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Standard Operating Procedure (SOP)

Title Isolation of Keratinocytes and Fibroblasts from Human Specimens			**Date** 2017-11-09
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Version	**valid from**	**Description of changes**
1	2014-04-24	Erstellung
2	2014-06-20	Selbst hergestellte Medien, mikrobielle Kontamination
3	2015-03-02	Trypsin-EDTA-Konzentration, Zählung
4	2015-12-30	Translation
5	2016-02-28	Trypsin-EDTA-working and -stock solutions
6	2017-11-09	NHK freezing

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Scope

Scientific projects C02 (Schäfer-Korting, Hedtrich) and Z01 (Kleuser, Ma, Schäfer-Korting) of the CRC 1112; scientific projects “Development of human-based skin disease models” (Schäfer-Korting) and “Integration of Langerhans cells into reconstructed human skin” (Weindl) of BB3R.

Directory

AIMS

Standardized cell isolation from human skin specimens with maximal cell yield.

SCOPE

This SOP applies to the scientific projects C02 (Schäfer-Korting, Hedtrich) and Z01 (Kleuser, Ma, Schäfer-Korting) of the collaborative research center 1112 as well as to the scientific projects “Development of human-based skin disease models” (Schäfer-Korting) and “Integration of Langerhans cells into reconstructed human skin” (Weindl) of the Berlin-Brandenburg research platform BB3R with integrated graduate education.

MATERIALS

Equipment

Designation	Manufacturer
Autoklave	Systec, Wettenberg
Centrifuge (Eppendorf)	Eppendorf, Hamburg
Centrifuge (Megafuge® 1.0R)	Thermo Fisher Scientific, Waltham, MA, USA
Cryotome (Leica CM 1510S)	Leica Microsystems, Wetzlar
Drying cabinet	Memmert, Schwabach
Fluorescence microscope (BZ-8000K)	Keyence, Osaka, JAP
Freezer (-20°C)	Siemens, München
Freezer (-80°C)	Thermo Fisher Scientific, Waltham, MA, USA
Freezing container (Nalgene, Mr. Frosty)	Thermo Fisher Scientific, Waltham, MA, USA
Neubauer counting chamber	Zeiss, Jena
Incubator (BB6220)	Thermo Fisher Scientific, Waltham, MA, USA
Magnetic stirrer RCT basic	IKA-Werke, Staufen
Microtome (Hyrax M40)	Zeiss, Jena
Nitrogen tank (Arpege 70)	Air Liquide, Paris, F
Paraffinization station (Microm EC 350)	Zeiss, Jena
Phase contrast inverted microscope (Axiovert 40C)	Zeiss, Jena
pH meter (766 Calimatic)	Knick, Nürnberg
Pipette (Eppendorf Reference®)	Eppendorf, Hamburg
Pipetting aid (Easypet®)	Eppendorf, Hamburg
Refrigerator (4°C)	Siemens, München
Sterile working bench (LaminAir®)	Thermo Fisher Scientific, Waltham, MA, USA
Water bath	Gesellschaft für Labortechnik, Burgwedel
Water processing unit (SG LaboStar)	SG Wasseraufberitung und Regenerierstation, Barsbüttel

Equivalent equipment of other suppliers can be used as well.

Consumables

Designation	Supplier
2-Propanol	VWR, Darmstadt
Calibration solutions (pH 7 / pH 9)	Hanna Instruments, Woonsocket, RI, USA
Cell culture flask (75 cm²)	TPP, Trasadingen, Schweiz
Cell strainer (70 µm pore size)	BD, Franklin Lakes, NJ, USA
Centrifuge tubes (50 mL)	TPP, Trasadingen, Schweiz
Cryo vial	Almeco, Esbjerg N, DK
Dimethyl sulfoxide (plant cell culture tested)	Sigma-Aldrich, München
Disodium hydrogenphosphate (pro analysi)	Merck, Darmstadt
Dispase II (Cat. No. 04.942.078.001)	Roche Diagnostics, Mannheim
Dulbecco's Modified Eagle Medium (DMEM)	Sigma-Aldrich, München
Ethylenediaminetetraacetic acid (EDTA)	Sigma-Aldrich, Schnelldorf
Fetal calf serum	Biochrom, Berlin
Forceps (anatomical)	Carl Roth, Karlsruhe
Keratinocytes Growth Medium (KGM)	Lonza, Köln
L-Glutamine	Sigma-Aldrich, München
Nitrogen (liquid)	Air Liquide, Paris, F
Parafilm (Nescofilm®)	Carl Roth, Karlsruhe
Penicillin-Streptomycin-solution (100x, 10.000 U/mL Penicillin, 10 mg/mL Streptomycin)	Sigma-Aldrich, München
Pipette tips	Eppendorf, Hamburg
Pipettes (5 mL, 10 mL, 25 mL)	Sarstedt, Nümbrecht
Potassium chloride (KCl, cell culture tested)	Sigma-Aldrich, München
Potassium dihydrogen orthophosphate (KH_2PO_4)	Carl Roth, Karlsruhe

