Cell Line: SA01
Lot: CA001

Table of Contents

This material was cultured and frozen using Cellartis' protocols. WiCell recommends that stem cells should be thawed and established in the conditions in which they were initially frozen prior to transfer to alternate culture platforms. The protocols that were used to produce these cells can be found on the following pages of this document.

If you have any questions or concerns please contact WiCell's technical support staff via our website side at www.wicell.org and we will be happy to assist you.

Thank you,

WiCell

P.O Box 7365 • Madison, WI 53707-7365 • Phone: 888.204.1782 • Fax: 608.441.8011
www.wicell.org

Certificate of Analysis - Amended

Depositor Distribution Lot

Product Description	(SA01) Depositor Distribution Lot
Cell Line Provider	Cellartis
Distribution Lot Number	SA01-DDL-01[1]
Date Vialed	[2]
Passage Number	[3]

The following testing specifications have been met for the specified product lot:

Test Description	Test Method	Test Specification	Result
Post-Thaw Viable Cell Recovery	SOP-CH-305C	Viable cells recovered	Pass
Identity by STR	SOP-SS-006B	Positive identity	Pass
Mycoplasma	Bionique Method M250	No contamination detected	Pass
Karyotype by G-banding	SOP-CH-003B	Normal karyotype	Pass

Cells distributed by the National Stem Cell Bank are intended for research purposes only and are not intended for use in humans.

Electronic versions lot certificates (CoAs) complete with electronic copies of individual reports, results, and procedures are available on our website, www.wicell.org. There are also archived CoAs for past cell lots.

Please visit the technical service portion of the website for assistance with your human ES Cells. The knowledgeable technical support staff can assist with embryonic stem cell culture concerns, training, and any other customer service concerns you may encounter.

Amendment(s):

Reason for Amendment	Date
CoA updated to include copyright information and electronic signature, and update to WiCell logo. Links updated.	See signature
Original CoA	23-Dec-2009

Date of Lot Release	Quality Assurance Approval
23-December-2009	1/3/2014 X AMC AMC Quality Assurance Signed by: [redacted]

[1] This material is Cellartis Lot CA001 and does not represent a lot of material grown by the NSCB. The test results shown on this CoA is to supplement the testing done by the provider.
[2] See Cellartis CoA for date vialed.
[3] See Cellartis CoA for passage number

UW Health

University of Wisconsin
Hospital and Clinics

Histocompatibility/Molecular Diagnostics Laboratory

Short Tandem Repeat Analysis*

Sample Report: 3125-STR | UW HLA#: 61995 | Sample Date: 11/06/09
Received Date: 11/06/09

Requestor: WiCell Research Institute
Test Date: 11/09/09 | File Name: 091110 | Report Date: 11/12/09

Sample Name: (label on tube) **3125-STR**

Description: DNA Extracted by WiCell
310.7 ug/mL; 260/280 = 1.86

Locus	Repeat #	STR Genotype
D16S539	5, 8-15	12,13
D7S820	6-14	8,10
D13S317	7-15	11,12
D5S818	7-15	12,13
CSF1PO	6-15	10,13
TPOX	6-13	8,9
Amelogenin	NA	X,Y
TH01	5-11	9.3,9.3
vWA	11, 13-21	16,18

Comments: Based on the 3125-STR DNA dated and received on 11/06/09 from WI Cell, this sample (UW HLA# 61995) matches exactly the STR profile of the human stem cell line SA01 comprising 15 allelic polymorphisms across the 8 STR loci analyzed. No STR polymorphisms other than those corresponding to the human SA01 stem cell line were detected and the concentration of DNA required to achieve an acceptable STR genotype (signal/noise) was equivalent to that required for the standard procedure (~1 ng/amplification reaction) from human genomic DNA. These results suggest that the 3125-STR DNA sample submitted corresponds to the SA01 stem cell line and it was not contaminated with any other human stem cells or a significant amount of mouse feeder layer cells. Sensitivity limits for detection of STR polymorphisms unique to either this or other human stem cell lines is ~5%.

11-17-09

, Manager Date
HLA/Molecular Diagnostics Laboratory

11/12/09

PhD, Director Date
HLA/Molecular Diagnostics Laboratory

* Testing to assess engraftment following bone marrow transplantation was accomplished by analysis of human genetic polymorphisms at STR loci. This methodology has not yet been approved by the FDA and is for investigational use only.

File: Final STR Report

Mycoplasma Testing Services

Safe Cells

BIONIQUE TESTING LABORATORIES, INC.

Document#: DCF3013D
Edition#: 10
Effective Date: 07/15/2003
Title: **M-250 FINAL REPORT SHEET**

M-250 FINAL REPORT

Direct Specimen Culture
Procedure 3008, 3011, 3013

TO: Wicell QA
WiCell Research Institute

BTL SAMPLE ID#: **58277** P.O.#: DATE REC'D: **08/06/2009**

TEST/CONTROL ARTICLE:

SA01 DDL 1 6 p27 NSCB#3125

LOT#: **NA**

DIRECT CULTURE SET-UP (DAY 0) DATE: **08/06/2009**

INDICATOR CELL LINE (VERO) SEE DNA FLUOROCHROME RECORD SHEET

			DATE
THIOGLYCOLLATE BROTH	DAY 7	+	08/13/2009
	DAY 28	+	09/03/2009
BROTH-FORTIFIED COMMERCIAL			
0.5 mL SAMPLE	DAY 7	+	08/13/2009
6.0 mL BROTH	DAY 28	+	09/03/2009
BROTH-MODIFIED HAYFLICK			
0.5 mL SAMPLE	DAY 7	+	08/13/2009
6.0 mL BROTH	DAY 28	+	09/03/2009
BROTH-HEART INFUSION			
0.5 mL SAMPLE	DAY 7	+	08/13/2009
6.0 mL BROTH	DAY 28	+	09/03/2009

(See Reverse)

Document#: DCF3013D
Edition#: 10
Effective Date: 07/15/2003
Title: M-250 FINAL REPORT SHEET

