages, analysing the binding between SRY-PCR products and an SRY-specific probe immobilized on the sensor chip. The results suggest that there is a statistically significant difference between samples collected by pregnancies carrying male or female fetus. Moreover cffDNA, obtained at early gestational ages (6^{th}-7^{th} week) and not detectable by conventional quantitative real-time PCR, can be discriminated with high accuracy and reliability using SPR-based biosensors (Breveglieri G et. al, Prenatal Diagnosis 2016, 36, 353–361) and pre-amplification steps. In conclusion these results should be considered as the basis of future developments for more extensive diagnostic trials.

SUBMITTED ABSTRACTS

2nd European Biosensor Symposium 2019, Florence (Italy), 18-21 February 2019.

A novel and efficient protocol for Surface Plasmon Resonance based detection of four β-thalassemia point mutations in blood samples and salivary swabs

Breveglieri G, D'Aversa E, Pellegatti P, Guerra G, Cosenza LC, Finotti A, Gambari R, Borgatti M.

Abstract

Optical affinity biosensors based on Surface Plasmon Resonance (SPR), such as the Biacore™ X100 instrument, are widely used to study biomolecular interactions in real-time with high sensitivity and specificity, and can be employed for the identification of single point mutations causing genetic diseases, such as thalassemia [1] and cystic fibrosis [2]. The aim of this study was to verify whether the Biacore™ X100 biosensor could be used for the real-time detection of point mutations affecting the human β-globin gene and responsible of β-thalassemia, a genetic blood disorder associated with absence (β^0) or reduction (β^+) of adult hemoglobin (HbA) and severe anemia. In particular we analyzed the four most frequent β-thalassemia point mutations in the Mediterranean area: $\beta^0$39, β^0IVSI-1, β^+IVSI-110 and β^+IVSI-6. We used a novel SPR-based interaction format, by immobilization on sensor chips of two oligonucleotide probes, one complementary to the normal sequence and the other to the mutated one. Then we injected asymmetric PCR product targets obtained from genomic DNA of analyzed subjects of different genotypes for each mutation, including healthy individuals, heterozygous β-thalassemia carriers and homozygous β-thalassemia patients. For the development of the diagnostic approach genomic DNA samples were obtained from blood samples or salivary swabs of 71 total subjects. In all cases the genotypic identification was achieved [3]. The results obtained allow proposing a new SPR-based protocol for β-thalassemia single point mutations by using blood samples and salivary swabs as a source of genomic DNA. The same approach has been used for the molecular detection of the HbS point mutation responsible for sickle cell disease (SCD), another hemoglobinopathy characterized by strong anemia and vessel occlusion, fully confirming applicability of the developed protocol to other genetic diseases (supported by AIFA, Wellcome-Trust and by EU projects ULTRAPLACAD and THALAMOSS).

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[2] G. Feriotto, R. Corradini, S. Sforza, N. Bianchi, C. Mischiati, R. Marchelli, R. Gambari, Lab. Invest., 81, 1415-1427 (2001).

[3] G. Breveglieri, E. D'Aversa, T.E. Gallo, P. Pellegatti, G. Guerra, L.C. Cosenza, A. Finotti, R. Gambari, M. Borgatti, Sens. Actuators B Chem., 260, 710-718 (2018).

Development and characterization of a cellular biosensor for the screening of globin gene inducers useful in β-thalassemia

Breveglieri G, Salvatori F, Finotti A, Cosenza LC, Zuccato C, Bianchi N, Lampronti I, D'Aversa E, Tupini C, Borgatti M, Gambari R.

Abstract

The screening of molecules able to induce the production of fetal or adult hemoglobin in erythroid cells isolated from β-thalassemia patients is very important for the identification of compounds of interest for the pharmacological therapy of β-thalassemia, a genetic disease characterized by insufficient production of β-globin chains of adult hemoglobin (HbA) and severe anemia. In particular the stimulation of human γ-globin gene expression and increase of production of fetal hemoglobin (HbF) is considered a potential therapeutic approach both in β-thalassemia [1] and in sickle cell disease (SCD). In this study we report the development and characterization of a cellular biosensor for the identification of inducers of γ- and β-globin gene promoters. To this aim we first produced a vector containing green (EGFP) and red (RFP) fluorescence protein genes under the control of human γ-globin and β-globin gene promoters, respectively. Human K562 cells were used to obtain stable transfectants, where the transcriptional activity of globin promoters is proportional to the fluorescence signal produced by the respective protein [2], assessed by FACS (fluorescence activated cell sorting) analysis. The cellular model was then validated by treatment with hydroxyurea and sodium butyrate, two compounds known for their HbF inducing effects on thalassemic patients. Similar results were obtained after the administration of other compounds known for their stimulating effects on γ-globin gene in vitro and in vivo, such as mithramycin and ara-C, and new HbF inducers, demonstrating that our experimental system is suitable for a preliminary and fast screening of high numbers of compounds potentially active on the transcription of γ-globin and β-globin genes, possibly useful for β-thalassemia therapy. A good correlation was demonstrated between the results obtained using the EGFP/RFP clones and experiments performed on erythroid precursor cells from β-thalassemic patients (supported by AIFA, Wellcome-Trust and by EU projects ULTRAPLACAD and THALAMOSS).

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[1] R. Gambari, Blood Transfus., 8, 5-7 (2010).

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2nd European Biosensor Symposium 2019, Florence (Italy), 18-21 February 2019.

SPR-based studies of the binding efficiency of LYAR protein to the (+25 G→A) $^{A}\gamma$-globin gene sequences mutated in β-thalassemia

Breveglieri G, Finotti A, Gemmo C, Lampronti I, Cosenza LC, Zuccato C, Fabbri E, Bianchi N, D'Aversa E, Gasparello J, Zurlo M, Papi C, Borgatti M, Gambari R.

Abstract

Transcription factors regulating γ-globin gene transcription are known to interact with several elements of the γ-globin gene promoter. Interestingly, several of them (MYB, BCL11A) are strong repressors of γ-globin gene transcription. This issue is of great interest since the conclusions reached predict that the possible inhibition of these repressors might lead to the activation of γ-globin gene expression and production of fetal hemoglobin (HbF) in erythroid cells. Increased HbF has been firmly demonstrated to ameliorate the clinical severity of β-thalassemia and sickle-cell anemia patients. LYAR (Ly-1 antibody reactive clone) protein is a novel repressor of γ-globin gene transcription, which binds to a region corresponding to the γ-globin gene 5'-UTR. One of the possible effects of the $^{A}\gamma$-(+25 G->A) mutation in β-thalassemia is a decrease of the LYAR binding efficiency to its 5'-GGTTAT-3' binding site [1,2]. The present SPR-based study was undertaken to determine whether the β-thalassemia mutations at this $^{A}\gamma$-globin gene region alter the binding to the target DNA sequences by LYAR and LYAR-complexes by performing a BIAcore analysis. SPR-based experiments support the concept that LYAR binds to the mutated site present in β-thalassemia (5'-GAGATTATCA-3', mutation underlined) with lower efficiency. This is expected to favor high level of transcription of γ-globin gene. Accordingly, the screening of β-thalassemia patients carrying this mutation would facilitate the identification of patients expected to respond efficiently to HbF inducers, such as sirolimus. This might be important in clinical trials in the step of recruitment of patients and in the development of personalized protocols in the field of precision medicine for β-thalassemia. Fully in agreement, the expression of LYAR decreases following treatment of erythroid cells (including precursor cells from β-thalassemia patients) with HbF inducers (supported by AIFA, Wellcome-Trust and by EU projects ULTRAPLACAD and THALAMOSS).

References:

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