

Contents

1. Description
 - 1.1 Background information
 - 1.2 Applications
 - 1.3 Reagent requirements
2. Protocol
 - 2.1 Protocol overview
 - 2.2 Preparation of complete medium
 - 2.3 Preparation of Matrigel®-coated plates
 - 2.4 Harvesting of human pluripotent and differentiated cells
 - 2.5 Detailed differentiation protocol

1. Description

This product is for research use only.

Components	36 mL StemMACS Medium I, human	72 mL StemMACS Medium II, human	60 mL StemMACS Medium, human	84 mL StemMACS Medium, human	Trilineage	MesoDiff	Trilineage	MesoDiff	Trilineage	EndoDiff	Trilineage	EctoDiff

Specifications	StemMACS Trilineage MesoDiff Medium I	StemMACS Trilineage MesoDiff Medium II	StemMACS Trilineage EndoDiff Medium	StemMACS Trilineage EctoDiff Medium
	Osmolality: 270–310 mOsmol/kg pH: 7.1–7.5	Osmolality: 260–300 mOsmol/kg pH: 7.1–7.5	Osmolality: 300–340 mOsmol/kg pH: 7.1–7.5	Osmolality: 270–310 mOsmol/kg pH: 7.1–7.5

Quality control Functionality assay: Differentiation of human pluripotent stem cells into mesoderm, endoderm, and ectoderm.

Capacity For 12 assays.

Storage Upon arrival store the StemMACS Trilineage Media protected from light at –20 °C. Aliquots of the media can be stored at –20 °C. Avoid repeated freeze-thaw-cycles. Once thawed, media bottles or aliquots should be kept at 2–8 °C and used within 2 weeks. The expiration date is indicated on the vial label.

1.1 Background information

Pluripotency is the ability to differentiate into the three embryonal germ layers, ectoderm, mesoderm, and endoderm, and is a defining characteristic of pluripotent stem cells (PSCs). Therefore, basic characterization of PSC lines includes typically a test for pluripotency in addition to surface marker expression and morphology. However, traditional pluripotency assays such as embryoid body and teratoma formation are both time-consuming and difficult to quantitate.

The StemMACS Trilineage Differentiation Kit, human provides a functional assay that can be completed in 7 days. In contrast to other pluripotency assays, the StemMACS Trilineage Differentiation Kit allows either analysis of cells by immunocytochemistry or quantitative analysis by flow cytometry. The kit enables parallel assessment of multiple PSC lines. The 12-well format is optimal for flow cytometric analysis. For immunocytochemistry, the 24-well format may be sufficient.

1.2 Applications

- Assessment of differentiation potential of human pluripotent stem cells.

1.3 Reagent requirements

- Dulbecco's phosphate-buffered saline (D-PBS) with Ca²⁺ and Mg²⁺
- Dulbecco's phosphate-buffered saline (D-PBS) without Ca²⁺ and Mg²⁺
- 0.05% Trypsin/EDTA
- Soybean Trypsin Inhibitor (0.5 mg/mL)
- Corning® Matrigel® hESC-Qualified Matrix
- DMEM/F12 with L-Glutamin, without HEPES
- ROCK inhibitor, e. g. StemMACS Thiazovivin (# 130-104-461) or StemMACS Y27632 (# 130-103-922)
- PSC cultivation medium, e.g. StemMACS iPS-Brew XF (# 130-104-368)

2. Protocol

2.1 Protocol overview

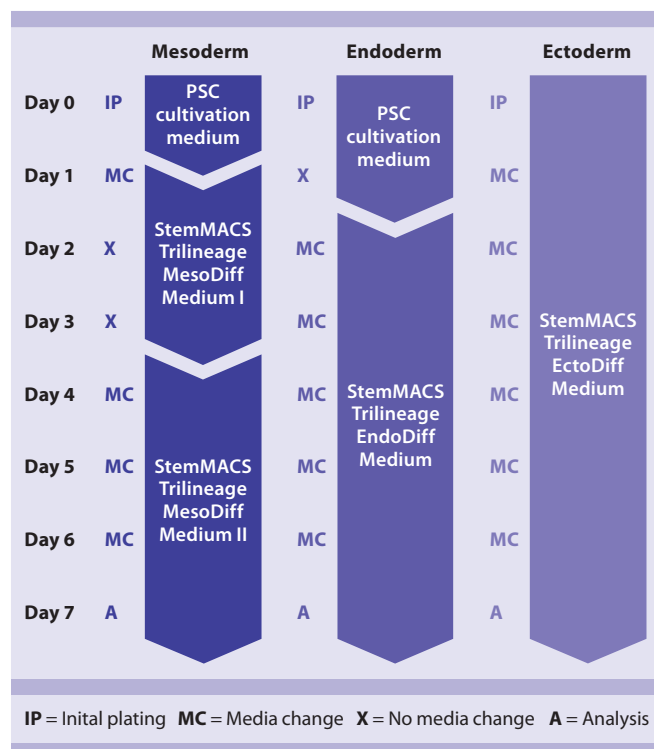


Figure 1: Overview of cell differentiation over 7 days using different media.

