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FACS-Based Isolation Progenitor-Like Keratinocyte Subpopulations from HaCaT Cells for Bulk RNA-Seq Profiling

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We use this protocol and it's working

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Abstract

This protocol describes a fluorescence-activated cell sorting (FACS) strategy to isolate progenitor-like keratinocyte subpopulations from HaCaT cultures based on cell-surface markers associated with basal, stem-like states (e.g., high CD49f/ITGA6 with distinct CD71 expression). The workflow covers sample preparation, antibody staining, gating, sorting, and post-sort handling under conditions that preserve RNA integrity for downstream bulk RNA-seq.
Using this protocol, users can obtain highly enriched progenitor-like and more differentiated keratinocyte fractions with high viability and RNA quality (RIN ≥ 9). These sorted populations are suitable for transcriptomic profiling of early keratinocyte commitment, proliferation control, and basal-layer dynamics in human epidermal models.

Protocol materials

- RNAprotect Cell Reagent **Qiagen Catalog #** 76526
- AccuBlue® Broad Range RNA Quantitation Kit **Biotium Catalog #**31073
- High Sensitivity RNA (NR1) **BiOptic Inc. Catalog #**C105111/C105211
- Direct-zol RNA Microprep Kit **Zymo Research Catalog #**R2062
- RNeasy Plus Micro Kit **Qiagen Catalog #**74034
- Pacific Blue™ anti-human/mouse CD49f Antibody **BioLegend Catalog #**313620
- APC/Cyanine7 anti-human CD71 Antibody **BioLegend Catalog #**334110

Before You Begin

1

- Brief background: importance of isolating progenitor-like keratinocytes.

Progenitor-like keratinocytes represent the stem- and transit-amplifying compartment of epidermal models and are essential for long-term renewal and controlled differentiation. In the HaCaT cell line, flow cytometry analysis of α6-integrin and CD71 has revealed a discrete α6^bright/CD71^dim subpopulation with clear stem-like features, including high self-renewal capacity, strong clonogenic potential, and elevated expression of core pluripotency regulators such as SOX2, OCT4, and NANOG (Domínguez-Catzín et al., 2017). Isolating this progenitor-enriched fraction is therefore critical to dissect early regulatory events in keratinocyte biology, as it reduces the masking effects of more differentiated cells and enables a more precise characterization of proliferation, commitment, and epidermal homeostasis.

Citation

Domínguez-Catzín V, Reveles-Espinoza AM, Sánchez-Ramos J, Cruz-Cadena R, Lemus-Hernández D, Garrido E
(2017)
. HPV16-E2 protein modifies self-renewal and differentiation rate in progenitor cells of human immortalized keratinocytes.
Virology journal.

https://doi.org/10.1186/s12985-017-0736-2 LINK

- Marker rationale: e.g., *CD49f (ITGA6)* and *CD71 (TFRC)* as indicators of basal and transit-amplifying populations

Note

The use of multiple markers is recommended to ensure the characterization of subpopulations such as: CD133, CD19, CD24, CD38

- Key objective: obtain subpopulations with RNA integrity (RIN > 9) suitable for transcriptomic profiling.

Materials and Reagents

2 1) Cell line and media:
HaCaT cells (a generous gift from Dr. Norbert Fusenig) were grown in culture dishes in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, CA, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, NY, USA), L-glutamine (2 mM), sodium pyruvate (1 mM), penicillin (50 U/ml), and streptomycin (50 µg/ml). Were incubated in a humidified atmosphere with 5% CO_2 at 37 °C and maintained in exponential growth phase.

37 °C 5 %

3 2) Antibodies:

Pacific Blue™ anti-human/mouse CD49f Antibody **BioLegend Catalog #**313620

APC/Cyanine7 anti-human CD71 Antibody **BioLegend Catalog #**334110

Note

The use of online tools such as FluoroFinder is recommended to design the panel that best suits the experiment, costs, and available equipment as the case may be.

Software

FluoroFinder© NAME

https://fluorofinder.com/flow-cytometry-panel-design/ SOURCE LINK

4 3) Buffers:
PBS (for 1L: KCl 0.2g + NaCl 8g + KH2PO4 0.2 g + Na2HPO4 1.42g)
FACS buffer (PBS 1x + 2% FBS)
Marking solution (PBS 1x + 2% FBS)
PBS+ (PBS + 2% FBS + 1% Hepes 1M pH 7.4)

5 4) Equipment:
Flow cytometer/sorter (BD FACSAria II)
Refrigerated centrifuge
RNA extraction kit RNeasy Plus Micro Kit **Qiagen Catalog #**74034

Direct-zol RNA Microprep Kit **Zymo Research Catalog #**R2062

RNA Quality High Sensitivity RNA (NR1) **BiOptic Inc. Catalog #**C105111/C105211

AccuBlue® Broad Range RNA Quantitation Kit **Biotium Catalog #**31073

- Qsep1 Manual.pdf 3.3MB
-

6 5) Optimal: RNAprotect Cell Reagent **Qiagen Catalog #** 76526

Equipment Setup

7
- Instrument BD FACSAria II configuration:

nozzle size: 80mm
flow rate: 1.0 - 2.6
flow threshold: max 8,000 events per second

- Laser and filter settings for Pacific Blue/APC Cy7 (or your fluorochromes).
- Compensation setup and controls (unstained, single-stained, FMO).

