Adjusted RosetteSep™ SepMate™ protocol

For enrichment, staining and filtration of CTCs on a micro-well chip

Version 1.0

Version 1.0

VyCAP

Reagents

Buffers

All buffers must be filtered with a 0.2µm filter prior to use.

- PBS
- PBS + 2% FBS (store at 4°C)
- Saponin (Sigma Aldrich, 47036) 0.20% in PBS for staining and permeabilization (make fresh once a week)

RosetteSep™ CTC Enrichment Cocktail and related products

- Small-cell carcinoma samples, including lung cancer samples
  - RosetteSep™ CTC Enrichment Cocktail Containing Anti-CD36 (STEMCELL Technologies, #15127)
- Breast cancer samples
  - RosetteSep™ CTC Enrichment Cocktail Containing Anti-CD56 (STEMCELL Technologies, #15137)
- Density gradient medium
  - Ficoll-Paque PLUS (GE Healthcare Life Sciences, 17-1440-02); **or**
  - Lymphoprep™ (STEMCELL Technologies, #07801)
- SepMate™-50 tubes (STEMCELL Technologies)
  - **Australia, Canada, Europe, USA**
    - SepMate™-50 (IVD) (#85450)
  - **Other countries**
    - SepMate™-50 (RUO) (#86450)

Staining solution for the detection of CTC

For staining the enriched cells after RosetteSep™ enrichment, a staining solution with a volume of **50 µL** is needed.

Component	Volumes (µL)
Saponin 0.20% in PBS	32.5
Anti-Pan Cytokeratin C-11 PE (Cell Signalling Technology, #5075S, clone C-11)	3
Anti-Pan Cytokeratin AE1/AE3 eFluor 570 (eBioscience, 41-9003-82, clone AE1/AE3)	1.5
Anti-EpCAM FITC (Abcam, ab8666, clone VU-1D9)	4
Anti-CD45 APC (eBioscience, 17-0459-41, clone HI30)	7.5
Hoechst 33342 100x diluted (Life Technologies, H3570)	1.5

VyCAP

Adjusted RosetteSep™ enrichment protocol with SepMate™ tubes

The following protocol is based on the original RosetteSep™ and SepMate™ protocol by STEMCELL Technologies (appendix I and II), with adjustments as listed below. Appendix I is based on the CD36-cocktail and is identical to the CD56-cocktail procedure. Ensure that sample, PBS + 2% FBS, density gradient medium, and centrifuge are all at room temperature (15 – 25°C).

Sample enrichment with RosetteSep™ cocktail

Follow steps 1 and 2 from the RosetteSep™ enrichment procedure, see appendix I. Continue with the following step:

3. Dilute sample with 3x sample volume of PBS + 2% FBS. Mix gently by slowly tilting the tube horizontally and back up.
    a. *Note: For example, dilute 7.5mL of sample with 22.5mL of PBS + 2% FBS. Keep the total capacity of the SepMate™ tube in mind!*

SepMate™ tube separation

Follow steps 1, 2 and 3 from the SepMate™ tube separation procedure, see appendix II. Continue with the following steps:

4. *Extra note for this step: Proper balancing of the centrifuge is essential, disbalance leads to difficulties in forming a proper plasma:density gradient medium interface. Do not centrifuge the sample more than once.*

5. Carefully aspirate 22.5mL of the top of the plasma layer to reduce the sample volume.
    a. *Note: Aspirate until the meniscus of the plasma layer hits the 25mL mark of the SepMate™ 50 tube. Do not disturb the plasma:density gradient medium interface!*

6. Pour the layer rapidly, with one smooth tilt, into a 15mL conical tube. Do not tilt the SepMate™ tube twice! Do not hold tube inverted for longer than 2 seconds.

