User Guide

ELIXIRGEN SCIENTIFIC

Quick-Endothelium™ Vascular - mRNA Kit (Small)

Catalog Number: VE-mRNA-S

Introduction

The Quick-Endothelium™ Vascular - mRNA Kit (Small) facilitates rapid and efficient differentiation of human iPS or ES cells into vascular endothelial cells in just 7 days. Our proprietary transcription factor-based stem cell differentiation method uses synthetic mRNAs to produce highly pure populations of vascular endothelial cells without a genetic footprint. Quick-Endothelium™ Vascular differentiated cell cultures display typical endothelial morphology and capillary tube formation and express endothelial cell markers, such as cluster of differentiation 31 (CD31) and von Willebrand factor (vWF) as well as the tight junction marker Claudin-5 (CLDN5). When handled and maintained according to the instructions in this user guide, vascular endothelial cells are viable long-term and are suitable for a variety of characterization and toxicity assays.

Scale: The Quick-Endothelium™ Vascular - mRNA Kit (Small) contains a set of reagents for use with a total 4 wells of a 24-well plate.

Related Products: Quick-Endothelium™ Vascular - Maintenance Medium, Catalog Number: VE-MM

Kit Contents

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

Reagents	Volume	Storage
QEV-mRNA	9 µl (4.5 µg) x 3	-80°C
Component J1	16.5 µl	-20°C or -80°C
Component J2	33 µl	-20°C or -80°C
Coating Material A	15.7 µl	-20°C or -80°C

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

Conditions 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 contact us at cs@elixirgensci.com or call +1 (443) 869-5420 (M-F 9am-5pm EST).

Last revised: February 24, 2021

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
Lipofectamine MessengerMAX	ThermoFisher	LMRNA001
Opti-MEM I Reduced Serum Medium	ThermoFisher	31985062
Minimum Essential Media (MEM) α, no nucleosides	ThermoFisher	12561056
KnockOut Serum Replacement (KSR)	ThermoFisher	10828010
Sodium Pyruvate (100 mM)	ThermoFisher	11360070
MEM Non-Essential Amino Acids Solution (100X)	ThermoFisher	11140050
Glutamax (100x)	ThermoFisher	35050061
Penicillin-Streptomycin	ThermoFisher	15140122
β-mercaptoethanol (β-ME)	ThermoFisher	21985023
StemFit Basic04 Complete Type, or StemFlex Medium	Elixirgen Scientific ThermoFisher	ASB04-C, or A3349401
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
Puromycin	InvivoGen	ant-pr-1
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich	D8418

*Can be substituted with our Cell Dissociation Reagent (Solution D1), Catalog Number: CDR.

Source hPSC Culture Conditions

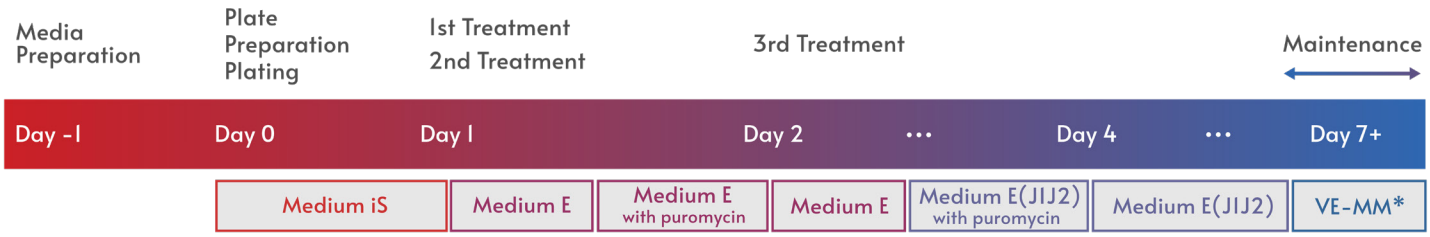
The Quick-Endothelium™ Vascular - mRNA Kit (Small) gives the best differentiation results when source human pluripotent stem cells (hPSCs) have been maintained in StemFit® Basic04, StemFlex™ Medium, or other similar culture media which enable the maintenance of cultures by single-cell passaging. This 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/cm2 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.

Drug Selection

Users should perform a puromycin kill curve for their cells to determine the minimum concentration required to kill all non-treated cells within ~60 hours. Based on Elixirgen Scientific's internal tests, the appropriate concentration ranges between 0.25 and 3 µg/ml. Successful results were obtained using 2 µg/ml puromycin for 16 hours after the second QEV treatment, and for 40-50 hours after the third QEV treatment.