SAMPLE ID#: 58277		AEROBIC	MICROAEROPHILIC	DATE
AGAR PLATES-FORTIFIED COMMERCIAL	DAY 7	+	+	08/13/2009
	DAY 14	+	+	08/20/2009
	DAY 21	+	+	08/27/2009
AGAR PLATES-MODIFIED HAYFLICK	DAY 7	+	+	08/13/2009
	DAY 14	+	+	08/20/2009
	DAY 21	+	+	08/27/2009
AGAR PLATES-HEART INFUSION	DAY 7	+	+	08/13/2009
	DAY 14	+	+	08/20/2009
	DAY 21	+	+	08/27/2009
BROTH SUBCULTURES (DAY 7)		DATE: 08/13/2009		
AGAR PLATES-FORTIFIED COMMERCIAL	DAY 7	+	+	08/20/2009
	DAY 14	+	+	08/27/2009
	DAY 21	+	+	09/03/2009
AGAR PLATES-MODIFIED HAYFLICK	DAY 7	+	+	08/20/2009
	DAY 14	+	+	08/27/2009
	DAY 21	+	+	09/03/2009
AGAR PLATES-HEART INFUSION	DAY 7	+	+	08/20/2009
	DAY 14	+	+	08/27/2009
	DAY 21	+	+	09/03/2009

RESULTS: No detectable mycoplasmal contamination

9/3/09
Date

Laboratory Director
Ph.D.

M-250 Procedural Summary: The objective of this test is to ascertain whether or not detectable mycoplasmas are present in an in vitro cell culture sample, be it a primary culture, hybridoma, master seed stock or cell line. This procedure combines an indirect DNA staining approach to detect non-cultivable mycoplasmas with a direct culture methodology utilizing three different mycoplasmal media formulations. The indirect approach involves the inoculation of the sample into a mycoplasma-free VERO (ATCC) indicator cell line and performing a DNA fluorochrome assay after 72-120 hours of incubation. The direct culture aspect of the test utilizes three different mycoplasmal media including both broth and agar formulations. The sample is inoculated into each of the 3 broth formulations and also onto duplicate plates (0.1 mL/plate) for each of the 3 agar formulations. Subculture from broth to fresh agar plates is carried out after 7 days incubation. Agar plates are incubated aerobically and microaerophillically in order to detect any colony forming units morphologically indicative of mycoplasmal contamination. Issuance of the final report with signature of the Laboratory Director signifies that the required controls were performed concurrently with the test sample(s) as detailed in the referenced SOPs and that all test conditions have been found to meet the required acceptance criteria for a valid test, including the appropriate results for the positive and negative controls.

Mycoplasma Testing Services

Safe Cells

MYCOPLASMA TESTING SERVICES

BIONIQUE TESTING LABORATORIES, INC

APPENDIX I

Document #:	DCF3008A
Edition #:	06
Effective date:	9/17/2003
Title:	DNA FLUOROCHROME ASSAY RESULTS

DNA-FLUOROCHROME ASSAY RESULTS

Procedures 3008, 3009, 3011

Sample ID # **58277** **M-250** Date Rec'd: **08/06/2009** P.O. #

Indicator Cells Inoculated: Date/Initials: 8/6/09 / BMB

Fixation: Date/Initials: 8/10/09 / JA

Staining: Date/Initials: 8/10/09 / JA

TEST/CONTROL ARTICLE:

SA01 DDL 1 6~~P27~~ P27 NSCB#3125 SA 9-2-09

LOT# **NA**

Wicell QA
WiCell Research Institute

DNA FLUOROCHROME ASSAY RESULTS:

__X__ **NEGATIVE:** A reaction with staining limited to the nuclear region, which indicates no mycoplasmal contamination.

_____ **POSITIVE:** A significant amount of extranuclear staining which strongly suggests mycoplasmal contamination.

_____ **INCONCLUSIVE:**

_____ A significant amount of extranuclear staining consistent with low - level mycoplasmal contamination or nuclear degeneration.

_____ A significant amount of extranuclear staining consistent with bacterial, fungal or other microbial contaminant or viral CPE. Morphology not consistent for mycoplasmal contamination.

COMMENTS: _____

Date: 8/10/09 Results Read by: JA Date of Review: 8-10-09 Reviewed by: SeA

WiCell Cytogenetics Report: 001224-070809
NSCB 1825

Report Date: July 17, 2009

Case Details:

Cell Line: *SA01-DDL-01 (1825)*

Passage #: *22*

Date Completed: *7/17/2009*

Cell Line Gender: *Male*

Investigator: *National Stem Cell Bank*

Specimen: *hESC on MEF feeder*

Date of Sample: *7/8/2009*

Tests,Reason for: *DDL Release Testing and MCB Pre-freeze*

Results: *46,XY*

Completed by , CLSp(CG), on 7/16/2009

Reviewed and interpreted by PhD, FACMG, on 7/17/2009

Interpretation: *No abnormalities were detected at the stated band level of resolution.*

Cell: *S01-05*

Slide: *C*

Slide Type: *Karyotyping*

Cell Results: *Karyotype: 46,XY*

# of Cells Counted: 20

# of Cells Karyotyped: 5

# of Cells Analyzed: 9

Band Level: 450-600

Results Transmitted by Fax / Email / Post **Date:**_____
Sent By:_____ **Sent To:**_____
QC Review By: _____ **Results Recorded:** _____

cellartis

hESC line SA001, LOT CA001

Cellartis AB
Arvid Wallgrens Backe 20
SE-413 46 Göteborg, SWEDEN

Human embryonic stem cell line SA001, LOT CA001

Background

For the purpose of *in vitro* fertilization (IVF) treatment of patients suffering from involuntary childlessness, human embryos are created at the IVF clinics using conventional IVF-techniques. Supernumerary embryos may, after written informed consent from the donors, be used for research purposes, such as for derivation of human embryonic stem cells (hESC). The hESC derivation process at Göteborg University and Cellartis follows all applicable laws in Sweden and is approved by the Local Research Ethics Committees at Göteborg University and Uppsala University.

