User Guide

ELIXIRGEN SCIENTIFIC

Quick-Trilineage™ Differentiation - SeV Kit (Small)

Catalog Number: TL-SeV-S

Introduction

The Quick-Trilineage™ Differentiation - SeV Kit (Small) facilitates rapid and efficient differentiation of human iPS or ES cells into cells of three germ layers in just 6 days. Our proprietary transcription factor-based stem cell differentiation method uses the Sendai virus to produce cells of three germ layers without a genetic footprint. Quick-Trilineage™ Differentiation cell cultures display typical differentiated cell morphologies for neurons and skeletal muscle. Neural markers such as tubulin beta 3 class III (TUBB3), neural cell adhesion molecule 1 (NCAM1), neurogenic differentiation 1 (ND1); skeletal muscle marker such as myosin light chain 1 (MYL1); and hepatocyte markers such as alpha-fetoprotein (AFP) and albumin (ALB) can be observed in Quick-Trilineage™ Differentiation cell cultures. When handled and maintained according to the instructions in this user guide, cells of three germ layers are viable and suitable for a variety of characterization assays.

Scale: The Quick-Trilineage™ Differentiation - SeV Kit (Small) contains a set of reagents to be used for a total of 4 wells in a 24-well plate, 2 wells per cell line of interest.

Contents

Upon receipt, store the reagents at the temperatures indicated in the table below. All reagents are shipped on dry ice.

Contents	Volume	Storage	Thaw
QTL-SeV*	50 µl	-80°C	On ice
Mesendoderm RNA	4.5 µl	-20°C or -80°C	On ice
Component P	50 µl	-20°C or -80°C	Room temperature
Coating Material A	15.7 µl	-20°C or -80°C	On ice or 4°C

This kit contains iMatrix-511 silk (Nippi, Inc.)

***IMPORTANT!** This kit contains Sendai virus (SeV) particles that are active at 33°C and become inactive at 37°C. SeV is non-pathogenic in humans, and humans are not natural hosts of SeV; however, Biosafety Level 2 (BSL-2) containment is required for its use. Please use a biological safety cabinet, laminar flow hood, and proper personal protective equipment in order to prevent mucosal exposure. More information on BSL-2 guidelines can be found at www.cdc.gov/labs/BMBL.html.

Condition of Use

This product is for research use only. It is not approved for use in humans or for therapeutic or diagnostic use.

Technical Support

For technical support please refer to the FAQ on our website.
You may also contact us at cs@elixirgensci.com or call +1 (443) 869-5420 (M-F 9am-5pm EST).

Last revised: July 18, 2023

Required Consumables

Item	Vendor	Catalog Number
24-well tissue-culture-treated polystyrene plate (e.g., Corning Costar Flat Bottom Cell Culture Plates)	Fisher Scientific	07-200-740
DMEM/F12	ThermoFisher	21331020
KnockOut™ Serum Replacement	ThermoFisher	10828010
GlutaMAX	ThermoFisher	35050061
Penicillin-Streptomycin	ThermoFisher	15140122
TrypLE Select Enzyme (1X)	ThermoFisher	12563011
0.02% EDTA in DPBS	Sigma-Aldrich	E8008-100ML
Phosphate-buffered saline (without Ca^{++} Mg^{++})*	ThermoFisher	20012050
ROCK inhibitor Y27632	Selleckchem	S1049
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich	D2650
Opti-MEM™ I Reduced Serum Medium	Fisher Scientific	31985062
Lipofectamine™ MessengerMAX™ Transfection Reagent (LMM)	Fisher Scientific	LMRNA001

* PBS should be used at room temperature unless otherwise specified.

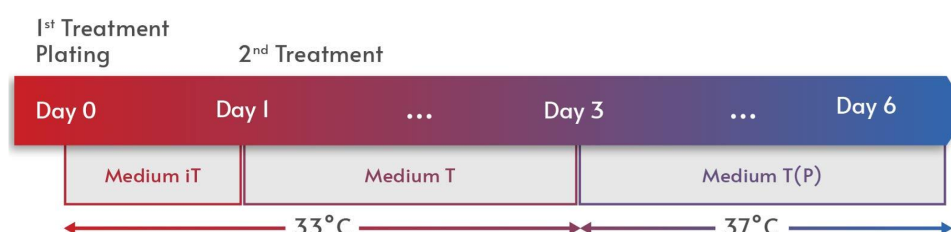
Source hPSC Culture Conditions

The Quick-Trilineage™ Differentiation - SeV Kit (Small) gives the best differentiation results when source human pluripotent stem cells (hPSCs) have been maintained in StemFit® Basic04, StemFit® AK02N, StemFlex™ Medium, or other similar culture media which enable the maintenance of cultures by single-cell passaging. This protocol also assumes that the source hPSCs are cultured in a 35-mm culture dish or one well of a 6-well plate. If iMatrix-511 silk is routinely used as a coating substrate, prepare one culture dish or well precoated with 0.25 μg/cm^2 iMatrix-511 silk diluted in 2 ml chilled PBS for this kit.

