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Standard Operating Procedure (SOP)

Title Production of Organotypic NMSC Models			**Date** 2015-12-30
Documentenno. SOP_nmsc-model	**First edition** 2012-06-01	**Issued by** Zoschke, C.	**Version** 4 **Page** 1 von 20

Version	**valid from**	**Description of changes**
1	2012-06-01	Erstellung
2	2013-10-15	Zellkultur, Kulturdauer, Zellidentifikation
3	2014-05-20	Kulturdauer, redaktionelle Überarbeitung
4	2015-12-30	Translation

Issued by	**Reviewed by**	**Approved by**
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Scope
Workgroup Prof. Dr. Monika Schäfer-Korting

Directory

AIMS

Standardized production of organotypic NMSC models with high reproducibility.

SCOPE

This SOP applies to the workgroup of Prof. Dr. Schäfer-Korting, Institute for Pharmacy, Freie Universität Berlin.

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
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.

Cells

Designation	Source
NHDF, passage 3, pooled of 3 donors	Isolation from juvenile preputium [1])
NHK, passage 3, pooled of 3 donors	Isolation from juvenile preputium [4])
SCC-12, passage ≤100	cell line

Consumables

Designation	Supplier
2-Propanol	VWR, Darmstadt
Acetone	VWR, Darmstadt
Adenine HCl monohydrate	Sigma-Aldrich, München
Amphotericin B	Biochrom, Berlin
Ascorbic acid	Merck, Darmstadt
Calcium chloride	Sigma-Aldrich, München
Calibration solutions (pH 7, pH 9)	Hanna Instruments, Woonsocket, RI, USA
Cell culture flask (75 cm², 150 cm²)	TPP, Trasadingen, Schweiz
Centrifuge tubes (15, 50 mL)	TPP, Trasadingen, Schweiz
Cholera toxin	Sigma-Aldrich, München
Collagen G (≥95% collagen type I, <5%: type III, IV, V)	Biochrom, Berlin
Coverslip	Carl Roth, Karlsruhe
Cryo vial	Almeco, Esbjerg N, DK
Cryomold Standard (25x20x5 mm) Specimen Molds Disposable Vinyl	Sakura, Zoeterwonde, NL
Deep-6-well-plate	Corning, Corning, NY, USA
Dimethyl sulfoxide (plant cell culture tested)	Sigma-Aldrich, München
Dulbecco's Modified Eagle Medium (DMEM)	Sigma-Aldrich, München
DMEM(10x)	Biochrom, Berlin
DMEM/F-12	Lifetechnologies, Darmstadt
DMEM+GlutaMax	Lifetechnologies, Darmstadt

[1]) according to SOP “Isolation of keratinocytes and fibroblasts from human specimens”

Consumables (continued)

Designation	Supplier
Embedding cassettes Rotilabo®	Carl Roth, Karlsruhe
Eosin solution	Carl Roth, Karlsruhe
Epidermal Growth Factor (EGF)	Lifetechnologies, Darmstadt
Ethanol, 96%, methylethyl ketone denaturated	Berkel AHK, Berlin
Ethanol, absolute	Merck, Darmstadt
Ethylenediaminetetraacetic acid (EDTA)	Sigma-Aldrich, Schnelldorf
EZ-DeWax™ for deparaffinization	BioGenex, San Ramon, CA, USA
Fetal calf serum	Biochrom, Berlin
Forceps (anatomical)	Carl Roth, Karlsruhe
Gentamicin	Lifetechnologies, Darmstadt
Glass bottles (0,25 L, 1 L, Boro 3.3, GL 45)	Schott, Mainz
4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer	Biochrom, Berlin
Hydrocortisone	Sigma-Aldrich, München
Inserts (polyethylene terephthalate membrane, 0.4 µm pore size)	Corning, Corning, NY, USA
Insulin	Roche, Grenzach-Wyhlen
Keratinocytes Growth Medium (KGM)	Lonza, Köln
L-Glutamine	Sigma-Aldrich, München
Mayer's Hem Alum solution	Carl Roth, Karlsruhe
Metal base mold for paraffinization (24x24x12; 14038832453)	Leica, Nussloch
Microtome blades (cryo)	Leica, Nussloch
Microtome blades SEC35 (paraffin)	Microm International, Walldorf
Nitrogen (liquid)	Air Liquide, Paris, F
Non-essential amino acids	Lifetechnologies, Darmstadt