Donor confidentiality

In order to protect the privacy and the confidentiality of the donors, all identifiers associated with the embryo donors have been removed. Thus, no information about the donors is accessible. Notably, the donation did not result in any financial gain for the donors.

Summary of characteristics of LOT CA001

Parameter	Passage	Result
Embryo source	--	Frozen, surplus from IVF
hESC line derived	--	March 20, 2001
Procedure for isolation of ICM cells	--	Immunosurgery
LOT preparation	p14	>100 vials
Thawing recovery rate	p14– p15	100 %
SSEA-1	p19	Negative
SSEA-3	p19	Positive
SSEA-4	p19	Positive
TRA-1-60	p19	Positive
TRA-1-81	p19	Positive
Oct-4	p19	Positive
Alkaline phosphatase	p32	Positive
Karyotype	p21, p32	46, XY
FISH (X, Y, 13, 18, and 21)	p21	Diploid, XY
Telomerase activity	p24, p26, p32	Positive
Pluripotency *in vitro*	p19	Endo-, ecto-, mesoderm
Pluripotency *in vivo*	p22	Endo-, ecto-, mesoderm
Mycoplasma	p14	Negative
Human Immunodeficiency Virus type 1 and 2	p14	Negative
Hepatitis B	p14	Negative
Hepatitis C	p14	Negative
Cytomegalovirus	p14	Negative
Herpes Simplex Virus type 1 and 2	p14	Negative
Epstein-Barr Virus	p14	Negative
Human Papilloma Virus	p14	Negative

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hESC line SA001, LOT CA001

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Details

Derivation of hESC line SA001

Establishment of hESC lines at Cellartis is performed according to the procedures described in Heins *et al.* (Stem Cells, May 2004) and in Patent application "A method for the establishment of a pluripotent human blastocyst-derived stem cell line" (PCT no. PCT/EP02/14895, Publication no. WO03/055992). For routine expansion, the hESC are cultured on top of a mouse embryonic feeder (mEF) layer using VitroHES™ medium provided by Vitrolife AB (Göteborg, Sweden).

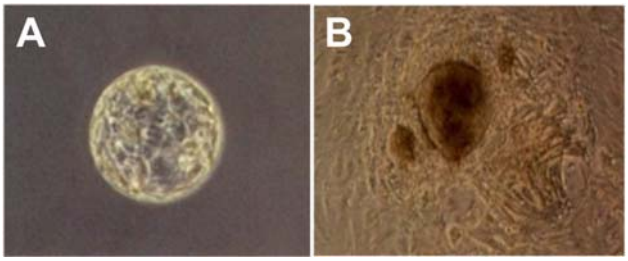

Figure 1. (A) Blastocyst from which hESC line SA001 was derived. (B) After Immunosurgery and plating on mEF.

Morphology

At the time of vitrification >100 vials were prepared from the hESC line SA001 in passage 14. Typical morphology of the hESC colonies, just prior to vitrification, is shown in Figure 2. After thawing and seeding of vitrified cells (*i.e.* LOT CA001), viable colonies proliferated and displayed the morphology that characterizes undifferentiated hESC (Figure 3). Subsequently, these cells were propagated and passaged according to standard procedures and representative illustrations of the hESC colonies in passage 15, 28 and 31 are shown in Figure 4.

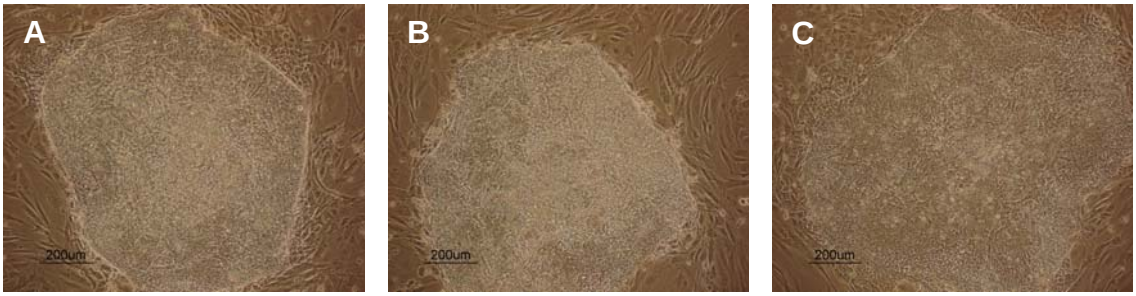

Figure 3 (A)-(C). Typical morphology of hESC cultured on mEF in passage 15 after thawing of vitrified cells (LOT CA001).

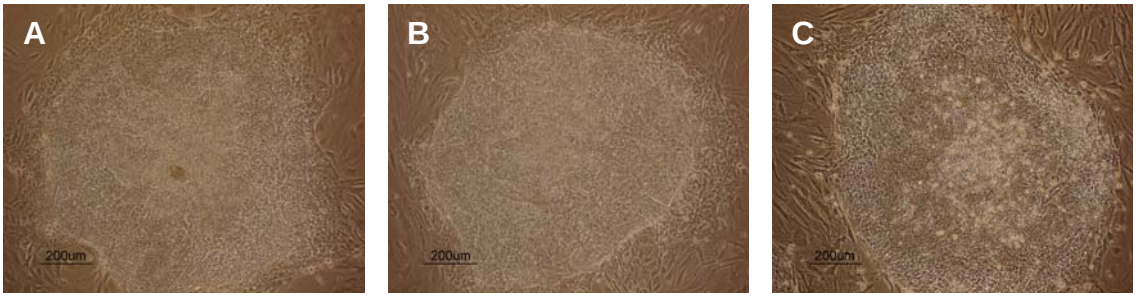

Figure 4. Typical morphology of hESC of LOT CA001 cultured on mEF in passage 15 (A), passage 28 (B) and passage 31 (C).