The assay starts with dissociation of the PSC culture into single-cell suspensions and seeding of defined cell numbers into 12- or 24-well plates in the presence of a ROCK inhibitor.

For mesoderm, cells are initially cultured for 1 day in PSC cultivation medium before mesoderm is induced using StemMACS Trilineage MesoDiff Medium I. Media changes are not required on day 2 and 3. From day 4 to day 6 media changes are performed daily using StemMACS Trilineage MesoDiff Medium II. On day 7, the differentiated samples are analyzed by either flow cytometry or immunocytochemistry.

For endoderm, cells are initially cultured in PSC cultivation medium for 2 days. Afterwards, endoderm differentiation is induced by exchanging the culture media with StemMACS Trilineage EndoDiff Medium followed by daily media changes.

For ectoderm, cells are differentiated in StemMACS Trilineage EctoDiff Medium with daily media changes.

2.2 Preparation of media

Thaw all media at 2–8 °C overnight.

2.3 Preparation of Matrigel®-coated plates

Coat plates according to the manufacturer's recommendation using a 1:80 dilution.

2.4 Harvesting of human pluripotent and differentiated cells

▲ It is mandatory to use single-cell suspensions for this assay. Volumes below are given for PSC maintenance cultivation in a 6-well plate. If using other culture ware adjust the volumes accordingly.

1. Aspirate cell culture medium and wash each well with 3 mL of D-PBS without Ca^{2+} and Mg^{2+} .
2. Add 1 mL of 0.05% Trypsin/EDTA per well. Gently rock the plate to ensure even distribution of the enzyme solution.
3. Incubate for 5 minutes at 37 °C in the dark.
4. Stop enzymatic reaction by adding 2 mL of Soybean Trypsin Inhibitor (0.5 mg/mL) per well.
5. Using a 5 mL serological pipette, dissociate to a single-cell suspension by carefully pipetting up and down.
6. Determine cell number.

2.5 Detailed differentiation protocol

Day 0

1. Plate cells by transferring the desired cell numbers into three 15 mL conical tubes. See table 1 for recommendations.

▲ **Note:** If using other culture ware use 40,000 cells/cm² for mesoderm, 66,000 cells/cm² for endoderm and 53,000 cells/cm² for ectoderm. Cell numbers might have to be adjusted depending on the cell line used.

Lineage	12-well plate	24-well plate
Mesoderm	150,000	80,000
Endoderm	250,000	130,000
Ectoderm	200,000	100,000

Table 1: Recommended starting cell numbers for mesoderm, endoderm, and ectoderm differentiation.

2. Centrifuge for 5 minutes at 200×g.
3. Aspirate supernatant.
4. Supplement media with ROCK inhibitor (10 μM StemMACS Y27632 or 2 μM StemMACS Thiazovivin) and resuspend the cell pellets using the media and volumes indicated in table 2.

▲ **Note:** Use Rock inhibitor only for initial plating.

Lineage	12-well plate	24-well plate	Medium
Mesoderm	1 mL	0.5 mL	PSC cultivation medium
Endoderm	1 mL	0.5 mL	PSC cultivation medium
Ectoderm	1 mL	0.5 mL	StemMACS Trilineage EctoDiff Medium

Table 2: Media and volumes used for plating.

5. Transfer the cells into the Matrigel-coated cell culture plate.

Media changesMesoderm

Day 1

After 24 hours (day 1), replace the PSC cultivation medium with 3 mL StemMACS MesoDiff Medium I per well in a 12-well plate (or 1.5 mL per well in a 24-well plate).

Days 2–3

Do not change the differentiation medium on days 2 and 3.

Days 4–6

On days 4, 5, and 6 replace the medium daily with 2 mL StemMACS MesoDiff Medium II per well in a 12-well plate (or 1 mL per well in a 24-well plate).

Endoderm

Day 1

Do not change the PSC cultivation medium on day 1.

Days 2–6

Wash the cells daily using 2 mL D-PBS with Ca²⁺ and Mg²⁺ and add 1 mL StemMACS EndoDiff Medium per well in a 12-well plate (or 0.5 mL per well in a 24-well plate).

Ectoderm

Days 1–6

Change medium daily using 1 mL StemMACS Trilineage EctoDiff Medium per well in a 12-well plate (or 0.5 mL per well in a 24-well plate).