- The protocols and reagents for StemFit® Basic04 and iMatrix-511 silk culture conditions are available at Elixirgen Scientific (Catalog Numbers: ASB04-C, NI511S).
- Differentiation should not be performed until the cells are at least 14 days post-thaw.
- We recommend preparing a minimum of 3.3×10^5 viable hPSC for use with this kit. This is usually obtained by using 1 well of a 6-well plate at 50-70% confluency.
- For optimal differentiation, hPSC confluency should be around 50% to 70%. Do not use wells more than 90% confluent.

Workflow

IMPORTANT! This workflow requires a humidified 33°C, 5% CO_2 incubator. Before starting this protocol, please make sure the incubator temperature is stable at 33°C.
Note: This protocol assumes that Day 0 is a Tuesday



Preparation

10 mM ROCK inhibitor Y27632 (iROCK)

1. Dissolve 10 mg ROCK inhibitor Y27632 in 3.12 ml DMSO.
2. Make aliquots of a convenient volume (e.g., 100 µl).
3. This solution, hereafter referred to as iROCK, can be stored at -20°C.

KnockOut™ Serum Replacement

1. Thaw KnockOut™ Serum Replacement overnight at 4°C.
2. Mix the bottle by inverting it several times.
3. Take out ~1 ml and place in a conical tube. Keep it at 4°C to make Medium T with this user guide.
4. Aliquot the rest of KnockOut™ Serum Replacement into convenient volumes (e.g., 1 ml and 10 ml) and store them at -20°C for future use.

0.5X TrypLE Select with EDTA (Solution D1)

1. Mix 1 ml TrypLE Select Enzyme (1X) with 1 ml 0.02% EDTA in DPBS.
2. This mixture, hereafter referred to as Solution D1, can be stored at 4°C for 2 weeks.

Medium T

1. Prepare Medium T using the reagents listed in the table below.
    - Warm all reagents at room temperature for 20-30 minutes.
    - Keep Medium T, and any subsequent media made with it, protected from light.
    - Store Medium T for up to 2 weeks at 4°C.

Reagents	Volume
DMEM/F12	13 ml
Knockout Serum Replacement	700 µl
GlutaMAX	140 µl
Penicillin-Streptomycin (10000 units/ml; 100x)	140 µl

Day -1

Note: This protocol assumes that Day 0 is a Tuesday so Day -1 is Monday.

Plate Preparation

1. Prepare diluted Coating Material A by mixing together the following components in a 15 ml conical tube.
    - Thaw Coating Material A for 20-30 minutes at the temperature indicated in the “Contents” table on page 1.
    - Keep Coating Material A on ice.
    - Make sure chilled PBS is used for this mixture.

Reagents	Volume
Coating Material A	6.0 µl
Chilled PBS	1.8 ml

2. Add 400 µl diluted Coating Material A to each new well of 4 wells.
3. Incubate the plate at 4°C overnight.
   Note: For best results we recommend precoating the plate 1 day or up to a week before use and keeping at 4°C. Alternatively plates can be precoated on Day 0 and placed at 37°C for at least 2 hours before use.

