

# Co-culturing Lonza Matched Donor Keratinocytes and Fibroblasts

A High-throughput, 2D Skin Model

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Of the various cell types that comprise the integument, keratinocytes are the most relevant cell type to study the physiology of human skin, from wound healing and diseases to toxicology, as they are the most abundant cell type in the epidermis of the skin and form a first line of defense against pathogens and other foreign substances. Keratinocytes initiate both wound healing as well as a variety of immune responses through chemical messaging and cellular cross-talk, especially with fibroblasts.<sup>1</sup>

Cell-to-cell interactions between fibroblasts and keratinocytes contribute to maintenance of the skin barrier, wound healing, and overall homeostasis.<sup>2</sup> These two cell types can be co-cultured to produce physiologically relevant *in vitro* model systems for studying interactions between these two cells and how they relate to processes such as wound healing, immune responses, skin sensitization, and diseases.<sup>3</sup>

In this study, we demonstrate a 2D mixed co-culture skin model that can be used to better simulate *in vivo* conditions by using human keratinocytes and fibroblasts taken from the skin of the same donor. We optimized seeding density, medium, and ICC visualization methods to produce a co-culture model with proper morphology and longevity suitable for applications such as wound healing and skin sensitization, as well as use in a high-throughput workflow.

## Methods

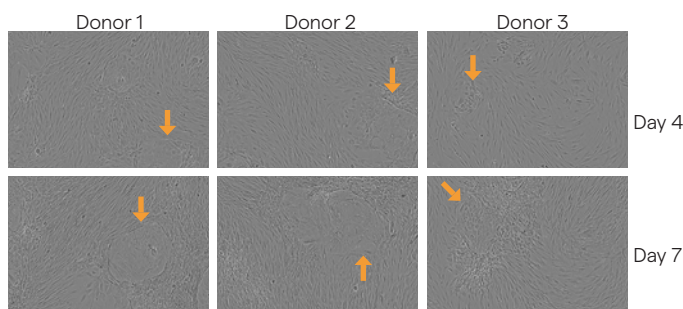
The optimized version of this model can be prepared according to the detailed instructions found in the [protocol published on the Lonza website](#).<sup>4</sup> The following steps briefly cover the protocol used in this study.

1. N=3 matched donor pairs of cryopreserved normal human dermal fibroblasts (part no. CC-2511) and cryopreserved normal human epidermal keratinocytes (part no. 00192627) were thawed according to manufacturer instructions and plated in separate T-150 flasks at a density of 3,500 cells/cm<sup>2</sup>.
2. Fibroblasts were plated in Lonza FGM<sup>®</sup> 2 complete medium (part no. CC-3132), while keratinocytes were plated in KGM<sup>®</sup> Gold complete medium (part no. 00192152).
3. Cells were incubated at 37°C and 5% CO<sub>2</sub>, with media changes occurring 1 day after plating and then every other day thereafter until 70–90% confluence was reached for both cell types (5–7 days).
4. After this point, matched donor monocultures of keratinocytes and fibroblasts were trypsinized and harvested.
  - a. If there were multiple flasks of one cell type (e.g., keratinocytes) for a donor, those cells were pooled.
5. All cells of each cell type were then transferred into a single tube (one tube for keratinocytes and one for fibroblasts).
6. Cells were then rinsed, resuspended in PBS, and counted.
7. Co-cultures were established by plating keratinocytes to fibroblasts in a variety of ratios and in a variety of media in 96-well plates to determine the best plating density and media combination for a high-throughput format.
8. Each cell type was also plated in monoculture as a control for each media treatment.
9. The resulting cultures were maintained for 7 days, after which they were stained with a fixable live/dead far red stain and fixed with 4% PFA in PBS.
10. Plates were then stained with two antibodies for visualizing keratinocytes (Alexa Fluor<sup>®</sup> 488 Anti-Cytokeratin 14) and fibroblasts (Alexa Fluor<sup>®</sup> 568 Anti-CD90 / Thy 1), incubated overnight in the refrigerator, then washed three times and stained with a combination DAPI + DABCO™ mounting medium.
11. Plates were then sealed with parafilm, imaged, and then visually assessed for proper morphology of both individual cell types as well as the co-culture as a whole.

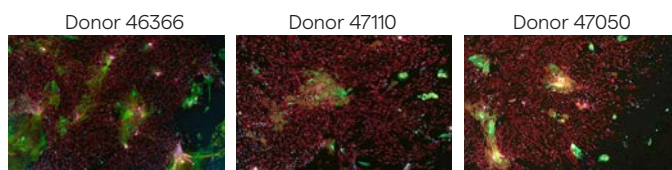
## Results

A healthy keratinocyte and fibroblast co-culture should show clusters of keratinocytes with small, tight cobblestone morphology (not misshapen or blebbed) surrounded by an even monolayer of elongated fibroblasts with no bulging nuclei. This morphology was attained by plating a 1:3 ratio of keratinocytes: fibroblasts at a total density of 4,000 cells/cm<sup>2</sup> (e.g., 1,280 keratinocytes and 3,840 fibroblasts added to each well) in CnT-Prime Epithelial/Stromal Co-Culture Medium (CellnTec part no. CnT-PR-CC; Fig. 1).