Consumables (continued)

Designation	Supplier
Paraffin No. 6	Thermo scientific, Braunschweig
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
Poly-L-Lysine HBr	Sigma-Aldrich, München
Potassium chloride (KCl, cell culture tested)	Sigma-Aldrich, München
Potassium dihydrogen orthophosphate (KH_2PO_4)	Carl Roth, Karlsruhe
Reaction tube	Eppendorf, Hamburg
Roti-Histofix 4%, pH 7	Carl Roth, Karlsruhe
Roti-Histokitt	Carl Roth, Karlsruhe
Roti-Histol	Carl Roth, Karlsruhe
Scalpel (No. 24)	Carl Roth, Karlsruhe
Slides (cryo, Polysine slides)	Thermo scientific, Braunschweig
Slides (paraffin, Superfrost Ultra Plus)	Thermo scientific, Braunschweig
Sodium chloride (NaCl, pro analysi)	Carl Roth, Karlsruhe
Sodium hydroxide (pellets)	Sigma-Aldrich, München
Stirring bar	Carl Roth, Karlsruhe
Syringe (10 mL, 20 mL)	BBraun, Melsungen
Syringe filter (Cellulose acetate, 0.2 µm pore size,[5])	Sarstedt, Nümbrecht
Syringe filter (Polyethersulfone, 0.2 µm pore size,[2])	Carl Roth, Karlsruhe
Tissue freezing medium	Leica, Nussloch
Transferrin	PromoCell, Heidelberg
Triiodothyronine	Sigma-Aldrich, München
Trypsin	Sigma-Aldrich, München

Equivalent consumables of other suppliers can be used as well.

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

Custom-made media

Description	Ingredients			Remarks
FGM	DMEM	500	mL	
	Fetal Calf Serum	50	mL	
	L-Glutamine	5	mL	
	Penicillin/Streptomycin	5	mL	4°C, 6 weeks [6])
PBS	KCl	200	mg	
	NaCl	8,000	mg	
	KH_2PO_4	200	mg	
	Na_2HPO_4	1,148	mg	autoclave, check pH [3])
	Aq. bidest.	1	L	4°C, 6 weeks [6])
SCC-12-	DMEM/F12	500	mL	
growth medium	Fetal Calf Serum	50	mL	
	Penicillin/Streptomycin	5	mL	4°C, 6 weeks [6])
Trypsin-EDTA	Trypsin	2,500	mg	c(Trypsin)=250 mg/mL
stock solution	EDTA	29.22	mg	sterile filtration,
	PBS	10	mL	Aliquot: 4°C, 6 weeks [6])
Trypsin-EDTA	Trypsin-EDTA stock solution	1	mL	c(Trypsin)=2.5 mg/mL = 0.25% c(EDTA)=1.0 mmol/L
working solution	PBS	99	mL	4°C, 2 weeks [6])

[3]) perform calibration of pH meter before use (pH 7, pH 9, stirring bar)

Custom-made media (continued)

Description	Ingredients	$c_{Aliquot}$	c_{CDM}		Remarks
CGM	Adenine HCl H_2O	$1.8\ 10^{-2}$	$1.6\ 10^{-6}$	mol/L	4°C, 6 weeks [3])
	Amphotericin B	0,3	$0.3\ 10^{-4}$	g/L	
	Choleratoxin	$1.1\ 10^{-6}$	$9.7\ 10^{-10}$	mol/L	
	DMEM+GlutaMax	--	44	%	
	DMEM/F-12	--	44	%	
	EGF	$10\ 10^{-3}$	$8.8\ 10^{-6}$	g/L	
	Fetal Calf Serum	100	8,8	%	
	Gentamycin	50	$4.4\ 10^{-2}$	g/L	
	Hydrocortisone	1	$3.5\ 10^{-3}$	g/L	
	Insulin	1	$0.4\ 10^{-3}$	g/L	
	Non-essential amino acids	100	0,4	%	
	Penicillin/Streptomycin	10^4 10	88 $8.8\ 10^{-2}$	U/mL g/L	
	Transferrin	4	$3.5\ 10^{-4}$	g/L	
	Triiodothyronine	$3\ 10^{-5}$	$1.9\ 10^{-9}$	mol/L	
CDM	CGM	--	99.3	%	
	Ascorbic acid	$5.7\ 10^{-2}$	$2.5\ 10^{-4}$	mol/L	
	$CaCl_2 \cdot 2H_2O$	1	$1.8\ 10^{-3}$	mol/L	