Sample Preparation

38m

8 38m
- Cell harvesting and counting
- Staining procedure with incubation time and temperature

Trypsinized cells are centrifuged in 1.5 mL Eppendorf tubes to form a pellet

3000 rpm, Room temperature 00:05:00

Blocking: Incubate with 1 mL of FACS solution 4 °C 00:15:00

Centrifuge 00:03:00 3000 rpm, 4°C

Remove supernatant by decanting Room temperature

Resuspend in 100 mL of labeling solution

Note

For the immunostaining and negative controls, the number of cells was always adjusted to one million (as suggested by the fluorochrome protocols).
This protocol is standardized for the following antibody concentrations
CD49F (1:100) CD71 (1:500)

	Tube	Condition	Staining Buffer	Antibody Volume	Total Volume
	1	Negative control	100 µL	0 µL	100 µL
	2	CD49f (1:100)	90 µL	10 µL	100 µL
	3	CD71 (1:500)	98 µL	2 µL	100 µL
	4	Double (CD49f + CD71)	88 µL	10 µL + 2 µL	100 µL

Table1. Example of immunostaining conditions for one million cells in each. From here, the volume is scaled up in the double monostaining as needed.

Incubate 4 °C
Gently agitate every 00:15:00 to avoid precipitation.
Centrifuge 00:03:00 3000 rpm, 4°C
Remove supernatant by decanting.
Wash with PBS+ solution. 600 µL
Centrifuge 00:03:00 3000 rpm, 4°C
Resuspend directly in PBS+ 600 µL into flow cytometry tubes

- Viability dye inclusion (if used).

Note

The transfer time between the wet lab and the sorting unit for this protocol is one and a half hours, so the samples (including the collection tubes) were transported on ice with the aid of gel antifreeze. It is recommended that each laboratory verify the stability of its cell line.

FACS Sorting Procesure

9

- Gating hierarchy (e.g., FSC/SSC → singlets → viable → CD49f/CD71 quadrants).
- Sorting speed, drop delay, and collection settings.
- Target populations (e.g., CD49f^bri/CD71^dim, CD49f^dim/CD71^bri).
- Purity check post-sort.

Post-Sorting Handling and Transport

10 1) Conditions for transport and time to RNA extraction.
The sorting process can result in a poor prognosis for genomic material extraction, especially RNA; therefore, the RNAprotect Cell Reagent **Qiagen Catalog #** 76526 reagent was used.

The protocol was standardized as follows:
For every 4 mL of volume recovered from the separated cells, 1 mL of RNAprotect Cell Reagent **Qiagen Catalog #** 76526 was added.
This ensured the protection of the RNA.

11 2) Temperature stabilization and container setup.

Despite using the RNAprotect Cell Reagent, the samples were transported back on ice 4 °C

12 3) Note that RIN > 9 was consistently achieved under these conditions.

13 4) Representative RIN trace image

RNA Extraction and Quality Control

14
- High Sensitivity RNA (NR1) **BiOptic Inc. Catalog #**C105111/C105211 The conditions of each of the extraction kits were followed.

HaCaT wt and HaCaT No Bri/dim

Direct-zol RNA Microprep Kit **Zymo Research Catalog #**R2062

HaCaT Bri/dim RNeasy Plus Micro Kit **Qiagen Catalog #**74034

- Integrity and quantification (RIN, A260/280, A260/230).

AccuBlue® Broad Range RNA Quantitation Kit **Biotium Catalog #**31073

Expected Results

15
- Representative gating plots or purity check images.
- RNA integrity examples (with RIN values).

HaCaT WT_HaCaTwt 1_S1S1_R1_RN... 183.6KB

HaCaT Bd _Bridim 1_S1S1_R1_RNA.p... 173.9KB

HaCaT No Bd _No Bridim 1_S1S1_R1... 179.5KB

Troubleshooting

16 List common issues and fixes:

	Problem	Possible Cause	Solution
	Low RNA integrity	Delayed processing	Use RNAlater or maintain at 4°C
	Poor separation	Incorrect compensation	Recheck FMO and gating strategy
	Cell clumping	Inadequate filtering	Use 40 µm strainers, add EDTA

Table2

Acknowledgments

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Citations

Step 1

Domínguez-Catzín V, Reveles-Espinoza AM, Sánchez-Ramos J, Cruz-Cadena R, Lemus-Hernández D, Garrido E. HPV16-E2 protein modifies self-renewal and differentiation rate in progenitor cells of human immortalized keratinocytes.
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