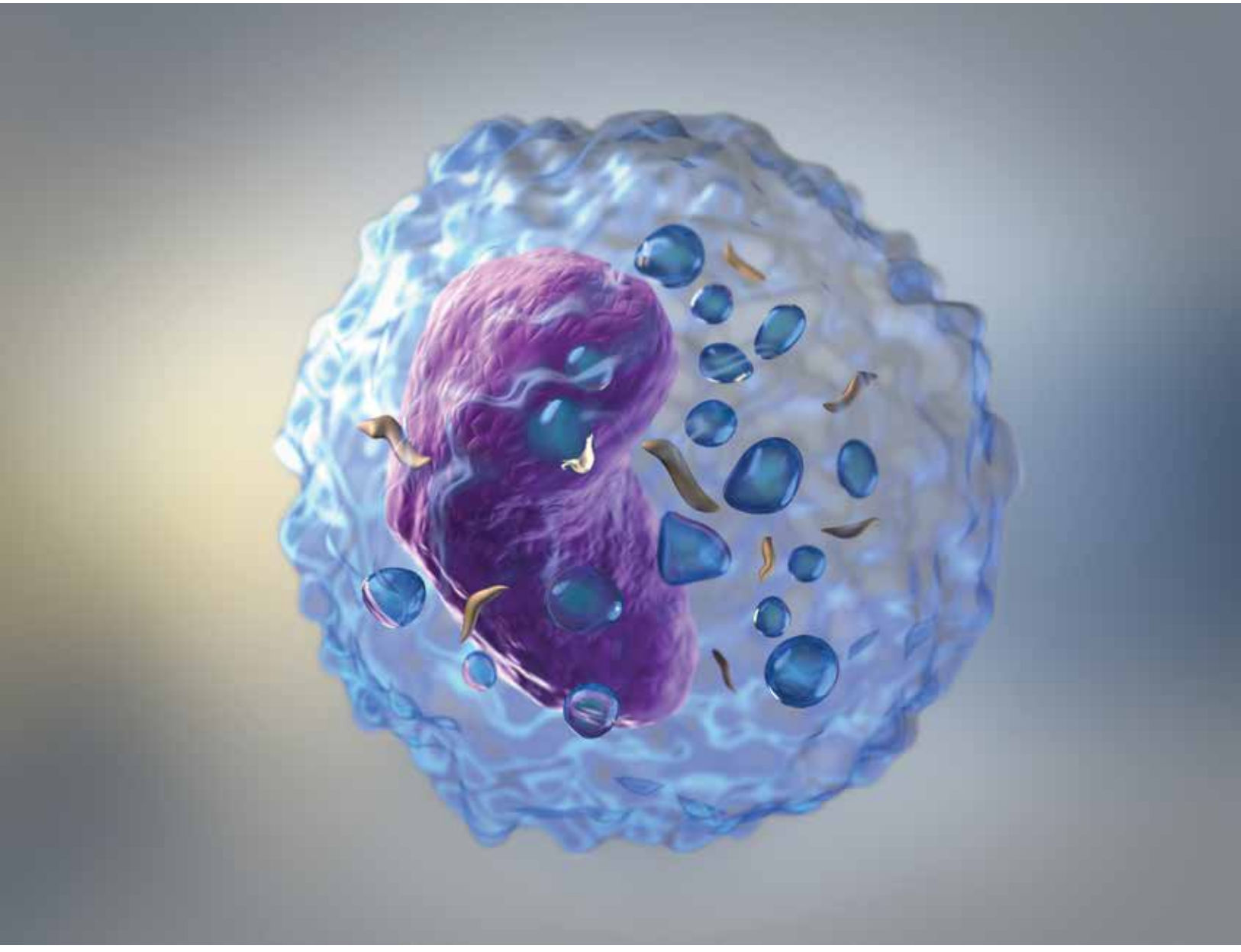


HIMEDIA®

For Life is Precious

HIMEDIA®

Cell Culture

Enabling Breakthroughs

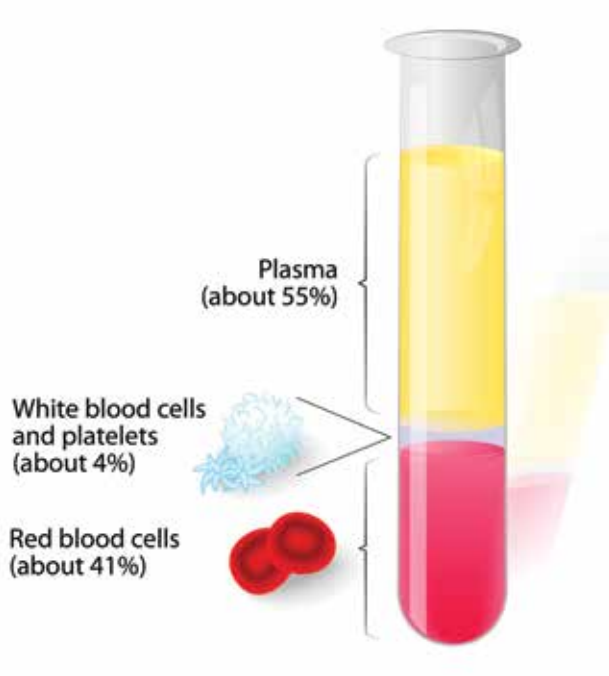
Why HiSep™ LSM ?

PBMCs - Peripheral Blood mononuclear cells - are a mixture of many subsets of lymphocytes and monocytes. These cells are essentially the ones that recognize, respond to, signal and deal with the threats to the body integrity: bacteria, virus-infected cells of the body, cancerous cells. State of the PBMC at any point in life is closely related to the infection history of the patient and correlates with his or her ability to effectively respond to such a threat in the future.

For this reason, isolation of PBMCs is fundamental to the immunology research. Isolation of PBMCs, especially the lymphocytes, is the intial step in the research process. Success of the process highly depends on quality of the final isolated population in terms of functional and morphological intactness of cells and presence of large number of a specific cell population. This isolated population should be pure or minimally contaminated with other cell types.

A major breakthrough was made by Boyum with the invention of density gradient centrifugation of blood cells for separation of lymphocytes. It works on the basis of differential migration of cells during centrifugation according to their buoyant density, which results in separation of different cell types into distinct layers.

HiSep™ Lymphocyte Separation Media are sterile, ready to use, density gradient media for isolating viable mononuclear cells from human blood and other animal species. They are made up of a polymer of sucrose with high synthetic molecular weight. Mononuclear cells (lymphocytes and monocytes) and platelets are contained in the banded plasma-LSM interphase due to their density and the pellet that is formed contains mostly erythrocytes and granulocytes, which have migrated through the gradient to the bottom of the tube.