(1) $c = \frac{m}{M_r \cdot V};\ \beta = \frac{m}{V} \Rightarrow c = \frac{\beta}{M_r}$ (2) $V_{Aliquot}$ *see Table 2* (3) $V_{CDM} = 1131\ mL$

(4) $c_{Aliquot} = \frac{m_{portion}}{M_r \cdot V_{Aliquot}}$ (5) $c_{CDM} = \frac{c_{Aliquot} \cdot V_{Aliquot}}{V_{CDM}}$

Prepare CGM without fetal calf serum in a 1 L-bottle and mix for 5 minutes using a stirring bar. Dispense the mixture to five 200 mL-bottles and add 20 mL fetal calf serum to each bottle. Gently mix the CGM, label with “CGM” and the date of production.

Prepare CDM by adding aliquots of ascorbic acid and $CaCl_2$ to the 200 mL-bottles shortly before using CDM. Gently mix the CDM and label with “CDM”.

STEP-TO-STEP PROTOCOL

Dermal Compartment | Day 01

Materials

Collagen G, deep-6-well-plate, DMEM(10x), forceps, HEPES buffer, insert, NHDF, materials for cell subculture and cell count

Step-to-step protocol

- Determine the number of constructs you need to grow
- Cultivate NHDF in an incubator. For each construct you need 0.8 x 10^6 NHDF
- Place inserts into deep-6-well-plate with forceps and label each tray with the batch no
- Prepare buffer for collagen gel. Mix appropriate volumes of DMEM(10x), HEPES buffer and NaOH solution in this order (Table 1). Store the prepared buffer on ice

Ingredients	**Proportion (%)**	**Quantity / 6 c (µL)[a]**	**Quantity / 6 c (µL)[b]**	**Quantity / 8 c (µL)[a]**	**Quantity / 8 c (µL)[b]**
Collagen G	80.0	6,400	18,080	8,800	24,080
DMEM(10x)	5.0	400	1,130	550	1,506
HEPES buffer	2.5	200	565	275	753
NaOH solution (0.7 mol/L)	2.5	200	565	275	753
FGM	10.0	800	2,260	1,100	3,010
NHDF (x10^6)	0.8	0	4.8	0	6.4
Σ	**100.0**	**8,000**	**22,600**	**11,000**	**30,102**

Table 1 Composition of dermal compartments including 20% addition. a) Quantities for acellular collagen gel. b) Quantities for cellular collagen gel. *c constructs*.

- Prepare acellular collagen gels by pouring collagen G into a centrifuge tube. Add the buffer and mix thoroughly, but avoid foaming. Then add FGM and mix thoroughly, but avoid foaming. Transfer 1 mL of collagen mix into each insert

Dermal Compartment | Day 01 (continued)

▲ **CRITICAL STEPs**

- Stick to the sequence DMEM(10x), HEPES buffer, NaOH solution to prevent the dermal compartments from crystals
- Keep collagen G chilled at 4-8°C, perform this step within 4 minutes to prevent early gelling
- Pure collagen G without foaming because air-bubbles damage dermal compartment

- Watch the pH value. If color of acellular collagen mix (phenol red in DMEM(10x)) still indicates acidic pH, add some microliters of NaOH (0.7 mol/L)
- Incubate the acellular collagen gel in the incubator for the next 2 h
- Trypsinize NHDF. Determine the number of NHDF, then centrifuge at 130 g for 5 min at 25°C, discard the supernatant. Wash NHDF with PBS, then centrifuge again at 130 g for 5 min at 25°C. Discard the supernatant, leave the pellet on ice for max. 30 min
- Resuspend NHDF pellet in appropriate volume of FGM (Table 1)
- Prepare cellular collagen gel by pouring collagen G into a 50 mL glass bottle (washed, autoclaved). Add the buffer and mix thoroughly, but avoid foaming. Then add NHDF (suspended in FGM) and mix thoroughly, but avoid foaming
- Transfer 3 mL of cellular collagen gel in each insert. The rest of cellular collagen gel can be used for gel control