Workflow



From Day 7, users may maintain differentiated cells in the maintenance medium best suited for their needs, though we recommend Quick-Endothelium™ Vascular - Maintenance Medium, Catalog Number: VE-MM.

Media Preparation

IMPORTANT! For the best possible delivery of QEV-mRNA into cells, we recommend Lipofectamine MessengerMax. If users prefer another transfection reagent, please make sure that the reagent provides a transfection efficiency of ≥80% prior to using this kit. QEV-mRNA mixed with Lipofectamine MessengerMax must be immediately applied to cultures and cannot be stored.

10 mM β-ME

1. Mix 80 µl 55 mM β-ME with 360 µl PBS.
2. Filter sterilize and store at 4°C.

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 is hereafter referred to as iROCK and can be stored at -20°C.

StemFit Basic04 Complete Type (Medium S)*

1. Thaw StemFit Basic04 Complete Type bottle overnight or multiple nights at 4°C.
2. Make aliquots of a convenient volume (e.g., 40 ml).
3. This solution is hereafter referred to as Medium S and can be stored at -80°C.

*Medium S can be substituted with StemFlex.

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.

*Can be substituted with our Cell Dissociation Reagent (Solution D1), Catalog Number: CDR.

Medium E

1. Prepare Medium E using the reagents listed in the table below.
   - Thaw all reagents at room temperature for 20-30 minutes.
2. Store Medium E for up to 2 weeks at 4°C.The leftover reagents can be discarded or saved for other uses.

Medium E Reagents	Volume
Minimum Essential Media (MEM) α, no nucleosides	28.8 ml
KnockOut Serum Replacement	1.6 ml
Sodium Pyruvate (100 mM)	320 µl
MEM Non-Essential Amino Acids Solution (100X)	320 µl
200 mM Glutamax (100x)	320 µl
Penicillin-Streptomycin (10000 units/ml; 100x)	320 µl
10 mM β-ME	320 µl

Day 0

~8 hr

Plate Preparation

1. Prepare diluted Coating Material A by mixing together the following components in a 15 ml conical tube.
    - Thaw Coating Material A on ice for 20-30 minutes (or at 4°C overnight one day before Day 0).
    - Make sure chilled PBS is used for this mixture.

Diluted Coating Material A Reagents	Volume
Coating Material A	11.9 µl
Chilled PBS	3.6 ml

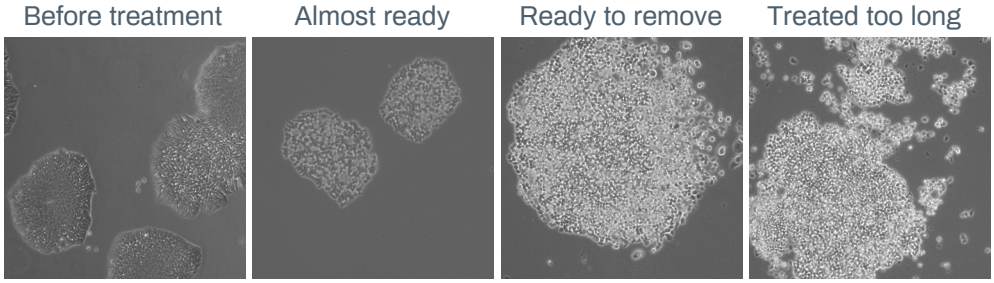
2. Add 400 µl diluted Coating Material A to each new well of 8 wells.
3. Incubate the plate at 37°C, 5% CO_2 for 2 hours (or 4°C overnight one day before Day 0).
4. Aspirate the supernatant from each well and add 500 µl PBS.
5. Incubate the plate at 37°C, 5% CO_2 until the hPSCs are ready for plating.

Plating

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

Medium iS Reagents	Volume
Medium S	5.5 ml
iROCK	5.5 µl

2. Tap the Solution D1 tube 5 times with a finger and centrifuge at maximum speed for 1 minute.
3. 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. Keep the rest of Solution D1 at 4°C.
5. Incubate the plate at 37°C, 5% CO_2 for 5 minutes. If all the cells are not rounded under a microscope, incubate at 37°C, 5% CO_2 for up to 5 more minutes in 1-2 minute increments (see images below).



6. Carefully pipet out Solution D1 from the culture using a P1000 pipettor and add 1 ml Medium iS to the well.
7. Disperse the medium over the bottom surface of the well by pipetting 8-15 times to detach cells.
8. Using the same pipette tip, collect the cell suspension in a 1.5 ml tube.