Analysis

On day 7, cells from all three differentiation pathways can be analyzed using either immunocytochemistry or flow cytometry.

For immunocytochemistry analysis fix cells, for example, with 4% paraformaldehyd (PFA) and stain according to manufacturer's recommendations. For marker recommendations see below.

For flow cytometry analysis harvest cells following the protocol in section 2.4 with half of the mentioned volumes by using a 1000 µL micropipette. A detailed protocol for flow cytometric staining and analyses is available at www.miltenyibiotec.com/130-115-660.

Marker recommendations

The following markers are suitable for analysis:

Mesodermal differentiation potential is described by the formation of endothelial cells (CD144⁺) and/or the presence of smooth muscle cells (CD140b⁺ or Smooth muscle 22 alpha⁺). The ratio of endothelial and/or smooth muscle cells may vary between cell lines.

Endodermal differentiation capacity is characterized by the presence of CD184 (CXCR4)⁺Sox17⁺ definitive endoderm cells. A further marker that can be used is FoxA2.

Ectodermal differentiation potential can be assessed by the presence of Pax6⁺Sox2⁺ neuroectoderm cells.

Refer to www.miltenyibiotec.com for all data sheets and protocols. Miltenyi Biotec provides technical support worldwide. Visit www.miltenyibiotec.com for local Miltenyi Biotec Technical Support contact information.

Legal notices**Limited product warranty**

Miltenyi Biotec B.V. & Co. KG and/or its affiliate(s) warrant this product to be free from material defects in workmanship and materials and to conform substantially with Miltenyi Biotec's published specifications for the product at the time of order, under normal use and conditions in accordance with its applicable documentation, for a period beginning on the date of delivery of the product by Miltenyi Biotec or its authorized distributor and ending on the expiration date of the product's applicable shelf life stated on the product label, packaging or documentation (as applicable) or, in the absence thereof, ONE (1) YEAR from date of delivery ("Product Warranty"). Miltenyi Biotec's Product Warranty is provided subject to the warranty terms as set forth in Miltenyi Biotec's General Terms and Conditions for the Sale of Products and Services available on Miltenyi Biotec's website at www.miltenyibiotec.com, as in effect at the time of order ("Product Warranty"). Additional terms may apply. BY USE OF THIS PRODUCT, THE CUSTOMER AGREES TO BE BOUND BY THESE TERMS.

THE CUSTOMER IS SOLELY RESPONSIBLE FOR DETERMINING IF A PRODUCT IS SUITABLE FOR CUSTOMER'S PARTICULAR PURPOSE AND APPLICATION METHODS.

Technical information

The technical information, data, protocols, and other statements provided by Miltenyi Biotec in this document are based on information, tests, or experience which Miltenyi Biotec believes to be reliable, but the accuracy or completeness of such information is not guaranteed. Such technical information and data are intended for persons with knowledge and technical skills sufficient to assess and apply their own informed judgment to the information. Miltenyi Biotec shall not be liable for any technical or editorial errors or omissions contained herein.

All information and specifications are subject to change without prior notice. Please contact Miltenyi Biotec Technical Support or visit www.miltenyibiotec.com for the most up-to-date information on Miltenyi Biotec products.

Licenses

This product and/or its use may be covered by one or more pending or issued patents and/or may have certain limitations. Certain uses may be excluded by separate terms and conditions. Please contact your local Miltenyi Biotec representative or visit Miltenyi Biotec's website at www.miltenyibiotec.com for more information.

The purchase of this product conveys to the customer the non-transferable right to use the purchased amount of the product in research conducted by the customer (whether the customer is an academic or for-profit entity). This product may not be further sold. Additional terms and conditions (including the terms of a Limited Use Label License) may apply.

CUSTOMER'S USE OF THIS PRODUCT MAY REQUIRE ADDITIONAL LICENSES DEPENDING ON THE SPECIFIC APPLICATION. THE CUSTOMER IS SOLELY RESPONSIBLE FOR DETERMINING FOR ITSELF WHETHER IT HAS ALL APPROPRIATE LICENSES IN PLACE. Miltenyi Biotec provides no warranty that customer's use of this product does not and will not infringe intellectual property rights owned by a third party. BY USE OF THIS PRODUCT, THE CUSTOMER AGREES TO BE BOUND BY THESE TERMS.

Trademarks

The Miltenyi Biotec logo and StemMACS are registered trademarks or trademarks of Miltenyi Biotec B.V. & Co. KG and/or its affiliates in various countries worldwide. All other trademarks mentioned in this publication are the property of their respective owners and are used for identification purposes only.

Copyright © 2023 Miltenyi Biotec and/or its affiliates. All rights reserved.