▲ **CRITICAL STEPs**

- Transfer the cellular collagen in the insert within 2 h after pouring the acellular collagen
- If the cellular collagen gel does not gel within 5 h at 25°C, discard all constructs of that batch and restart after checking pH of buffer solution (intended pH value: 7.9-8.05)

- Incubate the dermal compartment for 24 h in the incubator

Dermal Compartment | Day 02 (continued)

Materials

Materials for CGM (Table 2)

Step-to-step protocol

- Prepare CGM according to Table 2. For each construct approximately 80 mL are needed

Aliquots for CGM/CDM

Ingredients		V [mL]
Adenine HCl H_2O ("A")	155 mg Adenine HCl H_2O in (10 mL 0.1N HCl+ 40 mL PBS), (0.1N HCl: 1 mL 1N HCl + 9 mL Aq. bidest.) **stf (CA)**	**0.1**
Amphotericin B ("AmB")	According to manufacturer	**1**
Choleratoxin ("CT")	1 mg in 10 mL PBS	**1**
EGF	100 µg in 10 mL PBS	**1**
Fetal Calf Serum ("FCS")	Heat inactivation 30 min, 56°C H_2O	**50**
Hydrocortisone	50 mg in 50 mL EtOH (100%), **stf (PES)**	**50**
Insulin ("I")	10 mg in 10 mL 0.01N HCl (0.01N HCl: 0.1 mL 1N HCl+9.9 mL Aq.bidest.), **stf (CA)**	**0.5**
Transferrin ("T")	100 mg in 25 mL PBS	**0.1**
Triiodothyronine ("T3")	1 mg in 1 mL 1N NaOH; **stf (CA),** dilute with CGM 1:50	**0.07**
Ascorbic acid	100 mg in 10 mL PBS; **stf (CA)** (black tubes)	**1**
$CaCl_2 \cdot 2H_2O$	1.47 g in 10 mL Aq. bidest., **stf (CA)**	**10**
All others	Ready to use	--

CGM

Ingredients	🌡 [°C]	V [mL]	get	add
Glass bottles	RT			
DMEM+GlutaMax	4	500		
DMEM/F-12	4	500		
Adenine HCl H_2O	- 20	0.1		
Amphotericin B	- 20	1		
Choleratoxin	- 20	1		
EGF	- 20	1		
Fetal Calf Serum	- 20	100		
Gentamycin	RT	1		
Hydrocortisone	4	4		
Insulin	- 20	0.5		
N-e. amino acids	4	5		
Pen/Strep	- 20	10		
Transferrin	- 20	0.1		
Triiodothyronine	- 20	0.07		

CDM

CGM	4	1124		
Ascorbic acid	- 20	5		
$CaCl_2 \cdot 2H_2O$	4	2		

Table 2: Preparation of CGM/CDM. Left) Aliquots. Right) Final mixture*. stf sterile filtered, CA cellulose acetate, PES polyethersulfone*

▲ **CRITICAL STEP**

- Prevent the CGM from changing pH value by preparing aliquots of 250 mL

- Add 9.3 mL CGM outside the insert into each well
- Let the dermal compartment shrink until day 07

Change CGM | Day 05

Materials

CGM

Step-to-step protocol

- Aspirate CGM from inside and from outside of the inserts
- Fill 2 mL CGM onto the dermal compartment and 9.3 mL CGM into each well (outside of the inserts)

▲ **CRITICAL STEPs**

- All liquids must be removed from construct surface without damaging the dermal compartment
- Make sure that the medium level does not exceed the level of the construct surface

Epidermal Compartment | Day 07

Materials

CGM, NHK, materials for cell subculture and cell count

Step-to-step protocol

- Cultivate NHK to 60-80% confluence in the incubator; each construct requires 2-3x10^6 NHK

Carcinoma stage	SCC-12-ratio:NHK	SCC-12 (x10^6)	NHK (x10^6)
reconstructed normal human skin	0:100	0.0	3.0
reconstructed actinic keratosis	10:100	0.3	2.7
reconstructed invasive cSCC	50:100	1.0	2.0

Table 3 Composition of epidermal compartments.