Very low Endotoxin level <0.3 EU/ml

Manufactured in GMP, ISO 13485 and ISO 9001 certified facility

CE Marked for IVD (98/79/EC)

Select the Right Density

Code	Product Name	Density	Cell type separated	Species	Tissue Types
LS001-100ML LS001-500ML	HiSep™ LSM 1077	1.077 g/ml	Mononuclear cells (Lymphocytes and monocytes)	Human	Blood
LS002-100ML LS002-500ML	HiSep™ LSM 1073	1.073 g/ml	Lower density human mononuclear cells (eg- mesenchymal stromal cells or monocytes).	Human	Peripheral blood
LS003-100ML LS003-500ML	HiSep™ LSM 1084	1.084 g/ml	Mononuclear cells of higher density	Chicken, Rats, Mice and other Mammals.	Peripheral blood
LS004-100ML LS004-500ML	GranuloSep™ GSM 1119	1.119 g/ml	Mononuclear cells and granulocytes	Human	Peripheral blood
LS005-25ML LS005-50ML LS005-500ML	Ficoll 400-20%	Ficoll 400-20%	Commonly used to prepare density gradients	-	-

Quality Control

Physicochemical Testing	pH
	Osmolality
	Density
Performance Test	95 ± 5% mononuclear cells
	5 ± 2% red blood cells
	3 ± 2% granulocytes
	>90% viability of separated cells

Purity by Flow Cytometry

Quality of the lymphocyte separation medium depends solely on the purity of isolated lymphocytes with minimal granulocyte contamination. Because of the diversity of populations comprising PBMCs, flow cytometry represents the best method for studying functional and phenotypical properties of these cells. Each lot of LSM is rigorously tested for its ability to isolate pure population of lymphocytes by analyzing forward and side scatters. Forward scatter (FSC) correlates with cell size and side scatter (SSC) is proportional to the granularity of the cells.

