User Guide

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Ricoh Biosciences, Inc.

Quick-Muscle™ Skeletal - SeV Kit

Catalog Number: SM-SeV

Introduction

The Quick-Muscle™ Skeletal - SeV Kit enables the rapid and efficient differentiation of human iPS or ES cells into skeletal muscle cells within a mere 7 days. Our proprietary transcription factor-based stem cell differentiation method employs temperature-sensitive Sendai virus, yielding a highly pure population of skeletal muscle cells devoid of a genetic footprint. Quick-Muscle™ Skeletal differentiated cell cultures exhibit characteristic skeletal muscle morphology and express relevant markers, such as myosin heavy chain (MHC).

Scale: The Quick-Muscle™ Skeletal - SeV Kit contains a set of reagents for use with a total of 6 wells of a 6-well plate.

Related Products: Quick-Muscle™ Skeletal - Maintenance Medium, Catalog Number: SM-MM

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
QMS-SeV	110 µL	-80°C	On ice
Mesendoderm RNA-P	36 µL	-80°C	On ice
Component P	50 µL	-20°C or -80°C	Room temperature

***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 exclusively intended for research purposes. It has not been approved for human application 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@biosciences.ricoh.com or call +1 (443) 869-5420 (M-F 9am-5pm EST).

Required Consumables

Item	Vendor	Catalog Number
6-well tissue-culture-treated polystyrene plate (e.g., Corning Costar Flat Bottom Cell Culture Plates)	Fisher Scientific	07-200-80
Minimum Essential Medium (MEM) α, no nucleosides	ThermoFisher	12561056
KnockOut Serum Replacement	ThermoFisher	10828010
Sodium Pyruvate (100 mM)	ThermoFisher	11360070
MEM Non-Essential Amino Acids Solution (100X)	ThermoFisher	11140050
β-mercaptoethanol	ThermoFisher	21985023
Glutamax (100x)	ThermoFisher	35050061
Penicillin-Streptomycin	ThermoFisher	15140122
iMatrix-511 silk	Ricoh Biosciences	NI511S
TrypLE Select Enzyme (1X)	ThermoFisher	12563011
0.02% EDTA in DPBS	Sigma-Aldrich	E8008-100ML
Opti-MEM, reduced serum	ThermoFisher	31985062
Lipofectamine MessengerMax	ThermoFisher	LMRNA015
Phosphate-buffered saline (without Ca^{++} Mg^{++})	ThermoFisher	20012050
ROCK inhibitor Y27632	Selleckchem	S1049
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich	D2650
Puromycin (10 mg/ml)	InvivoGen	ant-pr-1

Source hPSC Culture Conditions

The Quick-Muscle™ Skeletal- SeV Kit 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 protocol for StemFit® Basic04 culture conditions and iMatrix-511 silk are available at Ricoh Biosciences (Catalog Number: NI511S).
- Differentiation should not be performed until the cells are at least 14 days post-thaw.
- We recommend preparing a minimum of 4 x 10^6 viable hPSC for use with this kit. This is usually obtained by using 3 wells 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.

Drug Selection

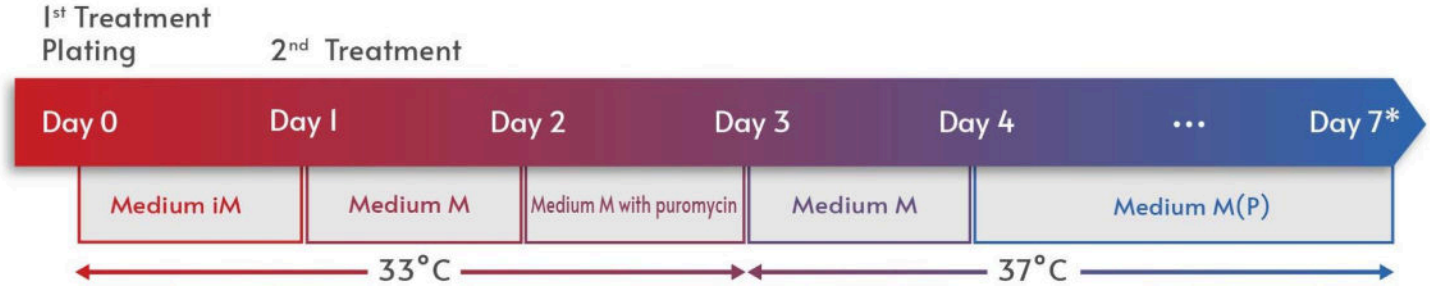
Users should perform a puromycin kill curve for their cells to determine the minimum concentration required to kill all non-treated cells within ~60 hours. Based on Ricoh Biosciences' internal tests, the appropriate concentration ranges between 0.5 and 2 μg/ml. We recommend maintaining 2 wells of untransfected iPSC (with standard StemFit conditions),

alongside the transfected wells, until after puromycin selection is performed. Treat 1 of those wells with puromycin at your selected concentration so as to confirm that the puromycin is effective at killing the untransfected cells in your experiment.