- Aspirate CGM from inside and from outside of the inserts
- Fill 9.3 mL CGM into each well

▲ **CRITICAL STEPs**

- All liquids must be removed from construct surface without damaging the dermal compartment
- Make sure that the medium level does not exceed the level of the construct surface

- Trypsinize NHK. Determine the NHK count, then centrifuge at 130 g for 5 min at 25°C. Wash the cell pellet with PBS, centrifuge again at 130 g for 5 min at 25°C, discard the supernatant and keep the pellet on ice for not longer than 30 min
- Resuspend the NHK pellet in KGM and gently pipette the cell suspension (total volume per model = 100 µL) onto the dermal compartment

Co-culture with SCC-12 cells | Day 08

Materials

CGM, SCC-12 cells, materials for cell subculture and cell count

Step-to-step protocol

- Aspirate KGM from the surface of the construct

▲ **CRITICAL STEPs**

- All liquids must be removed from construct surface without damaging the construct

- Trypsinize SCC-12 cells 24 h after NHK seeding. Determine the SCC-12 cell count, then centrifuge at 130 g for 5 min at 25°C. Wash the cell pellet with PBS, centrifuge again at 130 g for 5 min at 25°C, discard the supernatant and keep the pellet chilled for not longer than 30 min
- Re-suspend the SCC-12 pellet in CGM and gently pipette the cell suspension (total volume per model = 100 µL) onto the construct

Airlift | Day 09

Materials

CDM

Step-to-step protocol

- Aspirate CGM from the surface of the construct and outside of the insert
- Fill 9.3 mL CDM into each well (outside of the inserts)

▲ **CRITICAL STEPs**

- All liquids must be removed from construct surface without damaging the dermal compartment
- Make sure that the medium level does not exceed the level of the construct surface

- Change CDM 3 times a week

Application of test substances | Days 17, 19, 21

Materials

CDM, test substance

Step-to-step protocol

- Collect CDM, change CDM; apply 20 µL test substance homogenously per construct
- Cultivate constructs for 48 hours, collect CDM, and change CDM
  Apply 20 µL (10 µL/cm²) test substance homogenously onto each construct
- Cultivate constructs for 48 hours, collect CDM, and change CDM
  Apply 20 µL (10 µL/cm²) test substance homogenously onto each construct
- Cultivate constructs for 48 hours and then collect CDM

HISTOLOGICAL EVALUATION

Cryo sections | Day 23

Materials

Cryo mold, forceps, liquid nitrogen, scalpel, tissue freezing medium, ice-cold acetone

Step-to-step protocol

- Prepare cryo mold with freezing medium
- Cut the construct off the insert by taking the insert out of the deep-6-well-plate. Cut the insert membrane off the insert using a scalpel. Then place the construct and the insert membrane on a flat surface. Separate the construct from the insert membrane using forceps
- Place the construct into the freezing medium, cover the construct with tissue freezing medium; snap-freeze the construct in liquid nitrogen until it becomes a solid block

▲ **CRITICAL STEPs**

- Do not let the construct dry
- Cut each construct into halves and use 2 cryo molds per construct
- Avoid air-bubbles in the freezing medium and floating of the construct
- Snap-freeze construct on the surface of liquid nitrogen; do not submerse it
- Avoid freeze-thaw-cycles during histological evaluation
- Avoid storage of frozen constructs longer than 3 months

- Store cryo blocks at -80°C
- Cut cryo blocks using a cryotome. Use the trim (20-50 µm) to cut the clutter. Then cut the construct into slices of 5 (or 7) µm
- Fix the slices with ice-cold acetone; therefore, submerse slices (slides) in ice-cold acetone for 10 min and let the acetone vapor for 30 min at room temperature

Paraffin sections | Day 23

Materials

DeWax™, Embedding cassette (labelled), ethanol, metal base molds for paraffinization, paraffin, scalpel