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Thawing recovery rate

The viability of hESC LOT CA001 was determined by measuring the thawing recovery rate. Briefly, out of the >100 frozen vials of LOT CA001, ten vials were sampled, thawed, and seeded in ten separate dishes containing mEF and VitroHES™ medium. The number of hESC clusters that were seeded, attached, proliferated, and displayed appropriate morphology was determined for each dish. The results are presented in Figure 5 and show that all ten vials (100%) gave rise to viable hESC colonies. These cells were subsequently passaged according to standard procedures and used for the characterization presented in this document.

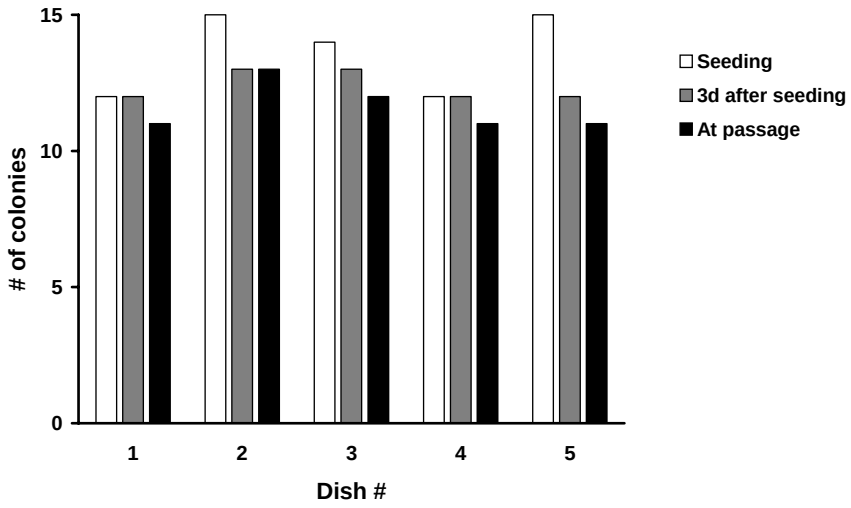

Figure 5. Thawing recovery rate of LOT CA001. Thawed hESC were seeded and the number of hESC clumps from each vial was determined (open bars) and subsequently the number of viable colonies was determined 2-3 days after seeding (grey bars) and at the time of passage (black bars).

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Immunohistochemical staining of undifferentiated hESC

Undifferentiated hESC colonies of LOT CA001 were fixed in PFA and subsequently permeabilized using Triton X-100. After consecutive washing and blocking steps, the cells were incubated with the primary antibody (as indicated in the figure legend). Conjugated secondary antibodies were subsequently used for detection. The nuclei were visualized by DAPI staining. The activity of alkaline phosphatase (ALP) was determined using a commercial available kit following the instructions indicated by the manufacturer (Sigma Diagnostics, Stockholm, Sweden). The passage number at which each analysis was performed is indicated within brackets in the figure legend. The results show that hESC of LOT CA001 are negative for SSEA-1 (B) and positive for SSEA-3 (C), SSEA-4 (D), TRA-1-60 (E), TRA-1-81 (F), Oct-4 (G), and ALP (H).

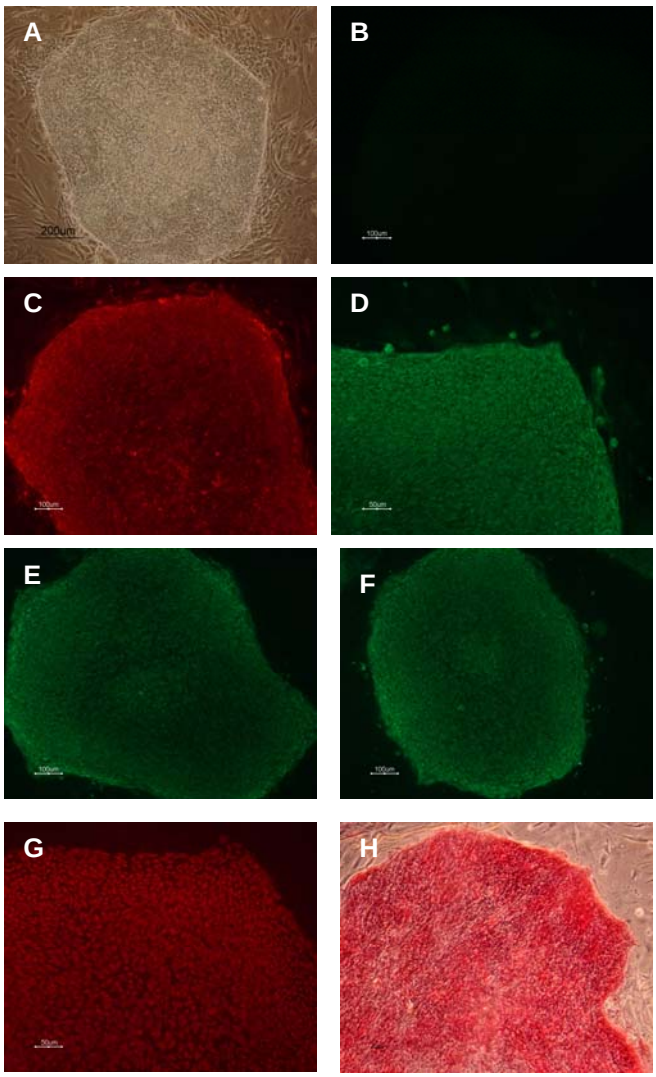

Figure 6 (right). (A) hESC colony [p15], (B) SSEA-1 [p19], (C) SSEA-3 [p19], (D) SSEA-4 [p19, (E) TRA-1-60 [p19], (F) TRA-1-81 [p19], (G) Oct-4 [p19], (H) ALP [p32]

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Karyotyping

The cells were incubated in the presence of Calyculin A and then washed with cell culture medium. The cells were collected by centrifugation and fixed using ethanol and glacial acetic acid. The chromosomes were visualized using a Trypsin-Giemsa staining (Figure 7) and no abnormalities were observed.