Workflow

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

Note: This protocol assumes that Day 0 is a Monday.



*From Day 7, users may maintain differentiated neurons in the medium best suited for their needs, though we recommend Quick-Muscle™ Skeletal - Maintenance Medium, Catalog Number: SM-MM.

Preparation

10 mM β-mercaptoethanol

1. Mix 280 µl 55 mM β-mercaptoethanol with 1.26 ml PBS.
2. Filter, sterilize, and store at 4°C.

10 mM ROCK inhibitor Y27632 (iROCK)

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

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 M

1. Prepare Medium M using the reagents listed in the table below.
    - Warm all reagents at room temperature for 20-30 minutes.

Reagents	Volume
Minimum Essential Medium (MEM) α, no nucleosides	90 mL
KnockOut Serum Replacement	5 mL
100 mM Sodium Pyruvate	1 mL
Non-essential amino acids (100x)	1 mL
Glutamax (100x)	1 mL
Penicillin-Streptomycin (10000 units/ml; 100x)	1 mL
10 mM β-mercaptoethanol	1 mL

- Keep Medium M, and any subsequent media made with it, protected from light.
- Store Medium M for up to 2 weeks at 4°C.

Medium M(P)

1. Prepare Medium M(P) using the reagents listed in the table below.
   - Thaw Component P for 20-30 minutes at the temperature indicated in the "Contents" table on page 1.
   - Warm Medium M at room temperature for 30 minutes.
   - Tap the Component P tube 3 times and then briefly spin it down before use.
   - Store Medium M(P) for up to 2 weeks at 4°C.
   - The rest of Medium M should be stored at 4°C for later use.
   - Leftover Component P can be discarded or saved at 4°C.

Reagents	Volume
Medium M	15 mL
Component P	7.5 µL

Day -3

< 1 hour

Note: This protocol assumes that Day 0 is a Monday so Day -3 is Friday.

Plate Preparation

1. Prepare diluted iMatrix-511 silk by mixing together the following components in a 15 ml conical tube.
   - Keep iMatrix-511 silk on ice.
   - Make sure chilled PBS is used for this mixture.

Reagents	Volume
iMatrix-511 silk	43.2 µL
Chilled PBS	13 mL

2. Add 2 mL diluted iMatrix-511 silk to each new well of 6 wells.
3. Incubate the plate at 4°C.
   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

4-6 hours

Note: This protocol assumes that Day 0 is a Monday.

Treatment

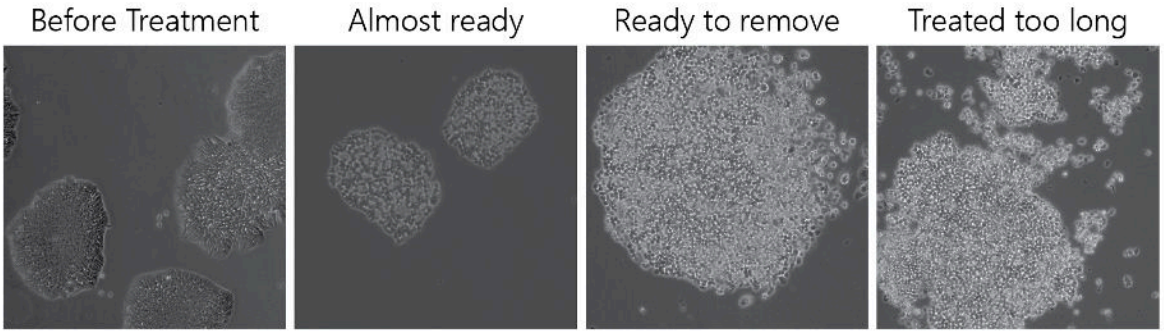
1. Determine the number of wells required to get 4 x 10^6 cells from the source hPSC 6-well plate.
2. Prepare Medium iM by mixing together the following components in a 15 ml conical tube.
   - Warm Medium M, iROCK, and Solution D1 at room temperature for at least 1 hour protected from light.
   - The rest of Medium N1 should be stored at 4°C for later use.