7. Wash enriched cells with PBS by topping the 50mL tube, mix by inverting it and centrifuge at 600 x g for 8 minutes at room temperature with brake on. Aspirate ⅔ volume after centrifugation. Repeat once:
    a. *<u>Second wash:</u> Add ⅔ volume of PBS and resuspend pellet by inverting the tube. Centrifuge and carefully aspirate until 100µl is left (without disturbing any visible pellet).*

8. Add 50µL of the previously noted staining solution to the 100µL of RosetteSep™ enriched sample. Mix by slowly pipetting up and down.

9. Incubate the sample at room temperature for 20 minutes.

10. Sample can now be filtered through a micro-well chip at 50mbar.
    a. *Note: To filter the sample, add 2ml of PBS onto the micro-well chip in the filter unit and filter until droplets pass through the chip. Pause the filtration by turning off the pressure and add the 150µl of sample to the PBS on top of the filter (microwell-chip). To ensure maximum recovery, it is strongly recommended to rinse the sample tube at least once with 500µl PBS to be filtered as well. Start the filtration again and filter until all fluids have passed the micro-well chip.*

	CellSave fixative	*Transfix fixative*
Recovery rate on chip (%)	*48*	*50*
Average quality single cell amplified (%)*		

**Quality determined by Ampli1 QC kit (#ref number) with xxng of input material.*

Appendix I: STEMCELL Technologies RosetteSep™ procedure

PROCEDURE

RosetteSep™

NEGATIVE SELECTION

STEMCELL™ TECHNOLOGIES

CTC ENRICHMENT COCKTAIL CONTAINING ANTI-CD36

CATALOG #15127 / 15167

DIRECTIONS FOR USE

Ensure that blood sample, phosphate-buffered saline with 2% fetal bovine serum (PBS + 2% FBS; Catalog #07905), density gradient medium (see Notes and Tips, reverse page), and centrifuge are all at room temperature (15 - 25°C).

1. Add RosetteSep™ CTC Enrichment Cocktail Containing Anti-CD36 at **50 µL/mL** of whole blood (e.g. for 2 mL of whole blood, add 100 µL of cocktail). Mix well.

   Note: If using samples other than fresh whole blood, please see Notes and Tips.

2. Incubate **20 minutes** at room temperature (15 - 25°C).
3. Dilute sample with an equal volume of PBS + 2% FBS and mix gently.
4. Layer the diluted sample on top of the density gradient medium

   OR

   Layer the density gradient medium underneath the diluted sample.

   Note: Be careful to minimize mixing of the density gradient medium and sample.

   See table below for volume recommendations. With 50 mL centrifuge tubes, we suggest using a minimum of 15 mL density gradient medium to make it easier to remove the enriched cell layer.

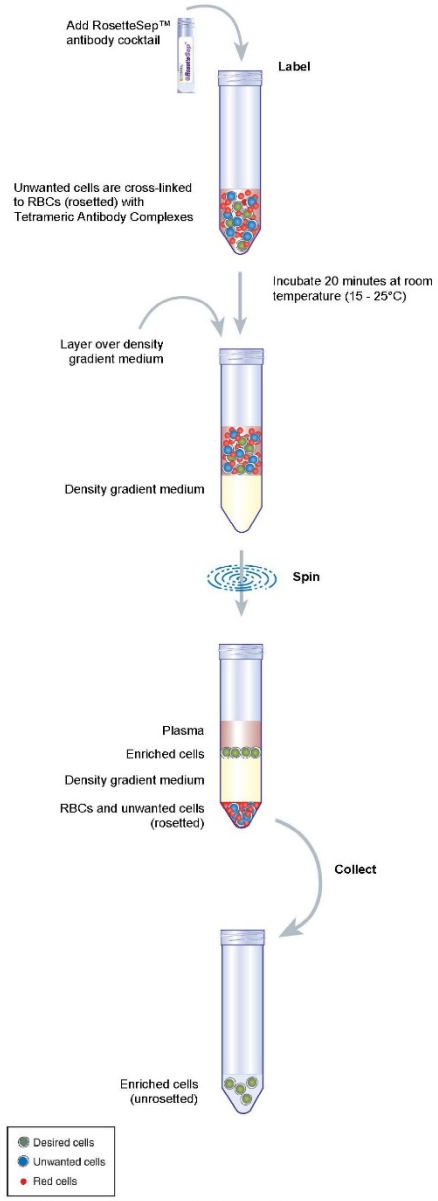
WHOLE BLOOD (mL)	PBS + 2% FBS (mL)	DENSITY GRADIENT MEDIUM (mL)	TUBE SIZE (mL)
1	1	1.5	5
2	2	3	14
3	3	3	14
4	4	4	14
5	5	15	50
10	10	15	50
15	15	15	50