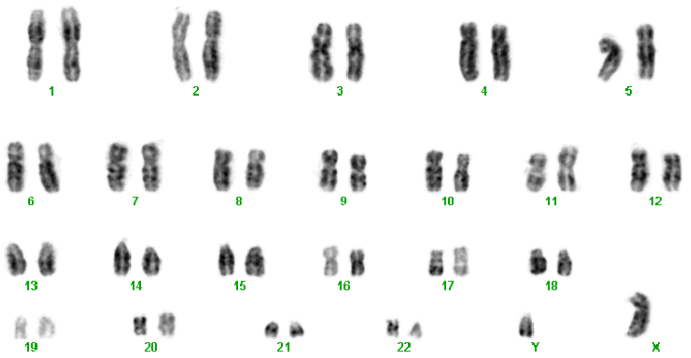

Figure 7. Karyotype of LOT CA001 in passage 21.

FISH

A commercially available kit containing probes for chromosome 13, 18, 21 and the sex chromosomes (X and Y) was used following the instructions from the manufacturer (Vysis. Inc, Downers Grove, IL, USA), with minor modifications. The slides were analyzed in an invert microscope equipped with appropriate filters and software (CytoVision, Applied Imaging, Santa Clara, CA, USA). The cells were XY and diploid for chromosome 13, 18, and 21.

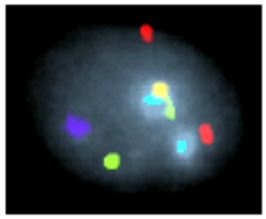

Figure 8. FISH analysis of hESC of LOT CA001 in passage 21, 13 (red), 18 (aqua), 21 (green) X (blue) and Y (gold).

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Multiplex Ligation-dependent Probe Amplification (MLPA)

To detect single or multiple deletions and amplifications in the subtelomeric regions, MLPA-technology was employed using the commercially available SALSA P019/P020 Telomers MLPA kit and following the instructions provided by the manufacturer (MRC-Holland, Amsterdam, The Netherlands). The probe mixes contain in total 72 probes. One probe for each of the 48 subtelomeric regions, as well as one probe directed to a sequence in the middle of each chromosome. The analysis was performed at Department of Paediatrics, Clinical Genetics, Sahlgrenska University Hospital/ÖS using hESC of LOT CA001 in passage 26 and 49. No deletions or amplifications were detected.

Telomerase activity

For analyzing the telomerase activity a *Te*lo TAGGG Telomerase PCR ELISA^{PLUS} kit (Roche, Basel, Switzerland) was employed according to the manufacturer's instructions. The assay uses the internal activity of telomerase, amplifying the product by PCR and detecting it with an enzyme linked immunosorbent assay (ELISA). hESC of LOT CA001 were analyzed in passage 24, 26 and 32 and displayed high telomerase activity.

Pluripotency in vitro

Undifferentiated hESC colonies were transferred to suspension cultures, using Stem Cell Cutting Tool (Swemed Lab, Göteborg, Sweden), to generate Embryoid bodies (EBs). Subsequently, these EBs were plated in tissue culture plates. Cells that spontaneously differentiated were subjected to immuno-histochemical evaluation. As illustrated in figure 9, positive staining was obtained using antibodies directed against β-III-tubulin (A), desmin (B), α-fetoprotein (C) and HNF-3β (D). Areas of spontaneously contracting cells, resembling cardiomyocytes, were also observed (not shown). Taken together, these results indicate that hESC of LOT CA001 are capable of differentiating *in vitro* to cells representing the three germ layers.

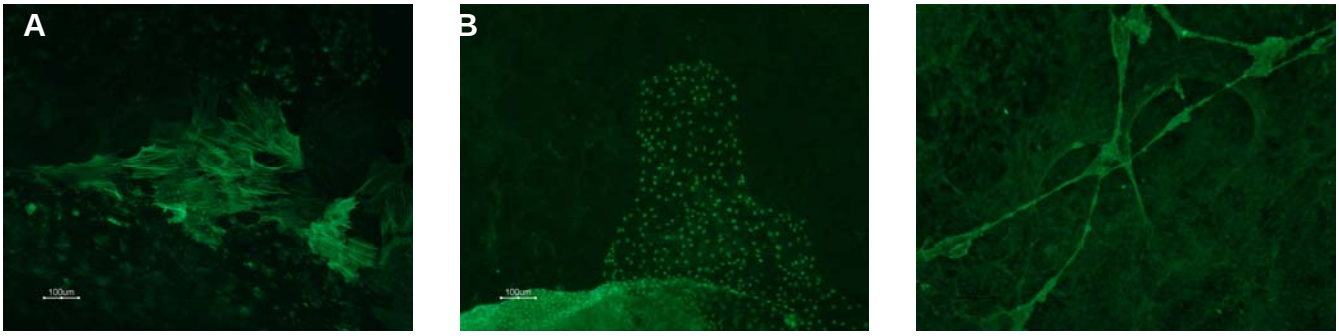

Figure 9. In vitro differentiation of hES cells, LOT CA001 in passage 19. (A) ASMA. (B) HNF-3β and(C) β-III-tubulin.

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Pluripotency in vivo

Undifferentiated hESC were surgically placed under the kidney capsule of severe combined immuno-deficient (SCID) mice. The mice were sacrificed after 8 weeks and tumors were dissected and fixed in PFA. Histological evaluation of hematoxylin-eosin stained paraffin sections (Figure 10) demonstrated the presence of tissues derived from endo- (A), meso- (B), and ectoderm (C).

All animal studies were reviewed and approved by the Institutional Animal Care and Use Committee in accordance with the policy regarding the use and care of laboratory animals. All research involving animals took place at the Laboratory for Experimental Biomedicine which is a specifically pathogen free, full barrier, animal facility at the University of Göteborg, Sweden. The University has a PHS Approved Animal Welfare Assurance number A5443-01.

