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Isolation and Functional Analysis of Human Neutrophils

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Abstract

This unit describes the isolation of human polymorphonuclear neutrophils (PMN) from blood using dextran sedimentation and Percoll or Ficoll-Paque density gradients. Assays of neutrophil functions including respiratory burst activation, phagocytosis, and microbial killing are also described.

Keywords

neutrophil; leukocyte; NOX2; phagocyte; innate immunity

Human polymorphonuclear neutrophils (PMN) are the most abundant nucleated cell in circulating blood (2 to 8 × 10^6 per ml). PMN possess characteristic cytoplasmic granules containing antimicrobial polypeptides and can produce antimicrobial reactive oxygen species (ROS, *e.g.*, H_2O_2 and hypochlorous acid). The life cycle of the PMN includes maturation in and release from the bone marrow, circulation in blood, loose adherence to vascular endothelia and clearance of senescent PMN by the reticuloendothelial system. If activated, peripheral PMN adhere to vascular endothelia and migrate out of the vasculature moving in the direction of infectious or inflammatory stimuli, a process that significantly alters their behaviors (Zarember and Kuhns, 2011). Upon encountering microbes, PMN may form neutrophil extracellular traps (NETs), release the contents of their granules, produce inflammatory mediators, produce reactive oxygen species (ROS), and engulf microbes through phagocytosis. Following activation, PMN can continue to contribute to the evolving inflammatory response or undergo apoptosis usually culminating in the phagocytosis of PMN themselves by tissue macrophages.

Considerations prior to isolation of cells

Given the relatively brief lifespan of the PMN in culture, it is essential to begin experiments without delay following their isolation from blood. Nevertheless, PMN can generally remain

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Key References

For more information on neutrophil biology, several excellent reviews by Nathan (Nathan, 2006), Nauseef (Nauseef, 2007), and Segal (Segal, 2005) are highly recommended.

viable in anti-coagulated blood (preferably using acid citrate dextrose as an anticoagulant) for up to 24–48 hrs after collection thereby permitting shipment of patient blood via overnight courier. If shipping patient samples, it is essential to also ship healthy donor specimens to account for effects of shipment and delayed processing on biological endpoints.

PMN are exquisitely sensitive to commonly occurring contaminants of media and labware. All media and materials that come into contact with the blood or cells should be pyrogen-free. Unless otherwise indicated, maintain blood and cells at physiologic pH, room temperature, and in the absence of divalent cations to help limit activation. Pyrogen-free polypropylene tubes are recommended to reduce adherence-induced activation of PMN. Pyrogen-free pipettes and pipet tips (preferably with aerosol barriers) should be used. The use of a biosafety cabinet with HEPA-filtered air may further decrease the likelihood of cellular activation. PMN responses can be 'primed', or functionally enhanced, by small amounts of activation during preparation and strong activation can result in formation of macroscopic clumps of cells. Consequently, basal or resting cells should be analyzed in each experiment to monitor such activation.

CAUTION: When working with human blood, cells, or potentially infectious agents, best biosafety practices should be used to limit risk of exposure to blood borne pathogens. Personal protective gear (gloves, lab coats, eye protection), biosafety cabinets, and aerosol control devices should be used at all times. Blood should be obtained under IRB approved protocols with documented informed consent.

Basic Protocol 1: Isolation of neutrophils using Percoll-based density gradient

The isolation of human peripheral blood PMN involves removal of red blood cells (RBC) using dextran-promoted rosette formation and hypotonic lysis of remaining RBC. PMN are separated from PBMC using density gradients of either Percoll-based isolation as described in this protocol or Ficoll-Paque premium as a density gradient (Alternate Protocol). PMN isolation using a discontinuous gradient of plasma/Percoll reduces PMN priming. Ficoll-Paque may result in low-level priming for functions such as the respiratory burst but is acceptable for most assays.

Remove red blood cells

1 Spin Vacutainer tubes at 400 × g for 10 min.

Anticoagulated blood can also be spun in larger tubes and volumes but may require modification in spin time or speed. This step serves to generate plasma used in the Percoll gradient.

2 Transfer plasma (upper phase) into new 15 ml conical tube and spin again to collect all leukocytes from plasma.

This optional step serves to boost yield and is especially important if low WBC counts are expected. The pellet from this low speed second spin can be added to the blood in step 4.