Reagents	Required medium volume based on # of wells of a 6-well plate		
	1 well	2 wells	3 wells
Medium M	8 mL	9 mL	10 mL
iROCK	8 µL	9 µL	10 µL

3. Referring to the table below, prepare the required volume of hPSC maintenance medium with iROCK in a new 15 ml conical tube. Mix well and allow to warm at room temperature for 20-30 minutes.

Reagents	Required volume per # of wells of a 6-well plate		
	1 well	2 wells	3 wells
hPSC maintenance medium	1.5 mL	3.0 mL	4.5 mL
iROCK	1.5 µL	3.0 µL	4.5 µL

4. Aspirate old medium from hPSC culture and add 1.5 mL of hPSC maintenance medium with iROCK to each well.
5. Incubate the culture at 37°C, 5% CO_2 for 1 hour before harvesting cells.
    - This is to decrease cell death on Day 1 and minimize the loss of cells.
    - During the incubation, start thawing QMS-SeV (undiluted) on ice and warming Solution D1 at room temperature.
6. Aspirate old medium from hPSC culture and add 2 mL PBS to each well being harvested.
7. Rock the plate 3 times, aspirate PBS from the culture, and add 300 µl of the cell dissociation reagent Solution D1 to each well.
8. Incubate the culture plate at 37°C, 5% CO_2 for 5 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).



9. Carefully pipet out Solution D1 from the culture and add 1 mL Medium iM to the well.
    - Follow steps 7-8 one well at a time if multiple wells are used.
10. Disperse the medium over the bottom surface of the well by pipetting 8-15 times to detach cells.
11. Using the same pipet tip, collect the cell suspension in a 15 ml tube.

Plating

1. Following this user guide, users will plate the hPSCs into 6 wells with 1 mL Medium iM per well. Please refer to the table below for our plating recommendations for each of the 6 wells. If the volume of the cell suspension needed to get the desired cell density is greater than 1 mL, centrifuge the excess volume of cell suspension at 200 x g for 4 minutes, remove the supernatant, and resuspend the pellet into 1 mL Medium iM per cell density.
2. Aspirate diluted iMatrix-511 silk from only one coated well at a time and add 1 mL cell suspension to the well.
    - Most of the diluted iMatrix-511 silk 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 iMatrix-511 silk is removed.
3. Repeat Step 2 for the rest of the cell suspensions.

4. Move the plate in 5 cycles of quick back-and-forth and side-to-side motions to evenly distribute treated cells in the cultures.

IMPORTANT! Before adding QMS-SeV, ensure that it is fully thawed. Do not centrifuge, vortex, or mix SeV with a pipettor; SeV is highly sensitive to physical stress.

5. Add the indicated volume of QMS-SeV into each well using a P200 pipettor.
    - By pushing the plunger, a small drop should be formed. As the drop is formed it can touch the medium surface. Repeat this process by making several drops to touch, e.g., 12, 3, 6 and 9 o'clock of the medium surface or until the tip becomes empty.

Cell density per well	QMS-SeV
4 x 10^5 cells	6.3 µL
5 x 10^5 cells	7.8 µL
6 x 10^5 cells	9.4 µL
7 x 10^5 cells	10.9 µL
8 x 10^5 cells	12.5 µL
9 x 10^5 cells	14.1 µL

6. Incubate the cultures at 33°C, 5% CO_2 overnight.

Day 1

~4 hours

IMPORTANT! The duration of incubation subsequent to the medium change is crucial for the second treatment. Should the confluency of any of the cultures range from 50-70%, the initial treatment may commence at any point, including immediately following the medium change, up to one hour thereafter. However, if any of the cultures exhibit less than 50% confluency, an incubation period of up to 4 hours is recommended. Conversely, if the cultures exceed 70% confluency, the first treatment ought to be performed within 1 hour of the medium change, although a reduced differentiation efficiency is anticipated.

Medium Change

1. Warm Medium M at room temperature for 20-30 minutes.
2. Pipet out the old medium from each well and add 1 mL Medium M to each well.
3. Incubate the cultures at 33°C, 5% CO_2 for 1-4 hours (see the note above).