Consumables (continued)

Designation	Supplier
Scalpel (No. 24)	Carl Roth, Karlsruhe
Sodium chloride (NaCl, pro analysi)	Carl Roth, Karlsruhe
Stirring bar	Carl Roth, Karlsruhe
Syringe (10 mL, 20 mL)	BBraun, Melsungen
Syringe filter (Cellulose acetate, 0.2 µm pore size,[1])	Sarstedt, Nümbrecht
Syringe filter (Reconstituted cellulose, 0.2 µm pore size,[1])	Carl Roth, Karlsruhe
Tissue culture dish	TPP, Trasadingen, Schweiz
Tissue culture test plate (6-well)	TPP, Trasadingen, Schweiz
Transport vial for preputial specimen	Sarstedt, Nümbrecht
Trypsin	Sigma-Aldrich, München

Equivalent consumables of other suppliers can be used as well.

[1]) Filtrate is sterile according to United States Pharmacopeia (USP)

Custom-made media

Description	Ingredients			Remarks
Dispase stock solution	Dispase	150	mg	c(Dispase)=10 U/mL
	PBS	15	mL	-20°C, 6 weeks [2])
Dispase working solution	Dispase stock solution	300	µL	c(Dispase)=1.2 U/mL
	PBS	2.5	mL	do not store
Freezing medium	FGM	45	mL	protect from light (tin foil)
	Dimethyl sulfoxide	5	mL	4°C, 6 weeks [2,3])
FGM	DMEM	500	mL	
	Fetal Calf Serum	50	mL	
	L-Glutamine	5	mL	
	Penicillin/Streptomycin	5	mL	4°C, 6 weeks [2])
PBS	KCl	200	mg	
	NaCl	8,000	mg	
	KH_2PO_4	200	mg	
	Na_2HPO_4	1,148	mg	autoclave, check pH [4])
	Aq. bidest.	1	L	4°C, 6 weeks [2])
Trypsin-EDTA-stock solution	Trypsin	2,500	mg	c(Trypsin)=139 mg/mL
	EDTA	197.3	mg	sterile filtration,
	PBS	18	mL	Aliquot: -20°C, 6 weeks [2])
Trypsin-EDTA-working solution	Trypsin-EDTA stock solution	1.8	mL	c(Trypsin)=2.5 mg/mL = 0.25% c(EDTA)=0.53 mmol/L
	PBS	99	mL	4°C, 2 weeks [2])

[2]) maximal duration of storage when media are stored at 2-8°C,
media need to be warmed to room temperature before use (except freezing medium)
[3]) use reconstituted cellulose filters for the filtration of DMSO
[4]) perform calibration of pH meter before use (pH 7, pH 9, stirring bar)

STEP-TO-STEP PROTOCOL

Prepare specimen | Day 01

Materials

Dispase working solution, forceps, Parafilm, PBS, scalpel, tissue culture dish

Step-to-step protocol

- Prepare specimens separately, one after each other
- Evaluate specimen and discard, if
  - specimen transport took longer than 48 h or
  - cooling chain was interrupted or
  - donor age exceeds 10 years (only applicable for preputial specimen) or
  - specimen shows pus, tumor, or necrosis
- Wash specimen (3-5 times) in transport vial with 10 mL PBS until the specimen is cleared from blood
- Transfer specimen in tissue culture dish
- Remove mucosal, vascular, and adipose tissue from preputial specimen using a scalpel
- Wash specimen (1-5 times) until PBS is clear
- Subdivide specimen into 3 mm wide pieces using a scalpel and forceps
- Place all pieces into another tissue culture dish with the dermis attaching the dish. Pieces should be placed without touching each other (remove any liquid)
- Pipette Dispase working solution into tissue culture dish, but not onto the pieces of the preputial specimen (2.8 mL per 60 cm^2 growth surface of the tissue culture dish)
- Label tissue culture dish with specimen number and time of adding Dispase working solution specimen number: year (yy) und serial number (xx) within the respective year (e.g. 16-1: first specimen in 2016)
- Seal the tissue culture dish and its lid with Parafilm and incubate the specimen for 18-20 h at 4°C