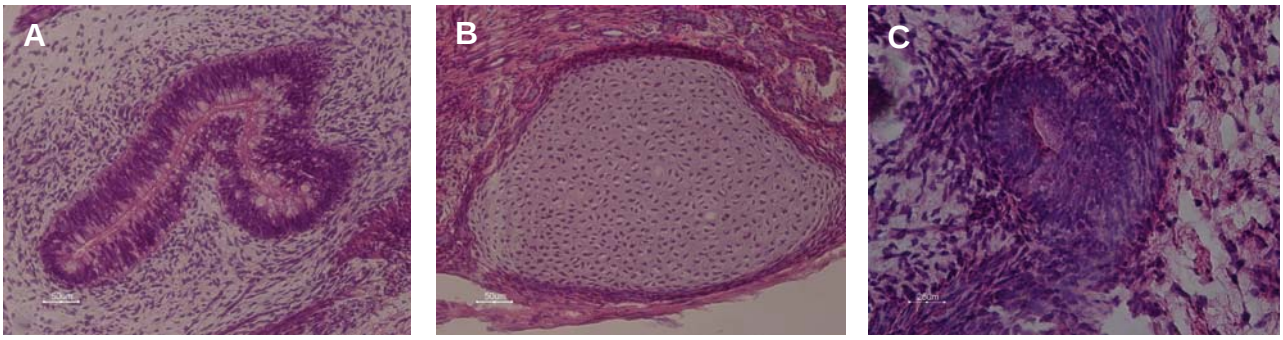


Figure 10. In vivo differentiation of hES cells, LOT CA001 in passage 22. (A) Endoderm (secretory epithelium). (B) Mesoderm (cartilage). (C) Ectoderm (neuroectoderm).

Mycoplasma

The presence of Mycoplasma in the hESC cultures of LOT CA001 was tested using PCR and Mycoplasma specific primers. The assay was performed at the DNA Laboratory at the Department of Clinical Bacteriology, Sahlgrenska University Hospital/SU, Göteborg. Sweden.
No Mycoplasma was detected.

Human viruses

hESC of LOT CA001 were tested for the presence of Human Immunodeficiency Virus type 1, Hepatitis B, Hepatitis C, Cytomegalovirus, Herpes Simplex Virus type 1 and 2, and Epstein-Barr Virus at the Department of Clinical Virology, Sahlgrenska Academy at the University of Göteborg, Sweden The presence of Human Papilloma Virus was analyzed at the Medical Microbiology Laboratory, University of Lund, Malmö, Sweden. Human Immunodeficiency Virus type 2 was analysed at SMI, Solna, Sweden
None of these viruses were detected.

Culturing of hES cells

General

The human embryonic stem (hES) cells are provided in straws containing approximately 10-12 pieces of hES cell colonies per straw. When culturing the hES-cells, we recommend the use of mouse embryonic fibroblast (mEF)-feeder cells seeded in centre-well organ culture dish. To reduce evaporation of the culture media in the centre-well of the culture dish, add 4 ml of medium supplemented with antibiotics to the outer well. The hES cells should be incubated at 37ºC in 5% CO_2.

Thawing of hES cells

Following instruction is designed for thawing cells in one straw; do not thaw more than one straw at a time.
NOTE! DO ALWAYS USE PROTECTIVE GLOVES AND FULL MASK WHEN THAWING.

Chemicals and material needed

Trehalose	P.No. T0167, Sigma-Aldrich
VitroPBS™	P.No. 10506, Vitrolife
hES culture medium	see Media preparation
Center-well organ culture dish	P.No. 353037 or 353653, BD Falcon
4-well dish	P.No: 176740, Nunc
Sterile filter, 0.22 µm	P.No: 166100-4433, VWR International
Stem Cell Cutting Tool	P.No: 190-210 S, Swemed by Vitrolife AB
Transfer Pipettes	P.No: H-190-210, Swemed by Vitrolife AB
Stainless steel Holder for Stem Cell Cutting Tool	P.No: H-9570, Swemed by Vitrolife AB
Plastic connector	Qosina, provided from Cellartis AB
Mitomycin C treated mEF-feeder cells	
Syringe (2 ml)	
Forceps (autoclaved)	
Pair of scissors (autoclaved)	
Cloth (autoclaved)	
Open container with liquid nitrogen (N_2)	
Container with water, 37ºC	
Stereo microscope	
Heated stage	

Method

1. Prepare 3 ml of a 0.2 M Trehalose solution in VitroPBS™ (Solution C).
2. Prepare 3 ml of a 0.1 M Trehalose solution in VitroPBS™ (Solution D).
3. Prior to sterile filtration, let 2 ml of Solution C and D respectively pass through the sterile filters and discard this volume.
4. Sterile filter Solution C and D. These solutions should be made immediately before use.
5. Pipette 0.5 ml of Solution C, 0.5 ml of Solution D and 0.5 ml hES medium into 3 separate wells in a 4-well dish.
6. Place the 4-well dish in 37˚C for 15 minutes.
7. Prepare a container with liquid N_2 and place it next to the permanent liquid tank or the transport vessel if thawed on delivery.
8. Put the Visiotube with the straw to be thawed in the container with liquid N_2.
9. Prepare a container with water, 37˚C.
10. Connect the plastic connector “Qosina” to the syringe and drawback, filling it with air. Place it on an autoclaved cloth or similar.
11. Place the 4-well dish on a heating stage under a microscope.
12. Uncap the Visiotube and use forceps to pull out the straw.

13. Hold the straw in the air in room temperature for 10 seconds.
14. Place the straw in the container with water, 37˚C for 2 seconds.
15. Wipe off the straw with an autoclaved cloth (soaked in 70% ethanol).
16. Hold the straw and use a pair of scissors to cut off the plugged seal next to the plug, in the column of air, (see Figure 2, Cut 1).
17. Connect the open end of the Plastic connector to the cut end of the straw.
18. Cut off the heat-sealed end, (see Figure 2, Cut 2). Syringe connected to straw, see Figure 3.
19. With some air in the syringe eject the cell colony pieces into Solution C. Use one well per straw.
20. Leave colonies in Solution C for 1 minute (on a heating stage).
21. Transfer the colonies to Solution D by using a transfer pipette or a Stem Cell Cutting Tool.
22. Place the 4-well dish in an incubator and leave the cell colony pieces in Solution D for 5 minutes.
23. Transfer the cell colony pieces as above to the hES medium, this is a washing step.
24. Transfer the cell colony pieces as above to plates coated with mEF cells and place in incubator.