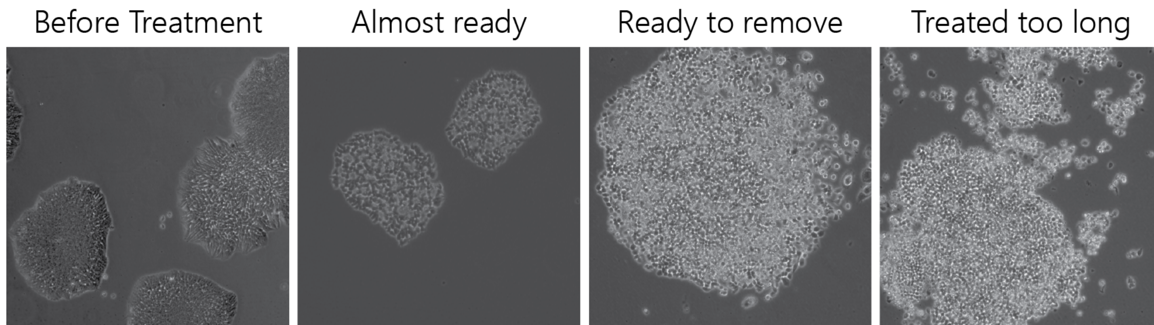
Day 0

First Treatment

1. Prepare Medium iT by mixing together the following components in a 15 ml conical tube.
   - Warm Medium T, iROCK, and Solution D1 at room temperature for at least 1 hour protected from light.
   - The rest of Medium T should be stored at 4°C for later use.

Reagents	Volume
Medium T	3 ml
iROCK	3 µl

2. Thaw QTL-SeV on ice.
3. Handling one cell line at a time, aspirate old medium from hPSC culture and add 2 ml PBS.
4. Rock the plate 3 times, aspirate PBS from the culture, and add 300 µl of the cell dissociation reagent Solution D1.
5. Incubate the culture plate at 37°C, 5% CO_2 for 10 minutes. If all the cells are not rounded under a microscope, continue to incubate at 37°C, 5% CO_2 in 1-2 minute increments (see images below).



6. Carefully pipet out Solution D1 from the culture and add 1 ml Medium iT to the well.

IMPORTANT! When Medium iT is added to Solution D1-treated cells, the cells begin to reattach to the bottom surface of the well. Since the dissociation of cells is harder to achieve the longer the cells are in Medium iT, begin resuspension immediately after adding Medium iT, and then promptly transfer into the 1.5 ml tube for counting.

7. Disperse the medium over the bottom surface of the well by pipetting 8-15 times to detach cells.
8. Using the same pipet tip, collect the cell suspension in a 1.5 ml tube.
9. Repeat Steps 3 to 8 for the other cell line.
10. Count cells and determine viability.

Plating

1. For each cell line, users will plate the hPSCs into 2 wells. Take out the volume of cell suspension needed to plate 1.4 or 1.6 x 10^5 cells per well and transfer each determined volume into a separate, labeled 1.5 ml conical tube.
   - Use total cell numbers (not only viable cells for this calculation).
   - If the volume of the cell suspension needed to get 1.4 or 1.6 x 10^5 cells exceeds 250 µl respectively, centrifuge the required volume of cell suspension at 200 x g for 4 minutes, remove the supernatant, and resuspend the pellet into 250 µl Medium iT.
   - If the required volume of the cell suspension needed to get 1.4 or 1.6 x 10^5 cells is less than 250 µl respectively, bring the volume up to 250 µl with additional Medium iT.

IMPORTANT! Before adding QTL-SeV, ensure that it is fully thawed. Do not centrifuge, vortex, or mix SeV with a

pipettor; SeV is highly sensitive to physical stress.

2. Aspirate diluted Coating Material A from one coated well at a time and add one cell suspension per well.
    - Most of the diluted Coating Material A should be aspirated but not completely to prevent the coated well from drying before adding the cell suspension. The cell suspension should be added to the well immediately after diluted Coating Material A is removed. Handle one well after another.
3. Using the table below, add the indicated volume of QTL-SeV into each of the wells using a P20 pipettor.
    - To add QTL-SeV, slowly push down the micropipette plunger to create a small drop at the end of the pipette tip. Repeat this process by making several drops to touch and distribute QLT-SeV in multiple locations, e.g., 12, 3, 6 and 9 o'clock of the medium surface until the tip becomes empty.

Cell Density per well*	QTL-SeV
1.4 x 10^5 cells	11 µl
1.6 x 10^5 cells	12 µl

4. Incubate the culture plate at 33°C, 5% CO_2 overnight.

Day 1

Medium Change

1. Warm Medium T and Opti-MEM at room temperature for 1 hour
2. Pipet out the medium from each well, leaving a small volume behind to avoid the cells drying out, and add 250 µl Medium T to each well.
3. Incubate the culture plate at 33°C, 5% CO_2 until it is time for transfection with Mesendoderm RNA.