Specimen documentation

- Record specimen number, date of receipt, and remarks (e.g. colored skin, female donor, specimen site) in the specimen documentation

Cell isolation | Day 01

Materials

Cell culture flask, cell strainer, centrifuge tube, FGM, forceps, KGM, PBS, tissue culture test plate, Trypsin-EDTA working solution, materials for cell count

Step-to-step protocol

- Dilute 4 mL Trypsin-EDTA working solution with 12 mL PBS
  (in centrifuge tube, do not store diluted Trypsin-EDTA working solution)
- Fill 5 mL PBS in the lid of the tissue culture dish
- Separate epidermis and dermis using forceps and collect the epidermal pieces in the lid of the tissue culture dish
- Collect the epidermal pieces in diluted Trypsin-EDTA working solution in the centrifuge tube; epidermal pieces must not stick to the rim of the centrifuge tube
- Incubate the epidermal suspension for 20 min in the incubator. Wave the centrifuge tube twice every 5 min to re-suspend the epidermal pieces ↗
- Place dermal pieces upside down into the tissue culture test plate. Use one plate per donor and place max. 6 dermal pieces into one well
- Allow the dermal pieces to attach the bottom of the tissue culture test plate for at least 30 min at room temperature
- After at least 30 min, fill 1.5 mL FGM per well into the tissue culture test plate using an Eppendorf pipette without detaching the dermal pieces. Incubate the dermal pieces in the incubator

↗ Stop enzymatic reaction of Trypsin with 10 mL FGM and pipette the cell suspension three times up and down

- Filtrate the cell suspension using a cell strainer and wash the cell strainer with 5 mL PBS
- Centrifuge cell suspension at 130×g for 5 min at 25°C and discard the supernatant
- Re-suspend NHK pellet in 10 mL PBS
- Count cells and centrifuge cell suspension again

▲ **CRITICAL STEPs**

- Discard cells, if viable cell count is below 700,000 NHK or dead cell count exceeds 50%
- Otherwise use cells and
  - ⇨ Label cell culture flask with cell type, passage, and specimen number
  - ⇨ Re-suspend NHK pellet in 5 mL KGM and seed 2 x10^6 cells per cell culture flask (75 cm²)
  - ⇨ Use 12 mL KGM per cell culture flask (75 cm²), except on Friday (18 mL KGM)

Cell culture | Days 02-29

Materials

Cell culture flask, centrifuge tube, FGM, forceps, KGM, PBS, Trypsin-EDTA working solution, materials for cell count

Step-to-step protocol

Day 02-07

- Evaluate NHK (Figure 1a, c)
  - ⇨ adherent cells without microbial contamination⇨ change medium on Mo-Tu-Fr until day 08
  - ⇨ non-adherent cells or microbial contamination ⇨ discard cells

Day 07

- Evaluate NHDF (Figure 1b, c)
  - ⇨ adherent cells without microbial contamination⇨ aspirate FGM, remove dermal pieces using forceps, add 2 mL FGM per well but do not wash cells on day 07, change medium on Mo-Tu-Fr until day 29
  - ⇨ non-adherent cells or microbial contamination ⇨ discard cells

Day 08

- Evaluate NHK (Figure 1a)
  - ⇨ slack NHK colonies with cell membranes appearing as bright rims ⇨ subcultivate & seed 600,000 NHK per 75 cm² flask and culture for about 4 days freeze cells (label: p2)
  - ⇨ differentiated NHK appearing as cobblestones ⇨ discard cells

Day 29

- Evaluate NHDF (Figure 1)
  - ⇨ huge NHDF bundles ⇨ subculture cells from 6 wells in 3 cell culture flasks (75 cm²), label: p1
  - ⇨ small or absent NHDF bundles ⇨ discard cells

Cell count | Days 01, 08, 29

Materials

Cell suspension in PBS, Neubauer counting chamber

Step-to-step protocol

- Pipette 10 µL cell suspension into Neubauer counting chamber
- Determine the mean cell count of all four quadrants
- Calculate the absolute cell count per mL: mean cell count of all four quadrants x 10^4