IMPORTANT! In this protocol, users will plate the hPSCs into 8 wells with 500 µl Medium iS per well. However, if users know the best plating density already, prepare a cell suspension enough to plate 5 wells (4 wells plus 1 extra to account for the pipetting/plating error). We recommend aiming for 50-70% initial cell confluency on Day 1, with 20-50 cells per colony. Our data indicate that cell counts ranging from 1.0 - 2.4 x 10^5 viable cells per well are suitable. Cell count may vary based on cell health and the method used for cell counting.

If the confluency on Day 1 is

- above the target range, the differentiation efficiency will decrease.
- below the target range, more cell death will be observed.
- For first time users, we recommend plating the following numbers of cells into each of 8 wells: 1.0, 1.2, 1.4, 1.6, 1.8, 2.0, 2.2 and 2.4 x 10^5 cells. On Day 1, 4 wells showing 50-70% confluency should be selected for transfection.

9. Take out the volume of the cell suspension needed for each cell density, according to the note above, and place each in a new tube labeled with the corresponding density.
    - Bring the volume of the cell suspension in each tube up to 500 µl with Medium iS.
    - If the volume in the tube exceeds 500 µl, centrifuge the required volume of cell suspension at 200xg for 4 minutes, remove the supernatant, and resuspend the pellet in 500 µl Medium iS.
10. Aspirate PBS from each coated well and add 500 µl cell suspension to each well using a P1000 pipettor.
11. Place the plate in the incubator and rock it front to back and side to side for 15 seconds to make sure that the cells are evenly distributed.
12. Incubate the cultures at 37°C, 5% CO_2 overnight.

Day 1

~8 hr

IMPORTANT! Observe all wells under a microscope and select any 4 wells that show 50-70% confluency for transfections with QEV-mRNA. If none of the wells fall within the range of confluence, do not proceed.

First Treatment

1. Thaw 1 vial of QEV-mRNA on ice for 30 minutes and warm Opti-MEM and Medium E at room temperature for 20-30 minutes.
2. Aspirate the old medium from one well at a time and add 500 µl Medium E. Repeat this process for each of the selected 4 wells. Do not let cells dry out during the medium change.
3. Incubate the cultures at 37°C, 5% CO_2 for at least 10 minutes.
4. Prepare QEV by the following steps:
    - Prepare two 1.5 ml tubes with 220 µl Opti-MEM each.
    - Add 4.5 µl Lipofectamine MessengerMax (LMM) to one of the 1.5 ml tubes and mix by brief vortexing. Leave it at room temperature for 10 minutes (Mix 1). Keep the rest of LMM at 4°C for later treatments.
    - **IMPORTANT!** Immediately before 10 minutes pass (i.e., around 8 minutes), add 9 µl QEV-mRNA to the other 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 QEV. Leave QEV at room temperature for 5 minutes and no longer.

Mix 1 Reagents	Volume
Opti-MEM	220 µl
LMM	4.5 µl

Mix 2 Reagents	Volume
Opti-MEM	220 µl
QEV-mRNA	9 µl

5. Add 104 µl QEV to each culture by adding QEV dropwise with one hand while gently shaking the plate with the other hand.
6. Rock the plate front to back and side to side for 15 seconds to make sure that QEV is evenly distributed in the cultures.
7. Incubate the cultures at 37°C, 5% CO_2 for 2.5-3 hours.

Second Treatment

1. Pipet out the medium from each well using a P1000 pipettor and add 500 µl Medium E.
2. Incubate the cultures at 37°C, 5% CO_2 for 2 hours.
    - Put 1 vial of QEV-mRNA on ice when the incubation above reaches 1.5 hours and leave it for 30 minutes.
3. Repeat Steps 4-7 of the previous “First Treatment” section.

Drug Selection

1. Take 2.2 ml Medium E into a tube and add puromycin to it at the user’s optimal concentration.
2. Pipet out the medium from each well using a P1000 pipettor and add 500 µl Medium E with puromycin.
3. Incubate the cultures at 37°C, 5% CO_2 overnight.

Day 2

~6 hr

IMPORTANT! Observe the QEV-treated cultures to make sure that they are reaching confluency (≥70%). If the cultures are <50% confluent and show signs of cell death (e.g., many floating cells), users should skip the third treatment on Day 2 and proceed directly to the steps described in Day 3. The protocol will then be accelerated by one day. First time users, who have plated cells at different densities, should proceed to the third treatment for only two wells with higher plating densities. For the other two wells (with lower densities), skip the third treatment on Day 2 and proceed directly to the steps described in Day 3. The protocol will then be accelerated by one day for the lower density wells.