Mesendoderm Booster treatment

1. 45 minutes before the above incubation is completed, thaw Mesendoderm RNA-P on ice for 30 minutes and warm Opti-MEM at room temperature for 20-30 minutes.
2. Prepare Mesendoderm Booster by the following steps:
    - Tap the tube of Mesendoderm RNA-P 3 times and then briefly spin it down before use.
    - Prepare a 15 mL tube and a 1.5 mL tube with 800 µL Opti-MEM each. Label the 15 ml tube "Mix 1" and the 1.5 ml tube "Mix 2".
    - Add 33 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 later treatments.
    - **IMPORTANT!** Immediately before 10 minutes pass (i.e., around 8 minutes), add 36 µL Mesendoderm RNA-P 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 Mesendoderm Booster. Leave Mesendoderm Booster at room temperature for 5 minutes and no longer.

Mix 1 Reagents	Volume
Opti-MEM	800 µL
LMM	33 µL

Mix 2 Reagents	Volume
Opti-MEM	800 µL
Mesendoderm RNA-P	36 µL

3. Add 6 ml Medium M to Mesendoderm Booster (for a final volume of 7.6 mL) and pipet up and down 2-3 times to mix.
4. Working with up to 2 wells at a time, aspirate the old medium out and add 1.2 mL of Mesendoderm Booster mixture to each well. Repeat until Mesendoderm Booster mixture has been added to all wells.
5. Incubate the culture plate at 33°C, 5% CO_2 for 2.5 hours.

Medium Change

1. Warm Medium M at room temperature for 20-30 minutes.
2. Pipet out the medium from each well and add 2 ml Medium M.
3. Incubate the culture plate at 33°C, 5% CO_2 overnight.

Day 2

< 1 hour

Medium Change and Drug Selection

1. Warm Medium M at room temperature for 20-30 minutes.
2. Transfer 13 mL Medium M into a tube and add puromycin to it at the predetermined optimal concentration (see earlier section on “Drug Selection”).
3. Pipet out most of the medium from each well and add 2 mL Medium M with puromycin.
4. Incubate the culture plate at 33°C, 5% CO_2 overnight.

Day 3

< 1 hour

Medium Change and Temperature Shift

Note: This should be performed in the late afternoon.

1. Warm Medium M at room temperature for 20-30 minutes.
2. Pipet out most of the medium from each well and add 2 mL Medium M.
3. Incubate the culture plate at 37°C, 5% CO_2 overnight.

Day 4

< 1 hour

Maintenance

1. Warm Medium M(P) at room temperature for 20-30 minutes.
2. Pipet out most of the medium from each well and add 2 mL Medium M(P).
3. Incubate the cultures at 37°C, 5% CO_2 for 3 days.

Day 7

Assay or Continuous Maturation

- Elongated, spindle-shaped muscle cells can be observed on Day 4. For more mature skeletal muscle, we recommend culturing cells until Day 7. From Day 7, users may maintain differentiated skeletal muscle in the maintenance medium best suited for their needs, though we recommend Quick-Muscle™ Skeletal - Maintenance Medium, Catalog Number: SM-MM.
- Differentiation into skeletal muscle can be confirmed with an anti-MHC (myosin heavy chain) antibody.