Change cell culture media

Materials

FGM, KGM, pasteur pipettes, PBS

Step-to-step protocol

- Evaluate cell morphology (Figure 1)
- Aspirate KGM or FGM using pasteur pipettes without touching the cell monolayer
- Wash cells twice with 10 mL PBS (cell culture flask, 75 cm^2) or with 2 mL per well of a tissue culture test plate; do not pipette the liquids directly onto the cell monolayer

▲ **CRITICAL STEPs**

- Use 12 mL KGM/FGM per cell culture flask (75 cm^2), except on Friday (18 mL KGM/FGM)
- Use 2 mL KGM/FGM per well of a tissue culture test plate.
- Avoid foaming of media
- Change KGM three times a week (Monday-Wednesday-Friday)
- Change FGM twice a week (Monday-Thursday)

Cell subculture (passaging)

Materials

Cell culture flask, centrifuge tube, FGM, KGM, tissue culture test plates, Trypsin-EDTA working solution, PBS, materials for cell count

Step-to-step protocol

- Evaluate cell morphology
- Aspirate KGM or FGM using pasteur pipettes without touching the cell monolayer
- Wash cells twice with 10 mL PBS (cell culture flask, 75 cm^2) or with 2 mL per well of a tissue culture test plate; do not pipette the liquids directly onto the cell monolayer
- Incubate cells with 1.5 mL Trypsin-EDTA working solution per cell culture flask (75 cm^2) or 0.5 mL per well of a tissue culture test plate for 5 min in the incubator; close the cell culture flask gas tight to ensure optimal pH (pH 7.6-7.8) for Trypsin activity

Cell subculture (continued)

▲ **CRITICAL STEPs**

- To prevent cell damage to NHDF, Trypsin-EDTA working solution can be alternatively spread out to the cell monolayer and aspirated after 1 min using pasteur pipettes; close the cell culture flask gas tight and incubate for 5 min in the incubator
- Promote cell detachment by gently tapping at the side of the cell culture flask
- Check cell detachment microscopically (detached cells appear as floating spheres)
- Let as many cells as possible detach to prevent selection processes, but cells should be exposed not longer than 10 min to the Trypsin-EDTA working solution

- Inactivate Trypsin-EDTA working solution by adding FGM (FGM volume equals at least threefold of the Trypsin-EDTA working solution volume)
- Pipette the cell suspension into a centrifuge tube, wash the cell culture flask or the tissue culture plate with 10 mL PBS and transfer the wash fluid into the centrifuge tube
- Centrifuge the cell suspension at 130 g for 5 min at 25°C and discard the supernatant
- Re-suspend the cell pellet in 10 mL PBS and count cells
- Centrifuge the cell suspension again at 130 g for 5 min at 25°C and discard the supernatant
- Label cell culture flask with cell type, passage, and specimen number
- Re-suspend cells in 5 mL KGM or FGM, seed 2 x10^6 cells per cell culture flask (75 cm^2), and add KGM or FGM
- Allow the cells to attach to the cell culture flask and avoid agitation for the next 24 h
- Freeze passage 2 of NHK or NHDF and discard passage 4 or higher

Cell freezing

Materials

2-Propanol, cryo vial, freezing container, freezing medium, materials for cell subculture

Step-to-step protocol

- Change medium 24 h before cell freezing; evaluate morphology directly before cell freezing
- Fill 250 mL 2-Propanol into the freezing container (change 2-Propanol every 5 freeze-thaw-cycles)
- Label cell culture flask with cell type, passage (+1), specimen number, and date of freezing; (optimal cell count: $3x10^6$ NHK or $1.5x10^6$ NHDF per cryo vial)
- Subculture cells
- Re-suspend cells in freezing medium, fill every cryo vial with 1.8 mL cell suspension
- Store freezing container with closed cryo vials for 24 h in freezer (-80°C) and transfer cryo vials to nitrogen tank

Specimen and cell documentation

- Record date of freezing in the specimen documentation
- Record cell type, passage, specimen number, count, date of freezing in the cell documentation

