

Human Whole Blood Polymorphonuclear Cell Isolation (BN00719)

(Catalog # BN00719; for isolation of PMN cells from 30 ml whole blood; Store at 4°C)

I. Introduction:

Polymorphonuclear cells (PMN) have a multilobed nucleus and include the following cells types: mast cells, neutrophils, eosinophils and basophils. Upon isolation, these cells can be used for migration assays and determination of cytokine/chemokine production in response to pharmacological compounds. Assay Genie's PMN Cell Isolation Kit is unique in the market because it enables the user to isolate PMNs, assess viability of the obtained cell fractions and also determine purity with three simple, easy-to-use reagents! High yields, or 60-80% of total PMNs have been obtained yielding $\geq 5 \times 10^6$ cells per ml of human whole blood. Purity of the cell fraction is assessed with giemsa stain (included). Assay Genie's kit use results in highly enriched (>95%) cell fraction of polymorphonuclear cells with less than 3% red blood cell counts. More than 99% of the cells are viable, as determined by the Viability Stain.

II. Applications:

- Isolation of Polymorphonuclear cells from human whole blood.
- Isolated PMNs can be used studies of production and release of cytokines/chemokines and are commonly used in migration assays.

III. Sample Types:

- Fresh whole blood (collected less than 12 hours prior PMN isolation)

IV. Kit Contents:

Components	BN00719	Cap Code	Part Number
PMN Isolation Buffer (1X)	2 x 30 mls	NM	BN00719-1
PMN Density Gradient Media	2 x 20 mls	NM	BN00719-2
Viability Stain	200 μ l	Red	BN00719-3
Blunt-end needle 18 G; 1.5 in. (Sterile)	15 pieces	-	BN00719-4
Giemsa Stain	7 mls	Amber/NM	BN00719-5

V. User Supplied Reagents and Equipment:

- 3 ml or 5 ml syringes
- Hemocytometer
- 50 ml conical tubes (polypropylene or polyethylene)
- dH₂O for removal of excess Giemsa
- Centrifuge with Swinging bucket rotor
- Brightfield Microscope, Fluorescent Microscope with FITC/TRITC Filters
- 50 and 1.5 ml sterile centrifuge tubes
- Human blood 30 mls collected in EDTA (final concentration 1.5-2.0 mM)
- Glass Slides to evaluate purity of cell fraction obtained

VI. Storage Conditions and Reagent Preparation:

Store kit at 4°C, protected from light. Briefly centrifuge vials prior to opening. Read entire protocol before performing the assay.

- **PMN Isolation Buffer (1X):** store at 4°C and **PMN Density Gradient Media (PMN DGM)** should be opened and used in a *sterile environment* to prevent contamination. Bring to room temperature before use and mix well.
- **Viability Stain** store in dark, at 4°C.
- **Giemsa Stain:** store in dark at room temperature (RT).
- **Blunt-end needle 18 G; 1.5 in" (Sterile):** store at RT. The needle is not sharp, however it should be disposed of in an appropriate Sharps Collection & Disposal System.

VII. PMN Isolation and Viability Assay Protocol (30 mls total volume of whole blood):

Work in a sterile environment. Use universal precautions when handling blood products and human body fluids.

1. Sample Preparation:

- Bring PMN Density Gradient Media (PMN DGM) to RT and mix well by inverting bottle several times. Add 10 mls PMN DGM to a 50 ml centrifuge tube.
- To add whole blood: hold the conical tube at a 45° angle, place tip of pipette at the edge of the PMN DGM and slowly add 10 mls whole blood onto the PMN DGM (see Figure A). Carefully handle tube to prevent mixing of PMN DGM with blood. Centrifuge tube at 400 x g for 30 minutes at RT.

Note: It is preferable to spin tubes in a swinging bucket rotor without brake.
- Remove conical tube from centrifuge and observe the 6 layers (top to bottom): plasma, Peripheral Blood Mononuclear Cells (PBMCs), PMN DGM, PMNs, PMN DGM and RBCs at the bottom of the tube. It is normal/expected to see 2 layers of DGM.
 - PBMC removal:** with an 18 G 1.5" needle affixed to 3 ml syringe withdraw plasma to within 1 ml (1 cm³) of the PBMC layer. Discard plasma by expunging solution into waste container. With the same needle, withdraw the PBMC layer. Highest yields can be obtained by withdrawing approximately 1 ml above and 1 ml below the PBMC layer, which contains a small amount of plasma (above) or density gradient media (below). Expunge PBMC layer into a clean 50 ml centrifuge tube if further use is desired.
 - Isolation of PMNs:** with a clean needle, withdraw the PMN layer. Place the tip of the blunt end needle slightly above the PMN layer and circle the inside perimeter of the tube to extract the PMNs. The volume obtained is approximately 3 mls, expunge this solution with PMNs into a clean 50 ml centrifuge tube.

d. Wash PMNs: Add 6 mls of PMN Isolation Buffer (1X) to isolated PMNs. Centrifuge PMN suspension at 400 x g for 10 min. discard supernatant. Repeat wash, discard supernatant and resuspend in 5 mls of diluted PMN Isolation Buffer.