Second Treatment

1. Thaw Mesendoderm RNA on ice for 20-30 minutes.
    - Tap the Mesendoderm RNA tube 3 times and then briefly spin it down before use.
2. Prepare Mesendoderm Booster by mixing the following reagents according to the volumes provided in the table below:
    - Prepare two 1.5 ml tubes with the required volume of Opti-MEM in each tube. Label the 1 tube "Mix 1" and the other tube "Mix 2".
    - Add 5 µl Lipofectamine MessengerMax (LMM) to the Mix 1 tube and mix by brief vortexing. Leave it at room temperature for 10 minutes (Mix 1). Keep the rest of LMM at 4°C for future experiments.
    - **IMPORTANT!** Immediately before 10 minutes pass (i.e., around 8 minutes), add the required volume of Mesendoderm RNA to the 1.5 ml tube with Opti-MEM (Mix 2). Mix by tapping 5 times. Do not vortex.
    - 10 minutes after mixing LMM with Opti-MEM, add Mix 2 into Mix 1, and pipet up and down 8-10 times. This mixture is called Mesendoderm Booster. Leave Mesendoderm Booster at room temperature for 5 minutes and no longer.

Mix 1 Reagents	Volume
Opti-MEM	125 µl
LMM	5 µl

Mix 2 Reagents	Volume
Opti-MEM	125 µl
Mesendoderm RNA	~4.5 µl

3. After the 5 minute incubation, take 103 µl of the Mesendoderm Booster mixture using a P200 pipettor and evenly distribute it dropwise into a set of 2 wells with Medium T.
4. Repeat Step 3 for the second set of wells.
5. Incubate the culture plate at 33°C, 5% CO_2 for 2.5 hours.

Medium Change

1. Pipet out the old medium from each well, leaving a small volume behind to avoid the cells drying out, and add 500 µl Medium T.
2. Incubate the culture plate at 33°C, 5% CO_2 overnight.

Day 2

Medium Change

1. Warm Medium T at room temperature for 30 minutes.
2. Pipet out the medium from each well, leaving a small volume behind to avoid the cells drying out, and add 500 µl Medium T.
3. Incubate the culture plate at 33°C, 5% CO_2 overnight.

Day 3

Medium Change and Temperature Shift

Note: This should be performed in the late afternoon.

1. Prepare Medium T(P) using the volumes indicated in the table below.
    - Warm Medium T at room temperature for 30 minutes.
    - Thaw Component P for 20-30 minutes at the temperature indicated in the “Contents” table on page 1.
    - Tap the Component P tube 3 times and then briefly spin it down before use.

Reagents	Volume
Medium T	4.4 ml
Component P	2.2 µl

2. Pipet out the medium from each well, leaving a small volume behind to avoid the cells drying out, and add 1 ml Medium T(P) to each well.
3. Incubate the culture plate at 37°C, 5% CO_2 for 3 days.

Day 6

Assay

- Differentiated cells of three germ layers can be observed on Day 6.
- Differentiation into cells of three germ layers after using Quick-Trilineage™ Differentiation - SeV Kit can be confirmed by using RT-qPCR to determine the expression of neuronal markers such as TUBB3, NCAM1, Neuro D1; skeletal muscle markers such as MYL1; and hepatocyte markers such as AFP and ALB.