Cell thawing

Materials

2-Propanol (70%), cell culture flask, centrifuge tube, FGM, KGM, PBS

Step-to-step protocol

- Take cryo vial from nitrogen tank, vent cryo vials, and thaw them using a water bath (37°C)
- Wipe cryo vial with 2-Propanol (70%)
- Pipette cell suspension from cryo vial into centrifuge tube and add up to 20 mL PBS
- Centrifuge the cell suspension at 130 g for 5 min at 25°C and discard the supernatant
- Re-suspend the cell pellet in 10 mL PBS and count cells
- Centrifuge the cell suspension again at 130 g for 5 min at 25°C and discard the supernatant
- Label cell culture flask with cell type, passage, and specimen number
- Re-suspend cells in 5 mL KGM or FGM, seed 2 $x10^6$ cells per cell culture flask (75 cm^2), and add KGM or FGM
- Allow the cells to attach to the cell culture flask and avoid agitation for the next 24 h

Cell documentation

- Delete cells from cell documentation

Cells transport

Materials

cell culture flask, centrifuge tube, dry ice, FGM, KGM, PBS

Step-to-step protocol | send cells

- Use dry ice to ensure continuous cooling chain for 3 days and order overnight express
- Inform the recipient prior to cell delivery about cell type, passage, cell count, and culture conditions

▲ **CRITICAL STEPs**

- Observe country specific quarantine rules and guidance on dangerous goods

- If required, send cells in cell culture flasks
  - ⇨ Grow cells until 70% confluence
  - ⇨ Fill the cell culture flask completely with KGM or FGM
  - ⇨ Close the cell culture flask gas tight

Cell documentation

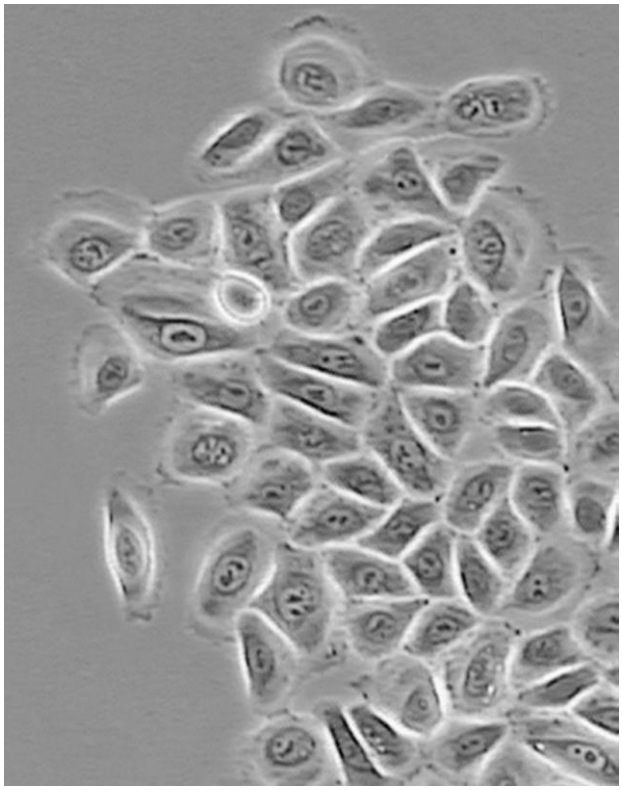
- Delete cells from cell documentation

Step-to-step protocol | receive cells

- Separate cells from routine cell culture to avoid cross contamination
- Culture cells according to the information of the sender
- If cells arrive in cell culture flasks
  - ⇨ Aspirate cell culture media from cell culture flask, but keep approximately 15 mL culture medium in a cell culture flask (75 cm^2)
  - ⇨ Change medium and evaluate morphology 24 h upon receipt

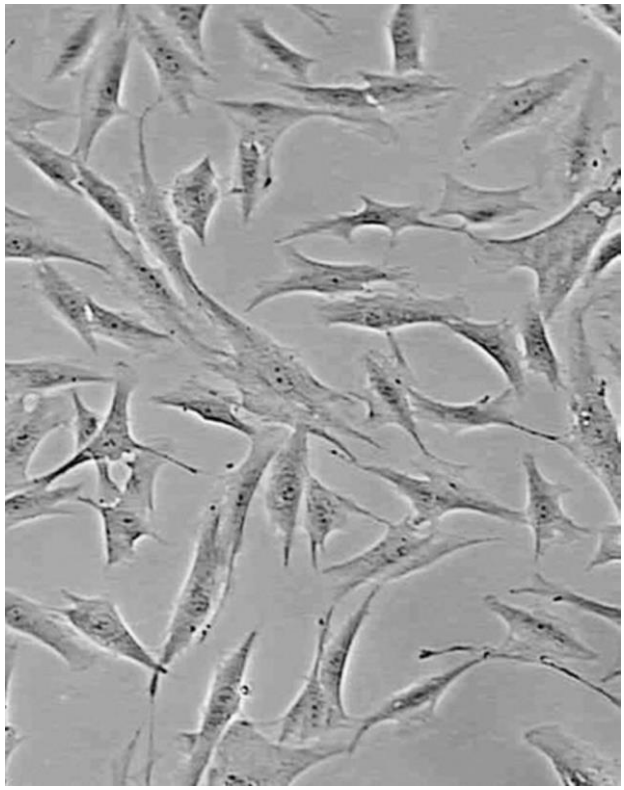
Cell morphology

a) NHK



(Gstraunthaler & Lindl, 2013)