Media Change of hES cells

Change medium every second or third day, starting on the second day after thawing or passaging. The total amount of VitroHES™ medium in the inner well should be 2.0 ml. Change 50% of the volume in the inner well organ culture dish each time. The medium in the outer well (4 ml) is changed once a week.

Chemicals and Material needed

VitroHES™ medium, (37˚C)	P.No: 10505, Vitrolife
bFGF, (4ng/ml)	P.No: 100-18B, Peprotech

Method

1. Do not change medium in more than 10 centre-well organ culture dishes at a time due to fall in temperature.
2. Remove 1 ml of medium from the inner well.
3. Add 1 ml of preheated medium to the inner well.
4. Place the centre-well organ culture dish in the incubator.
5. Discard left over preheated medium.

Culturing of hES cells

Passaging of hES cells

Passaging of hES-cell colonies should be done every 4th to 6th day. It is suitable to passage the colonies when at least 4 new pieces at the size of approximate 200 µm x 200 µm can be cut out from each undifferentiated colony. Only colonies with undifferentiated hES-cell morphology should be used. An undifferentiated hES appearance for a colony is that it has a homogenous structure. Avoid passage of the mEF feeder cells (see figure. 1).

Chemicals and Material needed

Mitomycin C treated mEF cells	
Stem Cell Cutting Tool	P.No: H-190-210 S, Swemed by Vitrolife AB
Holder for Stem Cell Knives	P.No: H-9570, Swemed by Vitrolife AB

Method

1. Place the dish with hES colonies under a stereomicroscope. Only cut one dish at a time, due to risk of temperature loss and pH change. The procedure of cutting one dish should typically take less than 10 minutes.
2. Focus the hES colonies one by one and cut a checked pattern (see Figure 1) as mentioned below.
3. Cut all colonies in one dish.
4. Use the knife to loosen all pieces one by one by carefully lifting a corner and then loosening them from the dish.
5. Use the Stem Cell Cutting Tool and holder for stem cell knives to transfer the pieces of hES cell colonies.
6. Place 10-16 pieces evenly in a new dish with mEF cells and place in incubator.

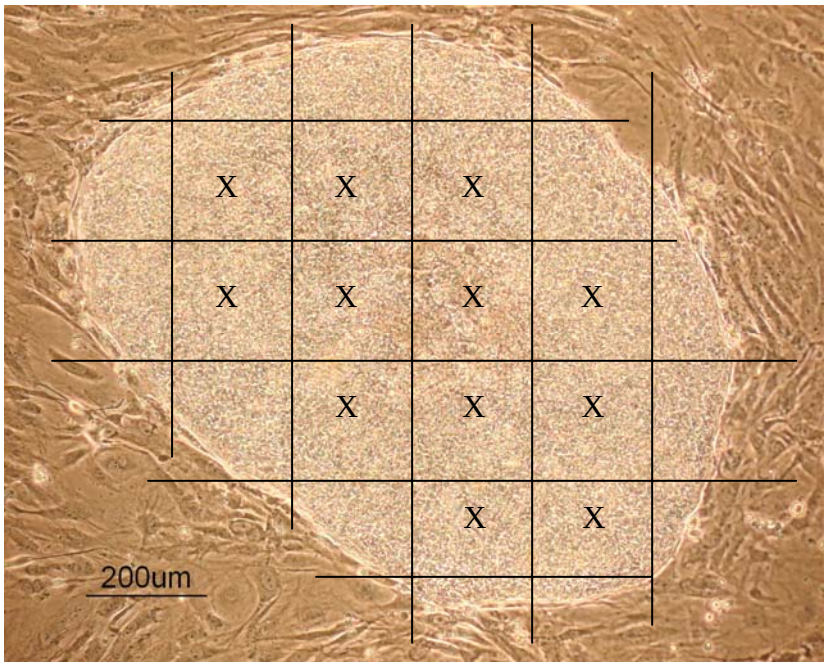


Figure 1. Preferable cutting pattern of a hES cell colony. It is recommended that only the colony pieces marked with “X” is transferred to a new culture dish, in order to avoid unwanted transfer of old mEF cells.

Culturing of hES cells

Freezing of hES cells

Following instruction is designed for freezing 3 straws.
Freeze 10-12 pieces (approximate 200µm x 200µm) of hES colonies in one straw.

Chemicals and material needed

VitroPBS™	P.No. 10506, Vitrolife
Trehalose	P.No: T0167, Sigma-Aldrich
Ethylene glycol	P.No: 102466,
DMSO	P.No: D2650, Sigma-Aldrich
Visiotube	P.No: 83000411, Air Liquide
Closed straws	P.No: 3589, Svensk Avel
Sterile filter, 0.22 µm, (DMSO safe)	P. No: 166100-4433, VWR International
4-well dish, Nunclon	P.No: 176740, Nunc
Stem Cell Cutting Tool	P.No: 190-210 S, Swemed by Vitrolife AB
Transfer Pipettes	P.No: H-190-210, Swemed by Vitrolife AB
Holder for Stem Cell Cutting Tool	P.No: H-9570, Swemed by Vitrolife AB,
Plastic connector	Qosina, provided from Cellartis AB
Syringe (2 ml)	
Forceps (autoclaved)	
Heat sealer	
Container with liquid N_2	
Cryo Pen	