5. Centrifuge for **20 minutes** at 1200 x *g* (see Notes and Tips) at room temperature (15 - 25°C), with the brake off.
6. Remove the enriched cells from the density gradient medium:plasma interface.

   Note: Sometimes it is difficult to see the cells at the interface, especially when very rare cells are enriched. It is advisable to remove some of the density gradient medium along with the enriched cells in order to ensure their complete recovery.

7. Wash enriched cells with PBS + 2% FBS. Repeat.
8. Use enriched cells as desired. We recommend that enriched samples are lysed with ammonium chloride to remove residual red blood cells (RBCs) prior to flow cytometric analysis (this can be done as one of the wash steps) or if residual RBCs will interfere with subsequent assays.

ROSETTESEP™ PROTOCOL DIAGRAM



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DOCUMENT #28583

Appendix I: STEMCELL Technologies RosetteSep™ procedure

CATALOG #15127	2 mL	For labeling 40 mL of whole blood
CATALOG #15167	10 mL	For labeling 200 mL of whole blood

RosetteSep™
PRODUCT INFORMATION SHEET

PRODUCT DESCRIPTION AND APPLICATIONS:

The RosetteSep™ CTC Enrichment Cocktail Containing Anti-CD36 is designed to enrich epithelial tumor cells from whole blood.

ROSETTESEP™ LABELING OF HUMAN CELLS

The RosetteSep™ antibody cocktail crosslinks unwanted cells in human whole blood to multiple RBCs, forming immunorosettes (Figure 1). This increases the density of the unwanted (rosetted) cells, such that they pellet along with the free RBCs when centrifuged over a density gradient medium. Desired cells are never labeled with antibody and are easily collected as a highly enriched population at the interface between the plasma and the density gradient medium.

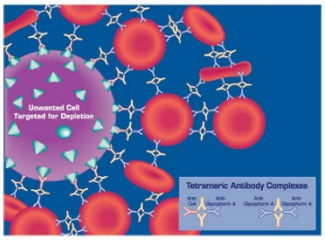


Figure 1 Rosette of unwanted cell and RBCs formed by RosetteSep™ Tetrameric Antibody Complexes (TACs)

NOTES AND TIPS

RECOMMENDED MEDIUM

The recommended medium is PBS + 2% FBS (Catalog #07905).

DENSITY GRADIENT MEDIUM

Density gradient medium refers to Lymphoprep™ (Catalog #07801), Ficoll-Paque™ PLUS, or other similar density gradient media.

CONVERSION of *g* to RPM

To convert *g* to rpm, use the following formula:

$$RPM = \sqrt{\frac{RCF}{(1.118 \times 10^{-5}) \times (Radius)}}$$

Where: RPM = centrifuge speed in revolutions per minute
RCF = relative centrifugal force (*g*)
Radius = radius of centrifuge rotor in centimeters (cm)

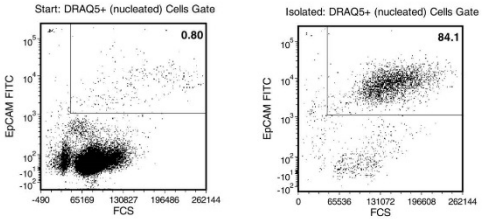
SAMPLES OTHER THAN WHOLE BLOOD

Although RosetteSep™ has been optimized for use with whole blood, cells can be enriched from other sources (i.e. buffy coat, leukapheresis). The concentration of nucleated cells in the sample should not exceed 5×10^7 cells/mL, and RBCs should be present at a ratio of at least 50 - 100 RBCs per nucleated cell.