b) NHDF



(Gstraunthaler & Lindl, 2013)

c) Microbial contamination

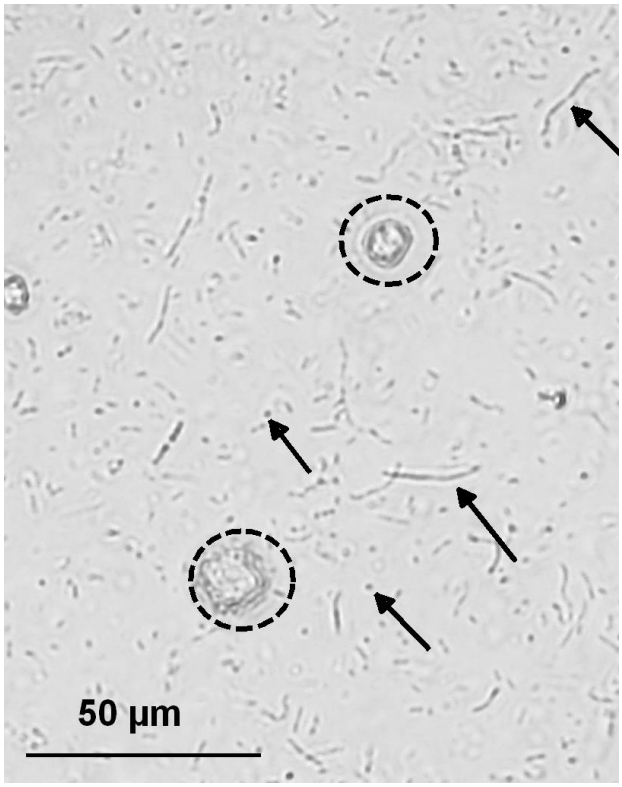


Figure 1 Morphology of cutaneous cells. a) polygonal, adherent NHK that grow in colonies. b) bipolar, elongated, adherent NHDF c) circles: round, adherent cells (NHK/NHDF) arrows: bacteria, suspended in medium. Scale bar = 50 µm.

SPECIMEN DOCUMENTATION

Specimen			Date of freezing	
No.	**Date of receipt**	**Remarks**	**NHK**	**NHDF**
yy-01				
yy-02				
yy-03				
yy-04				
yy-05				
yy-06				
yy-07				
yy-08				
yy-09				
yy-10				

CELL DOCUMENTATION

Box No. ....

	Cell type passage	**Vial count**	**Specimen number**	**Cell count (mio per cryo vial)**	**Remarks**	**Date of freezing**
1	*<NHDF p2>*	*<3>*	*<16-01>*	*<1.5>*		*<2016-01-02>*
2	*<NHK p2>*	*<3>*	*<16-01>*	*<3.0>*		*<2016-01-02>*
3						

TROUBLE SHOOTING

Problem	Solution
Epidermis cannot be separated from dermis	Incubate for additional 2 h (4°C) or 1 h (25°C)
Melanocytes grow within NHK monolayer	Discard cells, if the proportion of melanocytes exceeds 10%
Microbial contamination	Discard cells; if microbial contamination occurs in monolayers from more than one donor, than take actions to root out the causes
NHDF grow within NHK monolayer	• Aspirate KGM, using pasteur pipettes • Incubate cells with 2 mL Trypsin-EDTA working solution per cell culture flask (75 cm^2) until the NHDF detach ▲ Observe this process using a microscope • Wash cells with 10 mL PBS and discard the PBS • Incubate cells with 1.5 mL Trypsin-EDTA working solution per cell culture flask (75 cm^2) for 5 min in the incubator as described in cell subculture
Cells do not detach	Discard cells
Cells proliferate sparsely	≥ 30% confluence: subculture cells immediately < 30% confluence: discard cells