Third Treatment

1. Thaw 1 vial of QEV-mRNA on ice for 30 minutes and warm Opti-MEM and Medium E at room temperature for 20-30 minutes.
2. Aspirate the old medium from one well at a time and add 500 µl Medium E to the well. Repeat this process for the rest of the treated wells. Do not let cells dry out during the medium change.
3. Pipet out old medium from the other 4 wells that have not been treated.
4. Incubate the cultures at 37°C, 5% CO_2 for at least 10 minutes.
5. Prepare QEV by the following steps:
    - Prepare two 1.5 ml tubes with 220 µl Opti-MEM each.
    - Add 4.5 µl LMM to one of the 1.5 ml tubes and mix by brief vortexing. Leave it at room temperature for 10 minutes (Mix 1). Keep the rest of LMM at 4°C.
    - **IMPORTANT!** Immediately before 10 minutes pass (i.e., around 8 minutes), add 9 µl QEV-mRNA to the other 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 QEV. Leave QEV at room temperature for 5 minutes and no longer.

Mix 1 Reagents	Volume
Opti-MEM	220 µl
LMM	4.5 µl

Mix 2 Reagents	Volume
Opti-MEM	220 µl
QEV-mRNA	9 µl

6. Add 104 µl QEV dropwise to each culture with one hand while gently shaking the plate with the other hand.
7. Rock the plate front to back and side to side for 15 seconds to make sure that QEV is evenly distributed in the cultures.
8. Incubate the cultures at 37°C, 5% CO_2 for 2.5-3 hours.

Drug Selection

1. Prepare Medium E(J1J2) by mixing the reagents listed in the table below in a 50 ml conical tube.
    - Thaw Component J1 on ice for 20-30 minutes. Thaw Medium E and Component J2 at room temperature for 10 minutes.
    - Store Medium E(J1J2) for up to 2 weeks at 4°C.The leftover reagents can be discarded or saved for other uses.

Medium E(J1J2) Reagents	Volume
Medium E	16 ml
Component J1	8 µl
Component J2	16 µl

3. Take 4.4 ml Medium E(J1J2) into a tube and add puromycin at the user's optimal concentration. If more than 90% of the cells show resistance to puromycin at the concentration used on Day 1, consider increasing its concentration 1.5-fold. Store Medium E(J1J2) at 4°C for later use.
4. Pipet out the medium from each well using a P1000 pipettor and add 500 µl Medium E(J1J2) with puromycin.
5. Incubate the cultures at 37°C, 5% CO_2 overnight.

Day 3

~1 hr

1. Warm Medium E(J1J2) with puromycin at room temperature for 20-30 minutes.
2. Pipet out the supernatant from each well using a P1000 pipettor and add 500 µl Medium E(J1J2) with puromycin.
3. Incubate the cultures at 37°C, 5% CO_2 for 20-30 hours.

Day 4

~1 hr

1. Warm Medium E(J1J2) at room temperature for 20-30 minutes.
2. (Optional) If there are floating dead cells, pipet out the old medium from each well using a P1000 pipettor and gently add 500 µl PBS to it by adding PBS along the wall of the well.
3. Pipet out the supernatant from each well using a P1000 pipettor and add 2 ml Medium E(J1J2).
4. Incubate the cultures at 37°C, 5% CO_2 over the weekend.

Note: This protocol assumes a Monday start day such that Day 4 is Friday. Should users choose to start on another day or skip Day 2 treatment, feed with 500 µl fresh Medium E(J1J2) per well on Day 4 and incubate for up to 2 days. Feed with 2 ml fresh Medium E(J1J2) before the weekend.

Note: Cells should exhibit a typical cobblestone morphology beginning on Day 4. If cultures reach 80-90% confluency, fix cultures, let them form tubing shapes, or passage to a new plate (please refer to the user guide for Quick-Endothelium™ Vascular - Maintenance Medium, Catalog Number: VE-MM).

Day 7

Assay or Continuous Maturation

Differentiated vascular endothelial cells can be observed on Day 4. For more mature vascular endothelial cells, we recommend culturing cells until Day 7. From Day 7, users may maintain differentiated endothelial cells in the maintenance medium best suited for their needs, though we recommend Quick-Endothelium™ Vascular - Maintenance Medium, Catalog Number: VE-MM. Differentiation into vascular endothelial cells after using Quick-Endothelium™ Vascular - mRNA Kit can be confirmed with anti-CD31 (cluster of differentiation 31, a global marker for endothelial cells).