Step-to-step protocol

- Cut the construct off the insert by taking the insert out of the deep-6-well-plate. Cut the insert membrane off the insert using a scalpel. Then place the construct and the insert membrane on a flat surface. Separate the construct from the insert membrane using forceps
- Place the inserts into the labelled cages and immerse the construct overnight in formalin at 2-8°C
- Dehydrate the construct according to Table 4

Solution	Ethanol absolute (mL)	Aq. bidest. (mL)	Duration (minutes)
Aq. bidest.	0	506	30
Ethanol 50% (m/m)	218	288	20
Ethanol 70% (m/m)	319	187	20
Ethanol 80% (m/m)	374	132	20
Ethanol 90% (m/m)	435	71	20
Ethanol 96% (m/m)	476	30	20
Ethanol 96% (m/m)	476	30	20
Ethanol absolute - I	506	0	20
Ethanol absolute - II	506	0	20
Roti-Histol - I	0	0	30
Roti-Histol - II	0	0	30

Table 4 Solutions for construct dehydration.

(6) $V_{EtOHabsolute} = \frac{m_{EtOHabsolute} \cdot \omega_{EtOH}}{\varrho_{EtOHabsolute}}$ (7) $\varrho_{EtOHabsolute} = 0.79074$

- Immerse the constructs into paraffin for 90 minutes and into a second paraffin bath for 60 min
- Place the construct in metal base molds for paraffinization and embed the constructs into paraffin
- Freeze the paraffin blocks at -12°C for at least 15 min
- Store paraffin blocks at 2-8°C
- Cut paraffin blocks using a microtome; use the trim (20-50 µm) to cut the clutter. Then cut the construct into slices of 5-10 µm
- Remove the paraffin by immersing the slices into 2 DeWax™ solutions for 5 min each; carefully wash the slides with tap water

TROUBLE SHOOTING

Problem	Solution
Fetal calf serum floccu-lates after heat inactivation	Discard FCS. Use fresh FCS and mix more carefully
Collagen does not polymerize within 7 h	Discard dermal compartment. Prepare fresh NaOH solution and use fresh HEPES buffer
Air bubbles underneath the insert	Release any air bubbles trapped underneath the insert
Liquids on the construct surface	Remove any liquids from the construct surface. Record affected constructs in the lot documentation
Disrupted construct slices	Change cut angle, use new blade, or alter the cutting temperature

LOT DOCUMENTATION

Model | Lot	**cSCC, 5th generation | *yy-***	**Project**	**…**
Production	*name*	Recipient	*name*
NHDF Donor | Passage	**yy-xx | p**	Goals	…
NHK Donor | Passage	**yy-xx | p**		
Kokultur Zelllinie | Passage	**SCC-12 | p**	No. of constructs	**…**
Tray | Insert	deep-6-well | 0.4 µm pores	Remarks	…
CGM V_{out} | V_{in}	≤9.3 mL | 2 mL		
NHDF-Suspension	$0.8x10^6$ in 377 µL FGM		
NHK-Suspension	≤$3x10^6$ in 100 µL KGM		
SCC-12 Suspension	≤$1x10^6$ in 100 µL CDM		
NHK-seeding	day 07	Experiment - start	day *xx*
Airlift	day 08	Experiment - finish	day *xx*
SCC-12 co-culture	day 08		

Schedule

Date	**Mo**	**Tu**	**We**	**Th**	**Fr**	**Sa**	**Su**
Day							
Task				defreeze NHDF	changeFGM NHDF		
Date	**Mo**	**Tu**	**We**	**Th**	**Fr**	**Sa**	**Su**
Day				1	2	3	4
Task		changeFGM NHDF		Dermal com-partment	change CGM		
Date	**Mo**	**Tu**	**We**	**Th**	**Fr**	**Sa**	**Su**
Day	5	6	7	8	9	10	11
Task	change CGM		NHK seeding	SCC-12 co-culture	change CDM		
Date	**Mo**	**Tu**	**We**	**Th**	**Fr**	**Sa**	**Su**
Day	12	13	14	15	16	17	18
Task	change CDM			change CDM		CDM 1st treatment	
Date	**Mo**	**Tu**	**We**	**Th**	**Fr**	**Sa**	**Su**
Day	19	20	21	22	23		
Task	changeCDM 2nd treatment		changeCDM 3rd treatment		snap-freezing		

