

REAGENT AND EQUIPMENT DETAILS

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3 • Human primary mucosa-derived colon keratinocytes (freshly isolated from normal colorectal
4 mucosa (NCR), obtained from the resection margins of colectomy specimens from patients with
5 colorectal cancer). Human specimens must be collected with the written informed consent of
6 patients. . Human specimens were employed in agreement with the institutional biosafety protocol
7 requirements. Anonymously collected normal colorectal mucosa tissue samples were coded with
8 indications of anatomical area of the colorectum, age and gender. Written informed consent from
9 patients was obtained at the time of hospital admission. The County Ethics Committee, Ferrara,
10 approved the project, assigning the number 151078 to this study, including the methods employed.
11 All experiments were carried out in laboratories with the standard biosecurity and safety
12 procedures, according to institutional rules.

13 • Colon carcinoma human cell line (HTC116) was obtained from ATCC; cat. no. CCL-247)

14 • Normal human fibroblast (MRC-5) was obtained from ATCC; cat. no. CCL-171)

15 • Defined Keratinocyte Serum Free Basal Medium (SFM), 1X (dKSFM 1X) (Thermo Fisher
16 Scientific, Waltham, MA USA, cat. no. 10785-012).

17 • Defined Keratinocyte Serum Free Basal Medium (SFM) Growth Supplement (Thermo Fisher
18 Scientific, Waltham, MA USA, cat. no. 10784-015)

19 • Dulbecco's Modified Eagle's Medium/Ham's Nutrient Mixture F12 medium, (with L-glutamine,
20 15 mM HEPES and 3.151 g/L glucose) (Lonza, Milan, Italy, cat. no. BE12-719F)

21 • McCoy's 5A (Modified) Medium (1X) (GIBCO by Life technologies, Thermo Fisher Scientific,
22 Waltham, MA USA, cat. no.26600-023)

23 • Eagle's Minimum Essential Medium (Lonza, Milan, Italy, cat. no. BE12-125F)

24 • Fetal Bovine Serum (FBS) (EuroClone, Milan, Italy, cat. no. ECS0180L)

25 • Penicillin/Streptomycin (10,000 U ml⁻¹ penicillin, 10,000 U ml⁻¹ streptomycin) (Lonza, Milan,
26 Italy, cat. no. DE17-602E)

27 • Amphotericin B (250 µg ml⁻¹) (Lonza, Milan, Italy, cat. no. 17-836E)

- Dulbecco's Phosphate Buffered Saline (DPBS 1X, with calcium and magnesium) (Lonza, Milan, Italy, cat. no. BE17-513F)
- Dimethyl sulfoxide \geq 99% (DMSO) (Sigma-Aldrich, Milan, Italy, cat. no. M81802)
- Trypan Blue Stain, 0.4% (Thermo Fisher Scientific, Waltham, MA USA, cat. no. 15250061)
- Collagenase Type II (Worthington Biochemical Corporation, Lakewood, NJ, USA, cat. no. CSL-2)
- Trypsin/EDTA solution (Lonza, Milan, Italy, cat. no. BE17-161E)
- Ultrapure water (Milli-Q; Millipore Corporation, Vimodrone, Italy)
- Silicone rubber laboratory tubing (Sigma-Aldrich, Milan, Italy, cat. no. T2164)
- Neutral buffered formalin 10% (Bio-Optica, Milan, Italy, cat. no. 05-01005Q)
- Triton X-100 (Fluka Chemi AG., Buchs, Switzerland, cat. no. 93420)
- Bovine Serum Albumin (BSA) (Sigma-Aldrich, Milan, Italy, cat. no. A3294)
- Normal goat serum (sc-2043) (Santa Cruz Biotechnology, Inc. Dallas, Texas, U.S.A.).
- Primary antibody anti-Cytokeratin (clone MNF116) (Dako, Glostrup, Denmark, cat. no. M0821)
- Secondary antibody anti-mouse IgG (Fab specific) – FITC produced in goat (Sigma-Aldrich, Milan, Italy, F5262)
- 4',6-Diamidino-2-Phenylindole, Dihydrochloride, (DAPI) (Thermo Fisher Scientific, Waltham, MA USA, cat. no. D1306)
- Transparent nail polish.
- RNeasy Plus Mini Kit (Qiagen, Germany, cat. no. 74134)
- ImProm-II Reverse Transcription System (Promega, Italy, cat. no. A3800)
- Beta-2-microglobulin (b2m) gene expression assay (Bio-Rad, cat. no. 10031258)
- QX200 EvaGreen ddPCR Supermix (Bio-Rad, cat. no. 1864033)
- EvaGreen oil (Bio-Rad, cat. no. 1864112)