Appendix

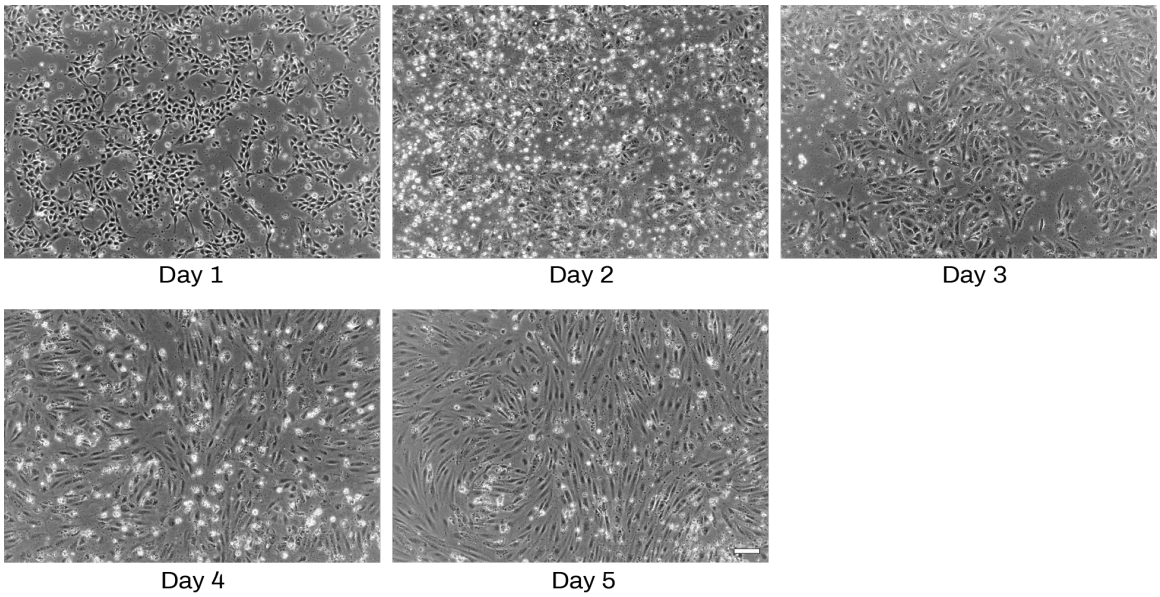


Figure 1. Representative phase contrast images of Quick-Endothelium™ Vascular - mRNA Kit cell cultures on days 1-5 post-differentiation (scale bar = 100 µm).

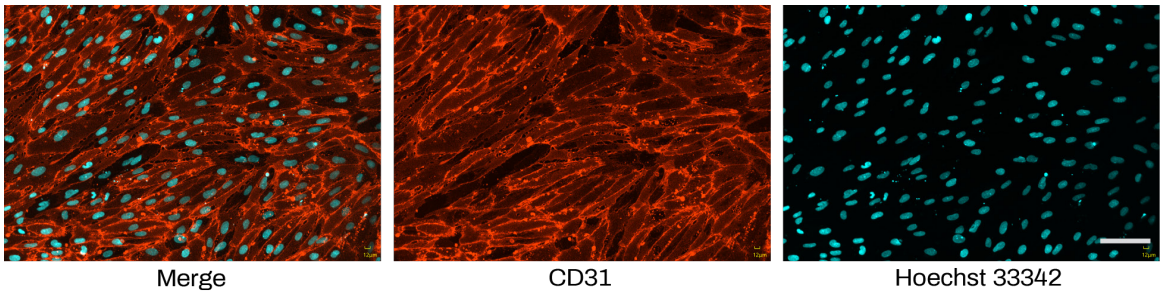


Figure 2. Immunofluorescent staining of Quick-Endothelium™ Vascular - mRNA Kit cell cultures shows typical vascular endothelium morphology and expression of CD31 on day 5 post-differentiation (scale bar = 100 µm). Staining conditions: Anti-CD31 primary antibody (Biolegend, Catalog Number: 303102, 1:50 dilution) in combination with a secondary antibody (Invitrogen, Goat anti-Mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 594, Catalog Number:A11032, 1:500 dilution) were applied. Nuclei were counterstained with Hoechst 33342.