LOT DOCUMENTATION (continued)

…….	Co-culture	Applied Substance	Cryo/paraffin	Evaluation
1				
2				
3				
4				
5				
6				
7				
8				
9				
10				
11				
12				

CELL LINE AUTHENTICATION REPORT

MULTIPLEXION

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Human Cell Line Authentication Report

Report ID	415	Order ID	493
Report Date	01.11.2013	Order Date	23.10.2013
		Purchase No.	510190F

Dear Monika Schäfer-Korting,
Many thanks for your order. The Multiplex human Cell line Authentication Test (MCA) was performed as described at www.multiplexion.de. Please find below the results.
Best regards,
Dr. Markus Schmitt

Information from Customer				Results			Summary			
Sample ID	Sample Name	Cell line name	If other: exact name	DNA quality	Best Hit with DataBase	Identity (%)	Present in Database?	Cross-Contamination?	Identity confirmed?	Genotype Code
668	SCC-12	other	SCC-12	ok	CC7	94	no	no	unique sequence	AATTAAAAATAWAAAAWTAAAATTTTATAAAAAATTTTAWTTTTNNWT

Legend:

DNA quality:	ok, good DNA quality detected; invalid, DNA was absent or degraded or from non-human species
Identity (%)	Identity of submitted cell line to best hit of data base, identical: 96% and above, not indentical: <96%
Present in database?	indicates whether submitted cell line is included in MCA data base. If your cell line is not included, than no identity confirmation can be made.
Cross-contamination?	indicates whether detected cell line is cross-contaminated by additional cells from another human cell line, the contaminating cell line cannot be specifically identified
Identity confirmed?	"identity confirmed", indicates whether identity was confirmed by MCA (96% and above); "identity not confirmed", submitted cell line is present in data base, but the genotype code is different than expected; "unique sequence", cell line is not present in data base and shows a genotype code that is not related to any cell line included in the data base; "false, match with known cell line", cell line is not present in data base but shows a genotype code that is identical to a cell line included in the data base
Genotype Code	48-letter code for 24 SNP locations; W, uncertain signal; N, no call

CELL CONTAMINATION TEST REPORT

MULTIPLEXION

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Monika Schäfer-Korting

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Multiplex cell Contamination Test Report

Report ID	415	Order ID	493
Report Date	01.11.2013	Order Date	23.10.2013
		Purchase No.	510190F

Dear Monika Schäfer-Korting,

Many thanks for your order. The multiplex cell contamination test was conducted for the following contaminations: Mycoplasma, Squirrel Monkey Retrovirus, Epstein-Barr-Virus, Human-, Macaca cynomolgus-, Mouse-, Rat-, Chinese hamster-, Syrian hamster, Feline-, Canine-, Rabbit-, Guinea pig and Drosophila cells. DNA Quality was determined by an internal DNA quality control. Positive und negative controls were included to monitor PCR performance. Empty "Result" cells indicate the absence of contaminations tested for.

Best regards,

Dr. Markus Schmitt

Information from Customer			Results				
Sample ID	Cell line name	Reported Species	DNA quality	Species	Viruses	Mycoplasma	Summary
983	SCC-12	Human	positive	Human,			clean

Legend:

DNA quality: "positive", good DNA quality detected; "QC failed", DNA was absent or degraded.

Species: If identical to "Reported Species", then species is confirmed,
If not identical, then contamination with other species detected as described.

Viruses: "SMRV", Env and Gag DNA sequences detected. Cell line should be discarded or used in S2/L2 only. "EBV", cell line is positive for Epstein-Barr-Virus. Please contact your biosafety officer for further information.

Mycoplasma: "Mycoplasma spec.", cell line is contaminated by Mycoplasma. Mycoplasma species is indicated. Cell line should be discarded or treated with antibiotics. Please contact your biosafety officer in the case of contaminations with pathogenic M. hominis, M. genitalium or M. pneumoniae for further information.