EQUIPMENT

- Incubator: $37^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$, $5.0\% \pm 0.5\%$ CO_2 in air (Eppendorf, model CO_2 Galaxy, cat. no. CO17111001)
- Laminar flow hood for cell cultures (Bio Air s.c.r.l., model AURA B3, cat. no. 407L01N0857)
- Water bath: $37^{\circ}\text{C} \pm 1^{\circ}\text{C}$ (LAE, model NG200)
- Fume hood (Labosystem, model 5001/2V, cat. no. 08/632/K120M)
- Sterile disposable tweezers (BioScientifica, cat. no. PT-1 5701S)
- Single-channel pipettes (Gilson)
- Serological pipettes (5, 10, 25, and 50 ml; StarLab, cat. nos. E4860-0511, E4860-1011 and E4860-2511)
- Cryotubes (Greiner bio-one, cat. no.122263)
- Polypropylene (PP) conical tubes (15 ml and 50 ml) (Greiner bio-one, cat. nos. 188261 and 227261)
- $0.22\ \mu\text{m}$ syringe filter (Corning, cat. no. 431224)
- 60 ml syringes (Rays, cat. no. 60LE)
- Cell culture flasks ($25\ \text{cm}^2$) (Greiner bio-one, cat. no. 690160)
- Cell culture dishes (100 mm) (Euroclone, cat. no. ET2100)
- 4-well cell culture plates (Thermo Scientific, cat. no. 176740)
- 6-well flat-bottom cell culture microtiter plates (Spl Life Sciences, cat. no. 30006)
- Sterile disposable scalpels (Paramount, cat. no. 132624)
- Cloning rings (height 10 mm, external diameter 9 mm, internal diameter 7 mm)
- Microscope slides (Menzel-Glaser, cat. no. 041300)
- Cover glasses (12 mm of diameter) (Hecht Assistant, cat. no. 1001/12)
- Burker's chamber for cell counting (Marienfeld, cat. no. 0640210)
- Centrifuge (Alc International, model. 4235A model)

- Laboratory balance (Ohaus Scout, cat. no. SC2020)
- Analytical balance (Sartorius, cat. no. 1601 MP8-1)
- Vortex (Velp Scientifica, cat. no. F202A0176)
- Inverted phase-contrast Microscope (Olympus CK2-TR, Japan)
- Fluorescent Microscope (Nikon Eclipse TE 2000- E; Nikon Instruments, Sesto Fiorentino, Italy)
- Digital camera (DXM 1200F; Nikon Instruments, Sesto Fiorentino, Italy)
- ACT-1 and ACT-2 software for DXM1200F digital cameras (Nikon Instruments)
- Nanodrop spectrophotometer (ND-1000; NanoDrop Technologies, Wilmington, DE, USA)
- Digital droplet PCR (ddPCR, Bio-Rad, Milan, Italy)
- Droplet generator cartridge (Bio-Rad, Milan, Italy cat. no. 1864108)
- QX200 Droplet Reader (Bio-Rad, Milan, Italy)
- QuantaSoft droplet reader software (Bio-Rad, Milan, Italy)
- Thermal cycler (SimplyAmp, Applied Biosystems, CA, USA)

REAGENT SETUP

The following solution must be prepared in sterile conditions.

NCR harvesting medium (50 ml) - In sterile conditions, combine 47.95 ml of DMEM F12 medium with 2 ml of 20,000 U ml⁻¹ Penicillin/Streptomycin (final concentration 800 U ml⁻¹), 50 µl of 250 µg ml⁻¹ Amphotericin B (final concentration 0.25 µg ml⁻¹). Prepare aliquots of 10 ml in 50 ml tubes and stored at 4°C for up to 3 weeks.