Note: Observe cell pellet at bottom of tube. PMNs may also cling to side of tube. *Do not disturb pellet when pouring off excess PMN Isolation Buffer.*

2. Determination of Live Cell Count:

a. Prepare a 1:10 dilution of viability stain with cell suspension by adding 2 μ l of Viability Stain to 18 μ l of washed PMN suspension in a 1.5 ml centrifuge tube. Tap to mix. Inoculate hemocytometer with 10 μ l of stained PBMC suspension. Determine and record the total cell count with a Bright-field microscope.

b. With the same Region of Interest (ROI) in view, illuminate the slide with light from the fluorescent lamp with the FITC/TRITC filter combination to count the fluorescent cells. *If a small amount of incidental white light illuminates the hemocytometer, the grid will be visible allowing the viewer to see the same ROI as visible with fluorescent light. Live cells will fluoresce green. Dead cells will fluoresce red.* Tally the number of green and red cells to complete the calculations using the equations in Measurement section below.

3. Evaluation of Cell Suspension Purity:

a. Remove 25 μ l of PMN suspension and add to clean glass slide. Allow the solution on slide to completely dry (15 min) or place on warming plate at 37°C to expedite drying process. Add 50 μ l of Giemsa Stain to stain cells and proceed with staining cells for 5 minutes. Wash slide until runoff is clear. Allow slide to dry. Cells and morphology are best viewed with oil immersion objective lens. Perform differential cell count using the formula described in **4. e Measurement** (below).

4. Measurement:

a. Number of RBCs = Total Cell Count (hemocytometer) – Total Number of Fluorescent Cells (both red and green cells)

b. Percent Live Cells = Number of Green Fluorescent Cells/Total Number of Fluorescent Cells \times 100

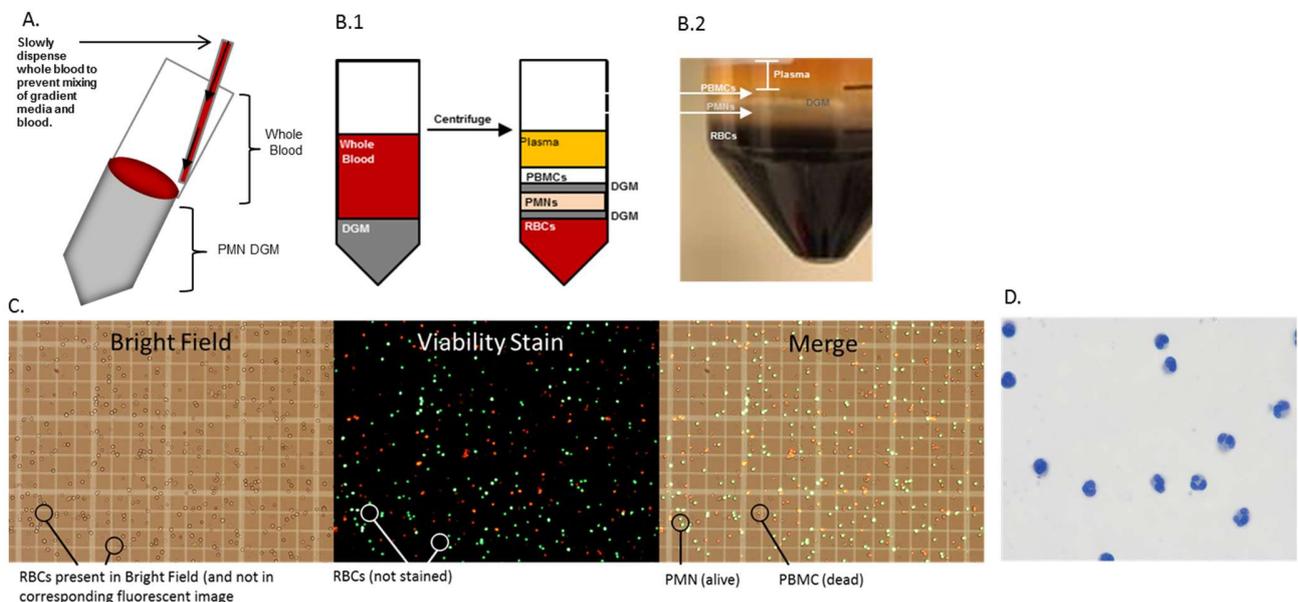
c. Percent Dead Cells = Number of Red Cells/Total Number of Fluorescent Cells \times 100

d. Percent RBCs = Number of RBCs/Total Cell Count (hemocytometer) \times 100

Note: Contamination of PMNs with RBCs may affect downstream applications including such as flow cytometry or neutrophil chemotaxis assays. Therefore, if RBCs are >10% of total PBMC count we recommend that RBC lysis be completed (See K5830).

e. Differential cell count: with a 40X objective, count a total of 200 cells. Observe the nucleus of each cell, and score those with multilobed nucleus as a PMN. Mononuclear cells or anucleate cells are scored as “not PMNs.” Determine the percent of PMNs and not PMNs using the formula:

$$\% \text{ PMNs} = \text{Number of Multilobed Cells} / \text{Number of Cells (200)} \times 100$$



Figures A. Illustration of conical tube held at 45 degrees while blood layered on top of PMN DGM. **B.1.** Layers of PMN DGM and whole blood prior to and after centrifugation showing the separation of layers in the conical tube. **B.2** Illustrates separation of six layers (plasma, PBMCs, PMN DGM, PMNs, PMN DGM and RBCs) obtained after spinning whole blood with PMN DGM. **C.** Bright-Field image of hemocytometer showing total cells (left); image from Fluorescent microscope with Rhodamine/FITC filters of same ROI showing live (green) and dead (red) cells (middle); merge of two panels (right). **D.** Polymorphonuclear Cells stained with Giemsa stain indicating purity of cell suspension obtained.

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