ASSESSING PURITY

Purity of epithelial tumor cells can be measured by flow cytometry after staining with a fluorochrome-conjugated tumor-specific antibody.

TYPICAL ROSETTESEP™ CTC ENRICHMENT PROFILE:



In the example above, CAMA (epithelial tumor cell line) cells were seeded into whole blood at a starting frequency of 0.8%. The CAMA cell content of the enriched fraction is 84%. Typically 3.4 to 4.7 log depletion of targeted CD45+ cells is attained.

COMPONENT DESCRIPTION:

ROSETTESEP™ CTC ENRICHMENT COCKTAIL CONTAINING ANTI-CD36

CODE #15127C

This cocktail contains a combination of mouse and rat monoclonal antibodies. These antibodies are bound in bispecific Tetrameric Antibody Complexes (TACs) which are directed against cell surface antigens on human hematopoietic cells (CD2, CD16, CD19, CD36, CD38, CD45 and CD66b) and glycophorin A on RBCs. The mouse monoclonal antibody subclass is IgG_1. It should be kept in mind that this product is a biological reagent, and as such cannot be completely characterized or quantified. Some variability is unavoidable.

STABILITY AND STORAGE:

ROSETTESEP™ CTC ENRICHMENT COCKTAIL CONTAINING ANTI-CD36

Product stable at 2 - 8°C until expiry date as indicated on label. Do not freeze this product. Contents have been sterility tested. This product may be shipped at room temperature (15 - 25°C), and should be refrigerated upon receipt.

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Appendix II: STEMCELL Technologies SepMate™ procedure

SepMate™

SepMate™-50
Catalog #85450 100 tubes
Catalog #85460 500 tubes

SepMate™-15
Catalog #85415 100 tubes
Catalog #85420 500 tubes

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Intended Use

SepMate™ is used to isolate mononuclear cells (MNCs, comprising lymphocytes and monocytes) from whole blood or bone marrow by density centrifugation.

For in vitro diagnostic use.

Product Description

MNCs are commonly isolated by density centrifugation. With this method, defibrinated or anticoagulant-treated blood is carefully layered on a density gradient medium and centrifuged for a short period of time. Differential migration during centrifugation results in the formation of layers containing different cell types. The bottom layer contains erythrocytes which have been aggregated by the density gradient medium and therefore sediment completely through the density gradient medium. The layer immediately above the erythrocyte layer contains mostly granulocytes, which at the osmotic pressure of the density gradient medium solution attain a density great enough to migrate through the density gradient medium layer. Because of their lower density, the MNCs are found at the interface between the plasma and the density gradient medium with other slowly sedimenting particles (platelets). The MNCs are carefully recovered from the interface and washed.

The specialized insert in SepMate™ minimizes mixing of the sample and the density gradient medium, thereby avoiding the need for careful layering and careful cell removal from the interface. Density gradient medium is pipetted through a central hole in the insert, partially filling the tube. Whole blood is then rapidly pipetted down the side of the tube to rest upon the density gradient medium. After centrifugation for 10 minutes with the brake on, the enriched cell layer is simply poured off into a new tube, while the density gradient medium, erythrocytes, and granulocytes are retained below the insert. The MNCs are washed and are then ready for use.

Storage and Stability

Store at ambient temperature. Product stable at ambient temperature until expiry date on label. Do not use if tubes are damaged.

Sterility

SepMate™ tubes are sterilized using irradiation. Sterile if package is unopened or undamaged. Do not re-use or re-sterilize.