Appendix

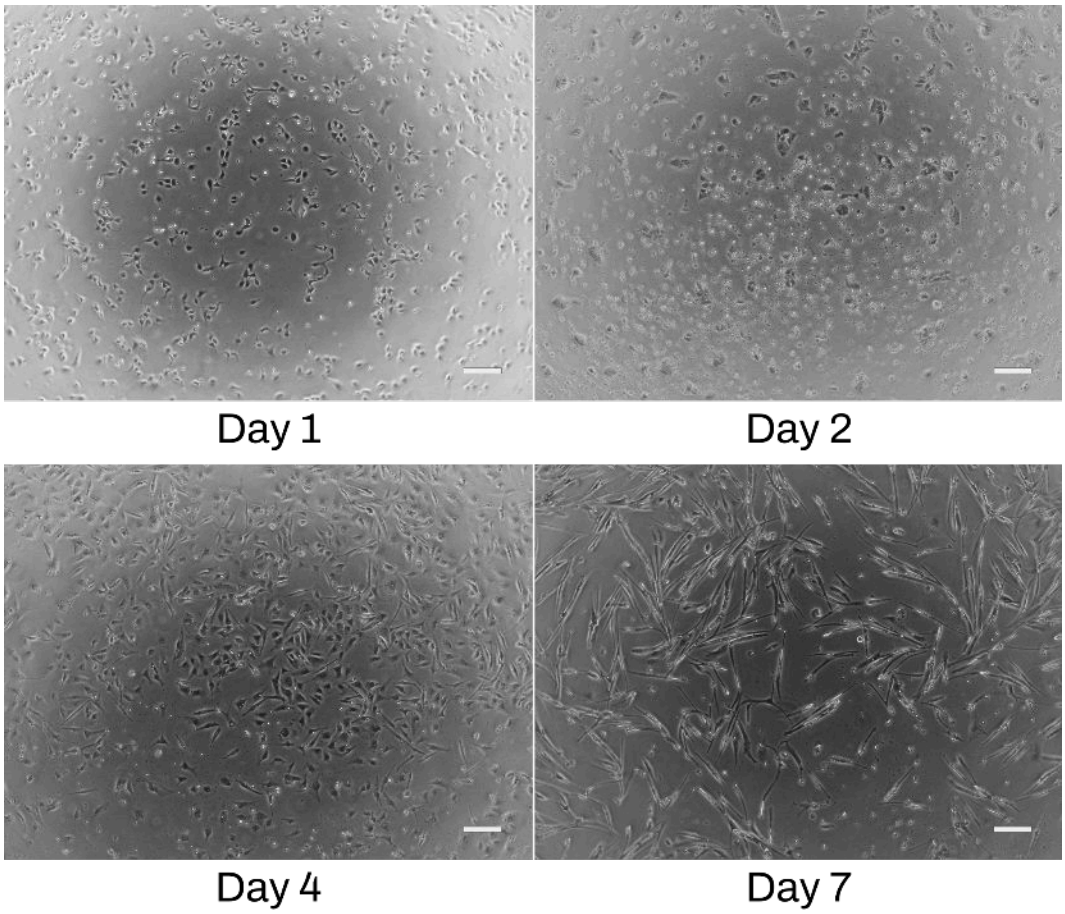


Figure 1. Representative images of Quick-Muscle™ Skeletal - SeV Kit cell culture (plated with 1.2 x 10^5 cells) on days 1, 2, 4, and 7 post-differentiation (scale bars = 100 µm).

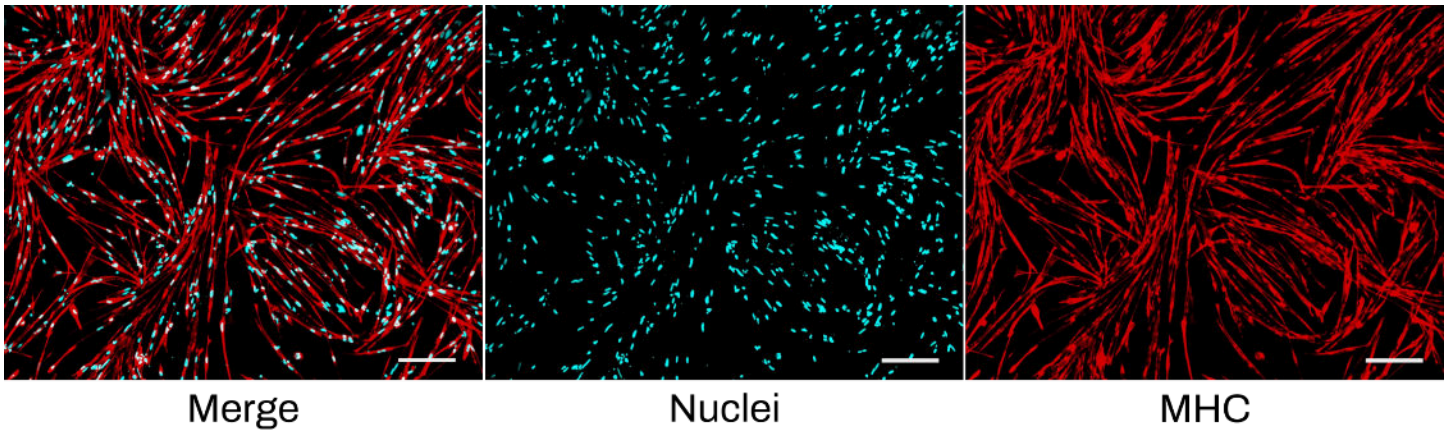


Figure 2. Immunofluorescent staining of Quick-Muscle™ Skeletal - SeV cell culture (plated with 1.2 x 10^5 cells) shows typical skeletal muscle morphology and expression of myosin heavy chain (MHC) on day 7. Nuclei were counterstained with Hoechst 33324. (scale bars = 200 µm)