Method

1. Prepare a container with liquid N_2.
2. Open the cap of the Visiotube and place in the container with liquid N_2 and submerge until the Visiotube is filled with liquid N_2.
3. Prepare 2 ml of 10% Ethylene glycol and 10% DMSO in VitroPBS™ (Solution A).
4. Prepare 2 ml of 0.3M Trehalose, 20% Ethylene glycol and 20% DMSO in VitroPBS™ (Solution B).
5. Prior to sterile filtration, let 2 ml of Solution A and B respectively pass through the sterile filters and discard this volume.
6. Sterile filter Solution A and Solution B. These solutions should be made immediately before use.
7. Pipette 0.5 ml of Solution A and 0.5 ml of Solution B in separate wells in a 4-well dish.
8. Place the 4-well dish in 37˚C for 15 minutes.
9. Cut colonies as described in Figure 1.
10. Pipette 2 drops (25 µl each) of Solution B to a sterile, hydrophobic surface for example the lid of a centre-well organ culture dish.
11. Pipette 1 ml of VitroPBS™ to the third well in the 4-well dish.
12. Connect the plastic connector to the syringe and the closed straw as described in figure 2.
13. Fill the straw with a 2-3 cm high column of VitroPBS™ by using a syringe.
14. Fill the straw with a 1-3 cm high column of air.
15. Fill the straw with a 0.5 cm high column of Solution B.
16. Transfer the cell colony pieces to Solution A.
17. Leave in Solution A for 1 minute.
18. Transfer the cell colony pieces to the first drop of Solution B and there after immediately to the second drop.
19. Immediately transfer the cell colony pieces from the second drop of Solution B into the straw, altogether Solution B with the colonies shall make a 2 cm high column.

The following steps (20-24) should be performed within 30 seconds.

20. Fill the straw with air until the VitroPBS™ makes the blue part of the plugged seal swell.
21. Use a forceps to flatten the straw 1-2 cm from the open end. Make a mechanical seal with the Heat sealer where the straw is flattened. Make two seals right next to each other (1-2 mm apart).
22. Loosen the syringe from the straw.
23. Label the straw on the "column of VitroPBS™", see Figure 2.
24. Place the straw in the Visiotube.
25. Cap the Visiotube and place it in a permanent liquid nitrogen tank for long term storage.

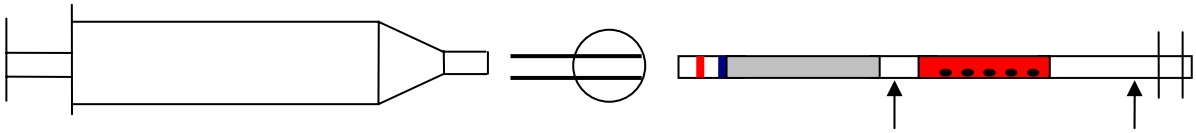
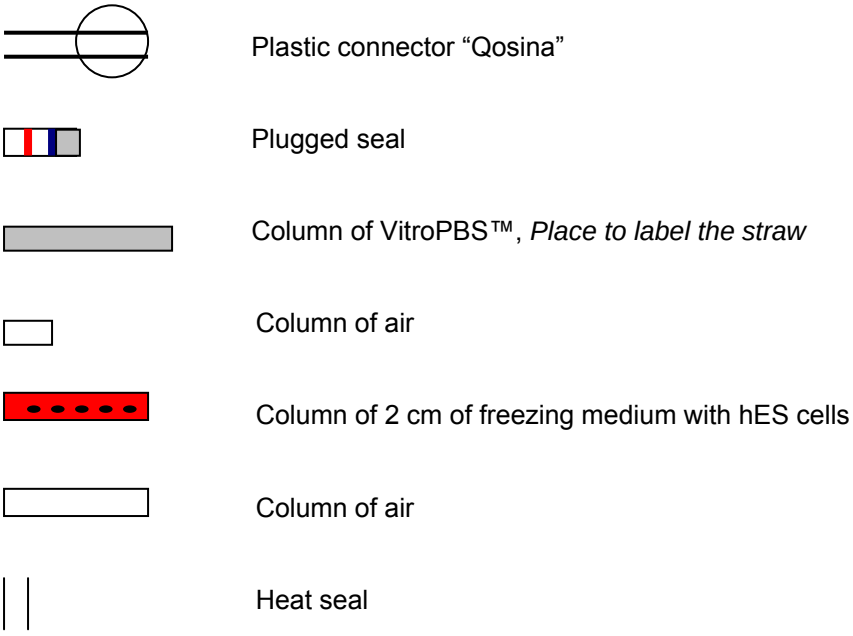


Figure 2. Syringe with closed straw.



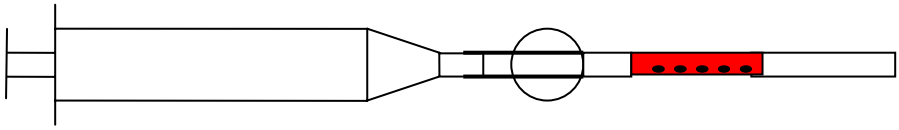


Figure 3. Syringe connected to straw.

Culturing of hES cells

mEF culture medium

Chemicals and Material needed

DMEM (Dulbecco's Modified Eagle Medium)	P.No. 61965-026, Invitrogen
FBS (Foetal Bovine Serum)	P.No. 10108-165 Gibco
PEST (Penicillin/Streptomycin)	P.No. 15140-122, Invitrogen

Method

Use mEF medium when thawing mEF cells and when preparing for seeding mEF cells in centre-well organ culture dishes. Prepare culture medium by adding 10% FBS and 1% PEST in DMEM.

hES medium

Chemicals and material needed

VitroHES™	P.No.10505, Vitrolife
bFGF	P.No. 100-18B, Peprotech

Method

Prepare the hES medium immediately before use, by adding bFGF (4ng/ml) to VitroHES™. Use the hES medium when seeding the Mitomycin C treated mEF cells, when thawing, changing medium and passaging of hES cells. Always use culture medium preheated to 37°C. Discard left over preheated medium.

Medium to the outer ring **(if using center-well organ culture dishes)**

Chemicals and material needed

Knock out DMEM	P.No. 10829-018, Gibco
PEST	P.No. 15140-122, Gibco

Method

Prepare medium to the outer ring by adding 1% PEST in Knock out DMEM.