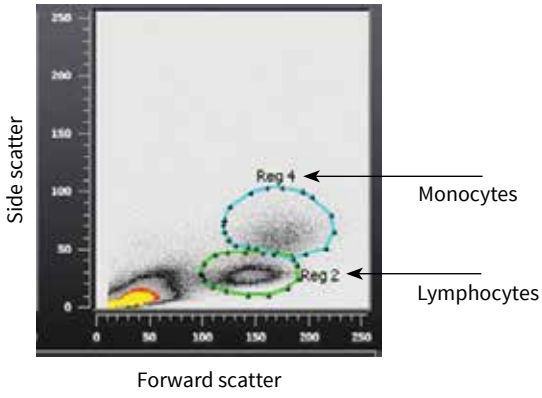


Fig 1 : PBMCs sample analyzed on flow cytometer showing lymphocytes and monocytes gated population based on forward scatter (FSC) and side scatter (SSC).

CD15⁺ Marker Analysis

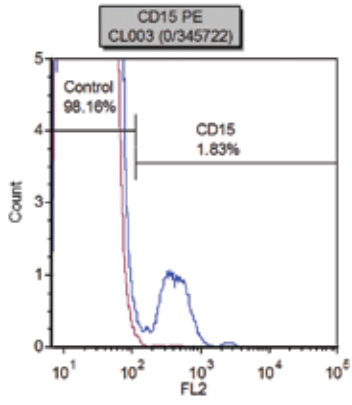


Fig 2 : Purity of each batch is tested using flow cytometry and staining for CD15⁺ cells.

Viability by Automated Cell Counter CASY

More than 90% viability of separated cells is determined by electronic cell counter CASY based on membrane potential of cells and trypan blue exclusion test on hemocytometer.

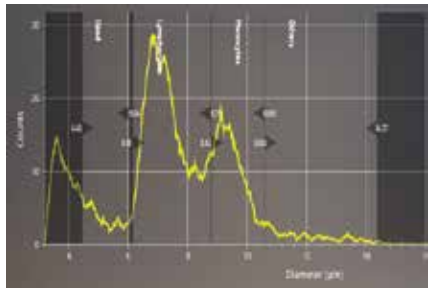


Fig 3 : PBMCs sample analyzed on CASY (electronic cell counter) showing lymphocytes and monocytes gated population based on diameter (µm) and cell counts.

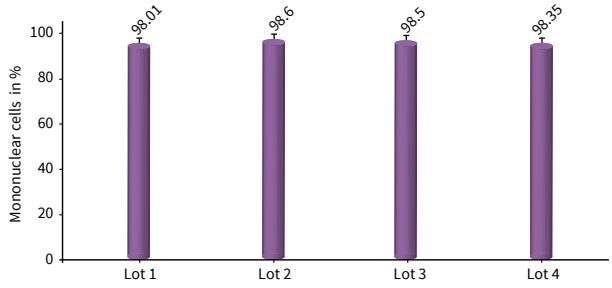


Fig 4 : Consistent performance of different lots of LS001 with respect to purity of isolated PBMCs

Applications

Immunology research

- Isolation of various immune cells subtypes and their functional studies
- Study of cell signaling pathway
- Cytokines research based on cell- cell/ pathogen infection
- Cell proliferation assay and cytokines profiling

Clinical research

- Regenerative therapy
- Cancer immunotherapy
- HLA therapy

Diagnostics

- Autoimmune disease
- Identification of various hematological malignancies and infectious diseases

Isolation of Mononuclear Cells

1. Aseptically transfer 2.5 ml of HiSep™ LSM 1077 to a 15ml clean centrifuge tube and overlay with 7.5ml diluted blood (1:1 or 1:2 with blood and DPBS). DO NOT MIX. The quality of the separation is dependent upon a sharp interphase between lymphocytes and the solution
2. Centrifuge at 1000 x g, at room temperature (15-25°C) for 30 minutes without brakes using swinging bucket rotor. Centrifugation will sediment erythrocytes and polynuclear leukocytes and buffy coat of mononuclear lymphocytes above HiSep™ LSM 1077.
3. Discard by aspirating most of the plasma and platelet containing supernatant above the interface band (granulocytes and erythrocytes will be in the red pellet).
4. Using Pipette carefully aspirate the mononuclear cells and transfer it to a clean centrifuge tube.
5. Add 10ml of isotonic phosphate buffered saline to mononuclear cells layer in the centrifuge tube and mix by gentle aspiration. Centrifuge at 160- 260 X g with brake off, at room temperature (15-25°C) for 10 minutes. This washing with isotonic phosphate buffered saline removes HiSep™ LSM and reduces the number of platelets.
6. Wash the cells again with isotonic phosphate buffered saline and re-suspend in an appropriate medium for your applications.