This morphology developed over the course of the entire 7 day culture, with a healthy fibroblast layer developing early, while islands of keratinocytes can be seen throughout as early as day 4 (Fig. 1). ICC staining and imaging further highlight the fibroblast layer (red) woven into the clusters of keratinocytes (green) with few dead cells throughout (pink; Fig. 2), indicating that the two cell types are forming the complex physical connections characteristic of epidermal tissues.



**Figure 1.** Phase contrast imagery (10X magnification) of 3 donor pairs of keratinocyte and fibroblast co-cultures plated at a total starting density of 4,000 cells/cm<sup>2</sup> and a ratio of 1:3 keratinocytes: fibroblasts. Keratinocytes (orange arrows) show a tight cobblestone morphology and form clusters that are visible as early as day 4 and grow into the layer of elongated fibroblasts by day 7.



**Figure 2.** ICC imaging of 3 optimized matched donor keratinocyte: fibroblast co-cultures. Even layers of fibroblasts (red) can be seen interwoven through the clusters of keratinocytes (green) while few dead cells can be seen (pink).

Other seeding densities, ratios of keratinocytes: fibroblasts, and media resulted in poor performance by one or both cell types, most often ending in fibroblasts dominating the culture at the expense of the keratinocytes (data not shown). A major factor influencing the difficulty of co-culturing keratinocytes and fibroblasts is the tension between the media needs of keratinocytes (which require minimal calcium levels for expansion) with fibroblasts (fibroblasts require serum for expansion, which contains calcium, on top of calcium in the medium itself).<sup>5,6</sup> Optimizing calcium in the co-culture medium is crucial, as keratinocytes begin to terminally differentiate above 0.1 mM (the “calcium switch”).<sup>6</sup>

It is possible that other media may be used to co-culture these two cell types, but further experimentation and optimization would need to be done to produce a healthy model that balances expansion of keratinocytes against the medium requirements of fibroblasts.

## Conclusions

This study demonstrates a robust and physiologically relevant skin model using keratinocytes and fibroblasts in co-culture. After extensive optimization of seeding density and media conditions, we produced a model with high viability, purity, and the expected morphology of both cell types through 7 days in culture. Keratinocytes and fibroblasts are plated at a 1:3 ratio and an initial total density of 4,000 cells/cm<sup>2</sup> in a 96-well plate using CnT-Prime Epithelial/Stromal Co-Culture Medium. This model can be used in a variety of high-throughput research applications, such as skin sensitization, wound healing, or disease modeling, to add high-throughput capabilities to dermal research applications.

## References

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## Ordering Information

Catalog No.	Description	Size
CC-2511	NHDF-Cryopreserved Normal Human Dermal Fibroblasts	≥ 500,000 cells
00192627	NHEK-Ad-Cryopreserved Normal Human Epidermal Keratinocytes; Single Donor	≥ 500,000 cells
00192060	KGM® Gold Keratinocyte Growth Medium BulletKit®	500 mL KBM® Gold Basal Medium plus KGM® Gold SingleQuots® supplements
00192151	KBM® Gold Basal Medium	500 mL bottle
00192152	KGM® Gold SingleQuots® Kit	1 kit
CC-3132	FGM® 2 Fibroblast Growth Medium BulletKit®	500 mL FBM® Basal Medium plus FGM® 2 SingleQuots® supplements
CC-3131	FBM® Basal Medium	500 mL bottle
CC-4126	FGM® 2 SingleQuots® Kit	1 kit
CC-5012	Trypsin/EDTA	100 mL bottle
CC-5002	Trypsin Neutralizing Solution (TNS)	100 mL bottle
CC-5024	HEPES Buffered Saline Solution (HEPES-BSS)	500 mL bottle

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CnT-Prime Epithelial/Stromal Co-culture Medium (CellnTec CnT-PR-CC) mentioned is a product of CellnTec.

LIVE/DEAD™ Fixable Far Red Dead Cell Stain Kit (Thermo Fisher L34974) mentioned is a product of Thermo Fisher Scientific.

Alexa Fluor® 488 Anti-Cytokeratin 14 antibody [EP1612Y] (Abcam ab192055) mentioned is a product of Abcam.

Alexa Fluor® 568 Anti-CD90 / Thy1 antibody [EPR3133] (Abcam ab201848) mentioned is a product of Abcam.

EMS Immuno Mount™ DAPI & DABCO™ (IFMDD) Mounting Medium (Electron Microscopy Sciences 1798997) mentioned is a product of Electron Microscopy Sciences.

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