Special Materials Required But Not Provided

Density Gradient Medium

Density gradient medium refers to Lymphoprep™ (Catalog #07801) or any similar medium with a density of 1.077 g/mL designed for the separation of mononuclear cells.

Recommended Medium

Phosphate-buffered saline with 2% fetal bovine serum (PBS + 2% FBS; Catalog #07905).

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CE IVD

Appendix II: STEMCELL Technologies SepMate™ procedure

SepMate™ STEMCELL™ TECHNOLOGIES

Directions for Use

Ensure that sample, phosphate-buffered saline with 2% fetal bovine serum (PBS + 2% FBS), density gradient medium (see Special Materials Required But Not Provided), and centrifuge are all at room temperature (15 - 25°C).

1. Add density gradient medium to the SepMate™ tube by carefully pipetting it through the central hole of the SepMate™ insert. Refer to Table 1 for required volumes. The top of the density gradient medium will be above the insert.

   NOTE: Small bubbles may be present in the density gradient medium after pipetting. These bubbles will not affect performance.
2. Dilute sample with an equal volume of PBS + 2% FBS. Mix gently.

   For example, dilute 5 mL of sample with 5 mL of PBS + 2% FBS.
3. Keeping the SepMate™ tube vertical, add the diluted sample by pipetting it down the side of the tube. The sample will mix with the density gradient medium above the insert.

   NOTE: The sample can be poured down the side of the tube. Take care not to pour the diluted sample directly through the central hole.
4. Centrifuge at 1200 x *g* (see Notes) for **10 minutes** at room temperature, with the **brake on**.

   NOTE: For samples older than 24 hours, a centrifugation time of 20 minutes is recommended.
5. Pour off the top layer, which contains the enriched MNCs, into a new tube. Do not hold the SepMate™ tube in the inverted position for longer than 2 seconds.

   NOTE: Some red blood cells (RBCs) may be present on the surface of the SepMate™ insert after centrifugation. These RBCs will not affect performance.

   NOTE: To reduce platelet contamination in the enriched MNCs, pipette off some of the supernatant above the MNC layer before pouring.
6. Wash enriched MNCs with PBS + 2% FBS. Repeat wash.

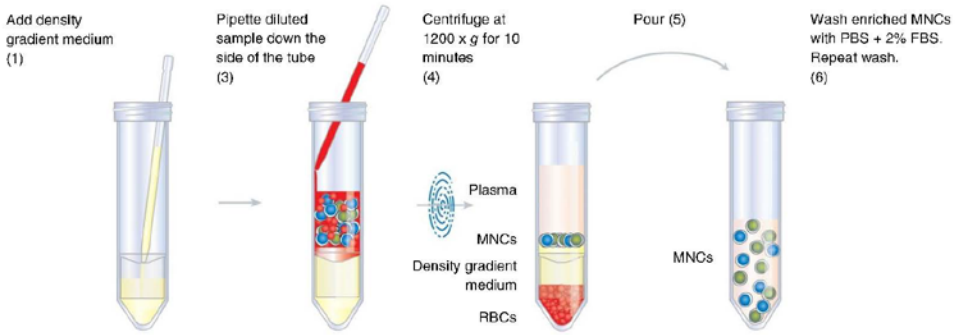
   NOTE: Centrifuging at 300 x *g* for 8 minutes at room temperature, with the brake on, is recommended.

   NOTE: To remove platelets from the enriched MNCs, perform one of the washes at 120 x *g* for 10 minutes at room temperature, with the brake off.

   NOTE: If the density gradient medium above the SepMate™ insert appears red after centrifugation (i.e. some RBCs have not pelleted), the SepMate™ tube can be spun at 1200 x *g* for another 10 minutes with the brake on. This step may be necessary when processing samples that are older than 24 hours.

SepMate™ Procedure

Numbers in brackets refer to steps under Directions for Use.