McCoy's 5A (Modified) complete culture medium (1X) (500 ml) - In sterile conditions, combine 439.5 ml of McCoy's 5A (Modified) complete culture medium with 10 ml of 20,000 U ml⁻¹ Penicillin/Streptomycin (final concentration 400 U ml⁻¹), 0.5 ml of 250 µg ml⁻¹ Amphotericin B (final concentration 0.25 µg ml⁻¹) and 50 ml (10% v/v) of FBS. This reagent is stable when stored at 4°C for up to 3 months.

Eagle's Minimum Essential Medium (EMEM) (500 ml) - In sterile conditions, combine 439.5 ml of Eagle's Minimum Essential Medium complete culture medium with 10 ml of 20,000 U ml⁻¹ Penicillin/Streptomycin (final concentration 400 U ml⁻¹), 0.5 ml of 250 µg ml⁻¹ Amphotericin B (final concentration 0.25 µg ml⁻¹) and 50 ml (10% v/v) of FBS. This reagent is stable when stored at 4° C for 3 months.

Dulbecco's Modified Eagle's Medium/Ham's Nutrient Mixture F12 (DMEM F12) complete culture medium (500 ml) - In sterile conditions, combine 439.5 ml of DMEM F12 medium with 10 ml of 20,000 U ml⁻¹ Penicillin/Streptomycin (final concentration 400 U ml⁻¹), 0.5 ml of 250 µg ml⁻¹ Amphotericin B (final concentration 0.25 µg ml⁻¹) and 50 ml (10% v/v) of FBS. This reagent is stable when stored at 4° C for 3 months.

Defined Keratinocyte Serum Free Medium (1X) (dKSFM 1X) complete culture medium (500 ml) - In sterile conditions, prepare 500 ml of dKSFM with 1 ml of the combined supplement, 10 ml of 20,000 U ml⁻¹ Penicillin/Streptomycin (final concentration 400 U ml⁻¹), 0.5 ml of 250 µg ml⁻¹ Amphotericin B (final concentration 0.25 µg ml⁻¹).

Dulbecco's Modified Eagle's Medium/Ham's Nutrient Mixture F12 - Defined Keratinocyte Serum Free Medium culture medium (1:1 ratio) (500 ml) – In sterile conditions, combine the 250 ml of DMEM F12 complete culture medium with 250 ml of dKSFM medium. The DMEM F12/dKSFM (1:1 ratio) medium is stable when stored at 4°C for up to 3 months.

NCR wash solution (50 ml) - Supplement 48 ml of DPBS with 2 ml of 20,000 U ml⁻¹ Penicillin/Streptomycin (final concentration 800 U ml⁻¹). Sterile-filter the solution through a 0.22 µm filter. Store the solution at 4°C for a maximum of 3 months.

0.7% Type II collagenase solution (10 ml) - Dissolve 0.07 g (20,000 U) of type II collagenase in 10 ml of ultrapure water, vortex and incubate at 4°C overnight. Filter-sterilize the solution through a 0.22 µm filter. Prepare aliquots of 500 µl and store them at -20°C for up to 1 year.

NCR cryopreservation solution – In sterile conditions, mix 1,350 ml of FBS and 150 µl of DMSO for each cryovial. We do not recommend storing this solution.

Cell permeabilization solution (10 ml) – Add 0.1% Triton X-100 (vol/vol) to 10 ml of DPBS. Vortex the contents well and store the solution at room temperature (20°C, RT) and use within 1 month.

Blocking solution - Dissolve 0.3 g of BSA in 10 ml of DPBS. Filter the 3% (wt/vol) BSA through a 0.2 µm filter. The solution (3% BSA/DPBS) should be stored at 4°C and can be used for up to 1 month. Before use, add normal goat serum at a 1:20 final dilution, maintaining the blocking solution at 4°C. We do not recommend storing the solution containing the serum.

Primary antibody solution – Before use, add the primary antibody anti-Cytokeratin to the 3% BSA/DPBS solution, at a 1:50 final dilution. We do not recommend storing this solution.

Secondary antibody solution – Before use, add the secondary antibody anti-mouse IgG (Fab specific) – FITC produced in goat to the 3% BSA/DPBS solution, at a 1:50 final dilution.