Isolation of Granulocytes

1. Aseptically transfer 3.0 ml of GranuloSep™ GSM 1119 to 15 ml clean conical centrifuge tube and slowly overlay with 3.0 ml of HiSep™ LSM 1077.
2. Overlay the upper gradient of the tube from step 1 with 6.0 ml of whole blood.
3. Centrifuge the tube at 700 x g for 30 minutes at room temperature (15-25°C) with brakes using swinging bucket rotor during centrifugation. Do not centrifuge at lower temperatures like 4°C, as it may result in cell clumping or poor recovery. The brake of the centrifuge should always be in the off position. Centrifugation should sediment erythrocytes. Granulocytes will be seperated on interphace of 1077/1119, whereas lymphocytes, other mononuclear cells and platelets are found at the plasma/1077 interphase.
4. After centrifugation carefully remove centrifuge tube. Two distinct upper and lower buffy coat layers should be observed at two interphases.
5. Aspirate and discard fluid to above of layer A. Transfer cells from this layer to a tube marked 'mononuclear'.
6. Aspirate and discard remaining fluid to above of layer B. Transfer cells from this layer to a tube labeled 'granulocytes'.
7. Add 10 ml isotonic phosphate buffered saline to the tubes to wash the cells. Centrifuge for 10 minutes at 200 x g. Remove the supernatant and discard.
8. Resuspend the cells by gentle aspiration with a Pasteur pipette.
9. Repeat steps 7 and 8 two times.
10. Add appropriate volume of isotonic phosphate buffered saline to re-suspend the cells.

    NOTE: *Count the cells and determine the number of viable cells by trypan blue exclusion staining. In case of low cell viability, phosphate buffered saline may be replaced with appropriate cell culture medium.*

Factors Affecting the Isolation of Mononuclear Cells

- **Blood**: The blood used for separation should be fresh and free of clots. Venous blood should be collected in a tube containing preservative-free anticoagulant. For best results process the blood as soon as possible. Loss of viability and lower cell recoveries may result, in case of delayed processing.
- **Temperature**: While performing density gradient centrifugation, the temperature variations play an important role. Room temperature, temperature of the density gradient media, centrifuge temperature, and the temperature of the liquid sample can hinder the isolation process and hence result in lesser yield.
- **Platelets**: To avoid platelet contamination while you are isolating mononuclear cells, increase a centrifugation step with a 4 – 20% sucrose gradient layered medium. This centrifugation will effectively remove any platelet contamination giving pure yield of mononuclear cells.
- **Yield & Purity**: The yield and purity of the isolated cells depend on the efficiency of the erythrocyte removal. Some mononuclear cells are seen as clumps and sediment with the erythrocytes when the erythrocytes in the whole blood are aggregated. One can solve this problem by diluting the whole blood.
- **Blood Volume**: The volume of blood and the diameter of the tube are crucial in determining the height of the blood sample in the tube. If the blood volume is more, there might be a chance of erythrocyte contamination. Hence, for a large volume of blood, preferably use a tube with a larger diameter, keeping the separation time constant.

Troubleshooting

HiSep™ LSM products if used as per the recommended procedure, are said to give trouble-free isolation of mononuclear cells. Deviations in experimental procedures or parameters, may lead to poor results. This troubleshooting table will assist in the rapid identification and rectification of the problem hindering the performance.

Problem	Possible Reason	Solution
Contamination of lymphocytes with red blood cells.	Low temperature	The densities of HiSep™ LSM are greater at low temperature. As a result, they are agitated less well. Raise the temperature to 18°C - 20°C.
	Low centrifugation speed	Use adequate x g force and if required increase the centrifugation speed.
	Stale blood	Process the blood as soon as possible.
Mononuclear cells with low yield and viability.	Must be due to high temperature	HiSep™ LSM products are less dense at high temperatures, therefore some lymphocytes may penetrate into the interface layer. Try and reduce the temperature to 18°C to 20°C. This might improve the cell viability too.
Mononuclear cells with low yield and normal viability.	The blood must not have been diluted 1:1 with balanced salt solution. High hematocrit.	Dilute the blood samples even further.
Mononuclear cells with low yield and increased granulocyte contamination.	Centrifugation rotor vibration that leads to the stirring of the gradient.	Check to see if the rotor is well balanced. Preferably choose the rotor speed to avoid natural resonant frequencies.
Mononuclear cells with low yield, low viability and contamination by other cell types.	Blood used might be non-human.	Use freshly collected human blood. Strictly do not use pathological blood, non-human blood samples, old blood samples or blood from sources other than peripheral blood.

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