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Version 1.0.0
2016

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Appendix II: STEMCELL Technologies SepMate™ procedure

SepMate™ STEMCELL™ TECHNOLOGIES

Table 1: Sample and Density Gradient Medium Volumes

SEPMATE™ TUBE	INITIAL SAMPLE (mL)	DENSITY GRADIENT MEDIUM (mL)
15	0.5 - 4.0	4.5
15	> 4 - 5	3.5
50	4 - 17	15

Supplementary Procedure

USE OF SEPMATE™ WITH ROSETTESEP™ COCKTAILS

SepMate™ tubes can be used with RosetteSep™ HLA cell enrichment cocktails to isolate specific cell types from human whole blood. For available RosetteSep™ cocktails please refer to www.rosettesep.com.

To use SepMate™ with RosetteSep™ cocktails:

1. Add RosetteSep™ cocktail to the whole blood sample using volumes recommended in the RosetteSep™ cocktail Product Information Sheet.
2. Incubate for 10 minutes at room temperature (15 - 25°C).
   NOTE: The 10-minute incubation time is specific to this procedure. It will have minimal effect on performance.
3. Follow the steps under SepMate™ Directions for Use on page 2.
   NOTE: Use density gradient medium recommended in the RosetteSep™ cocktail Product Information Sheet.

Warnings and Precautions

1. For professional users only.
2. This product is for in vitro diagnostic use. Not for use with therapeutic applications.
3. Do not re-use SepMate™ tubes.
4. Do not use SepMate™ tubes after the expiry date listed on the label.
5. SepMate™ is not intended for a specific diagnostic application. Validating SepMate™ for a specific diagnostic application is the responsibility of the end user.
6. This product should be handled by trained personnel observing good laboratory practices. Dispose of tubes and biologic waste in accordance with appropriate local, state or national biohazard safety regulations.
7. SepMate™ can be used with human whole peripheral blood, cord blood, and bone marrow samples. It is not intended for use with samples older than 48 hours.
8. Spin tubes at recommended settings in the centrifuge.

Appendix II: STEMCELL Technologies SepMate™ procedure

SepMate™ STEMCELL™ TECHNOLOGIES

Notes

SepMate™-15

SepMate™-15 is designed to process 0.5 - 5 mL of initial sample.

A minimum packed RBC volume of 0.25 mL is required. For samples with low hematocrits, the minimum sample volume may therefore be greater than 0.5 mL.

There is a maximum packed RBC volume of 3 mL. For samples with very high hematocrits, the maximum sample volume may therefore be less than 5 mL.

SepMate™-50

SepMate™-50 is designed to process 4 - 17 mL of initial sample.

A minimum packed RBC volume of 2 mL is required. For samples with low hematocrits, the minimum sample volume may therefore be greater than 4 mL.

There is a maximum packed RBC volume of 12 mL. For samples with very high hematocrits, the maximum sample volume may therefore be less than 17 mL.

Conversion of *g* to RPM

To convert *g* to rpm, use the following formula:

$$RPM = \sqrt{\frac{RCF}{(1.118 \times 10^{-5}) \times (Radius)}}$$

Where: RPM = centrifuge speed in revolutions per minute
RCF = relative centrifugal force (*g*)
Radius = radius of centrifuge rotor in centimeters (cm)

Technical Assistance

For technical support please contact us by email at techsupport@stemcell.com or call +00800.7836.2355 (Europe) or 1.800.060.350 (Australia). For more information please visit www.stemcell.com.

If you require a printed copy or a translated version of this document in a certain language please contact technical support.

Appendix II: STEMCELL Technologies SepMate™ procedure

REF Catalog or reference number	LOT Batch code	Use by: MM/YYYY
Caution, consult accompanying documents	IVD In Vitro Diagnostic Medical Device	STERILE R Sterilized using irradiation
CE Mark	Manufacturer's identification (name & address)	EC REP Authorized EC representative in the European Community
Consult Instructions for Use	Do not re-use	Contains sufficient for n tests