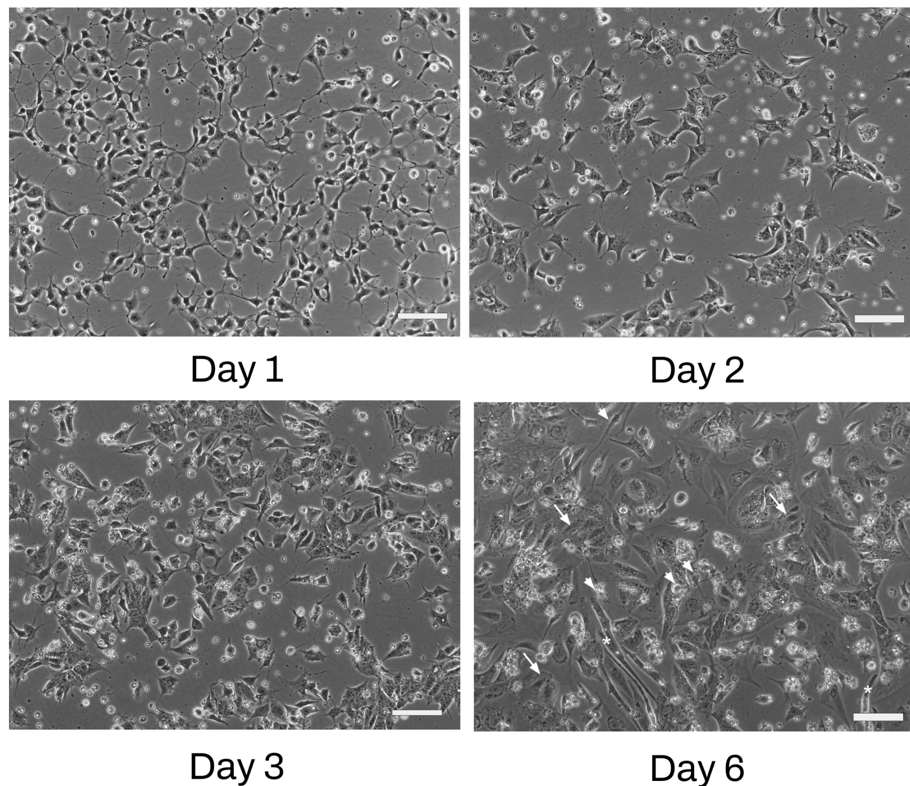


Figure 1. Representative images of Quick-Trilineage™ Differentiation - SeV cell culture plated with 1.6×10^5 cells on days 1, 2, 3, and 6 post-differentiation (scale bars = 100 µm). Typically day 6 cultures consist of groups of flat endodermal cells with dark nuclei (long arrows), spindle-shaped skeletal muscle cells (asterisks) and neurons with long processes (small arrows).

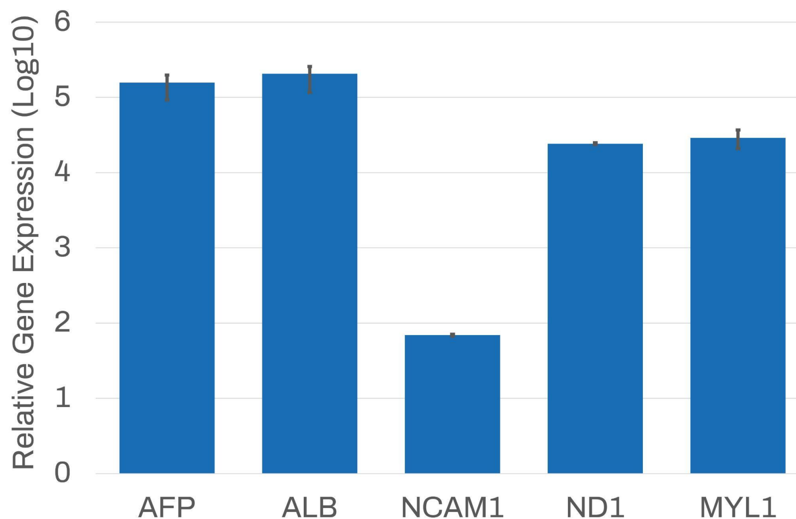
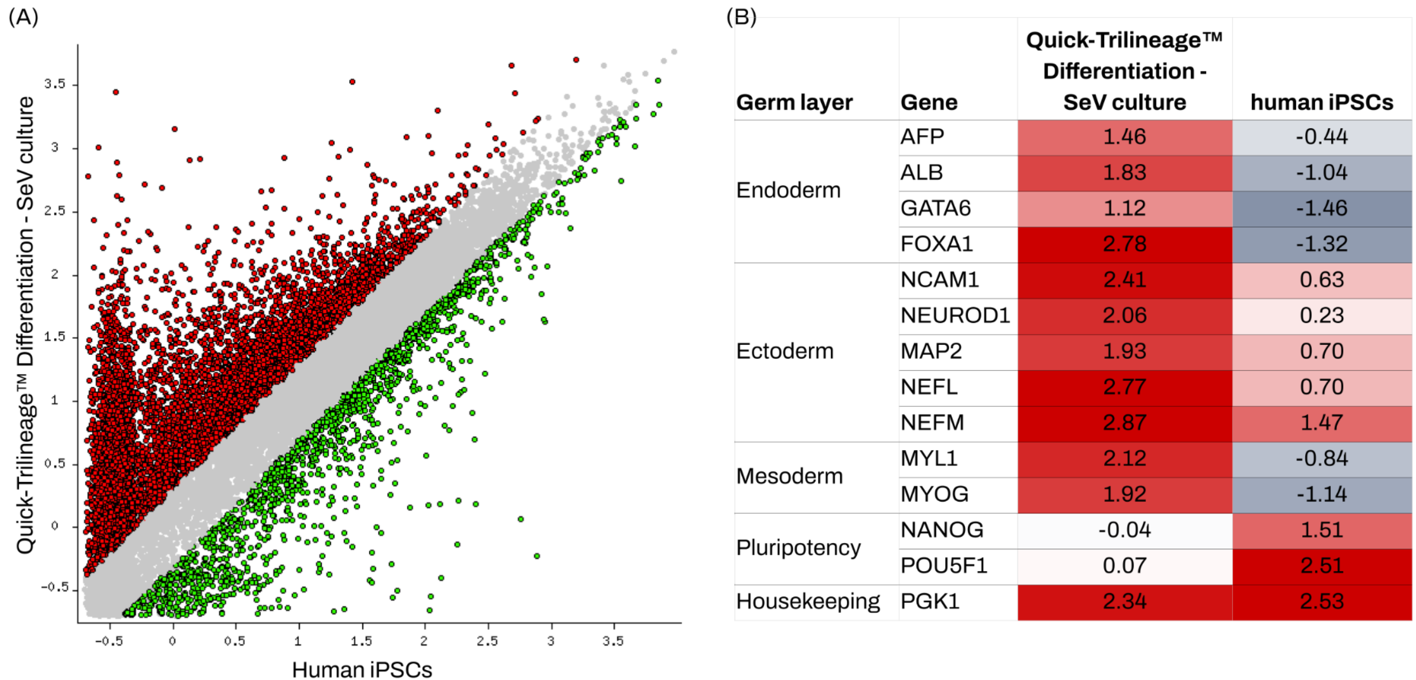


Figure 2. Real-time quantitative PCR analysis of expression levels of genes AFP, ALB, NCAM1, ND1, and MYL1 were examined. The graph shows gene expression in Quick-Trilineage™ Differentiation - SeV culture (1.6×10^5 cells plated) on day 6 post-differentiation.The relative gene expression is normalized to phosphoglycerate kinase 1 (PGK1), and then calculated as a fold induction relative to undifferentiated hPSCs as a control. Error bars show standard deviation. Primers used are listed in Table 1.

Table 1. List of PCR primers used in Figure 2

Gene	Forward primer	Reverse Primer	Primer Concentration
AFP	AAATGCGTTTCTCGTTGCTT	GCCACAGGCCAATAGTTTGT	250 nM
ALB	TGGCACAATGAAGTGGGTAA	CTGAGCAAAGGCAATCAACA	250 nM
NCAM1	CGACGCCGGCATTTACAAGTG	CTGACCACATCACACACAATCACG	250 nM
ND1	GAGACTATCACTGCTCAGGACC	TCATCTTCGTCCTCCTCCTCTCC	250 nM
MYL1	TGATGTCCTTCGAGCTCTGGG	GTTCAGCACCCATGACTGTGC	250 nM
PGK1	GTATGCTGAGGCTGTCACTCG	CCTTCCAGGAGCTCCAAACTGG	250 nM



Germ layer	Gene	Quick-Trilineage™ Differentiation - SeV culture	human iPSCs
Endoderm	AFP	1.46	-0.44
	ALB	1.83	-1.04
	GATA6	1.12	-1.46
	FOXA1	2.78	-1.32
Ectoderm	NCAM1	2.41	0.63
	NEUROD1	2.06	0.23
	MAP2	1.93	0.70
	NEFL	2.77	0.70
	NEFM	2.87	1.47
Mesoderm	MYL1	2.12	-0.84
	MYOG	1.92	-1.14
Pluripotency	NANOG	-0.04	1.51
	POU5F1	0.07	2.51
Housekeeping	PGK1	2.34	2.53

Figure 3. (A) Gene expression profiles of human iPSCs and Quick-Trilineage™ Differentiation - SeV Culture on day 6 were compared and the results are shown as scatter plots. The horizontal axis indicates the expression levels of genes in human iPSCs (obtained from the CIRM hPSC Repository for Medical Research: CW50065), whereas the vertical axis indicates the expression levels of genes in day 6 Quick-Trilineage™ Differentiation - SeV Culture. The levels of gene expression are shown based on transcripts per million (TPM) in the log10 scale. Red and green dots represent upregulated and downregulated genes (FDR<0.05), respectively, relative to their levels in human iPSCs. (B) TPM values in the log10 scale of representative genes from three germ layers in human iPSCs and day 6 Quick-Trilineage™ Differentiation - SeV Culture.