3 Remove platelet-rich plasma to new tube and spin for 10 min at ≥2000 × g to remove platelets. Platelet-poor plasma is used in step 15.

4 Pour RBC-containing layer from Vacutainer tubes (up to 3) into 50 ml conical tube. Rinse blood tubes with a few ml HBSS(−) to further boost yield and transfer rinses to 50 ml tube.

5 Pour HBSS(−) into the 50ml tube containing blood to the 25ml mark.

If >25 ml, split into a second tube and top off both to 25 ml with HBSS(−).

6 Add 25 ml of 3% dextran and invert 5 × to mix. Allow to sediment for 20–40 min.

Monitor sedimentation until a sharp line forms between the RBC layer and the leukocytes present in the supernatant. The difference in time required depends in part on the size of the dextran used but can also vary from donor to donor. It is important not to wait too long because PMN will begin to settle to the bottom of the tube and yield will be reduced.

7 Pipet the supernatant containing leukocytes into a fresh 50 ml conical tube leaving behind as much of the sedimented RBC layer as possible.

At this point, the supernatant contains total leukocytes, platelets and some RBC and can be subjected to Ficoll-Paque separation if desired (Alternate Protocol)

Lyse remaining RBCs

8 Top off tubes to 50 ml with HBSS(−) and centrifuge at 400 × g for 10 min.

9 Aspirate the supernatant and loosen the cell pellet by gently tapping tube.

10 Pipet 10 ml of 33mM NaCl into cells.

Hypotonicity of 33mM NaCl preferentially lyses RBC. However, it is essential to keep the time cells are exposed to hypotonic conditions LESS than 30 seconds.

11 Within 30 seconds, add 10 ml 267mM NaCl to normalize osmolarity, then top off tube with HBSS(−).

12 Centrifuge for 10 min at 400 × g and aspirate the supernatant.

During this step, prepare the Percoll gradient in step 15.

13 Repeat steps 9–12 again to reduce residual RBC.

14 Resuspend cell pellet containing leukocytes in 2 ml HBSS (−) by pipetting up and down.

If cells do not resuspend easily, it is possible that they have become activated in which case, they will not separate well and may behave abnormally in functional assays.

Prepare a discontinuous Percoll gradient and isolate PMNs

15 Prepare a discontinuous Percoll gradient as follows:

Bottom layer: Mix 1.133ml 90% Percoll solution and 0.867 ml autologous plasma (51% Percoll)

Separation between layers of Percoll is important. It may be useful to create a few small bubbles in the lower layer that then cushion the interface as the middle layer is applied. The denser Percoll is displaced downward by the less dense liquid on top.

Middle layer: Mix 0.933 ml 90% Percoll solution and 1.067ml autologous plasma (42% Percoll)

Layer drop-wise on bottom layer, leaving additional bubbles on top of layer and avoid moving tube.

Overlay 2ml resuspended leukocytes in HBSS(−) and centrifuge at 400 × g for 10 minutes.

16 PMN form a pellet at the bottom of the tube. Carefully aspirate upper layers leaving cell pellet at bottom. Resuspend PMN in 1 ml HBSS(−) by pipetting gently up and down. Transfer to fresh 15 ml conical tube containing 14 ml HBSS(−).

PMN pellets from normal subjects have a slight pale green color due to myeloperoxidase. Avoid adherent PBMC if present. Alternatively, if PBMC are desired, aspirate them from the interface of the top and middle layers before removing other layers.

17 Centrifuge PMN as before and resuspend in 1–2 ml HBSS(−)

18 Count the cells using an automated cell counter. Alternatively, dilute 10 μl of cells in 490 μl of 3% acetic acid and count in a hemacytometer.

1 to 2 × 10^6 PMN can be isolated per ml of whole blood with ≥95% purity and viability. Acetic acid enhances nuclear morphology permitting the differentiation of PMN from other leukocytes. PMN must be studied immediately after isolation. Maintain purified PMN in HBSS(−) at concentrations between 1 to 2.5 × 10^7/ml at room temperature.

19 To assess purity of isolated PMN, dilute cells to between 5 – 10 × 10^5/ml in HBSS(−) and load 100 μl in a cytocentrifuge funnel (*e.g.*, Cytospin 2, Shandon) following manufacturer's instructions. Spin for 1 minute at 1,000 rpm to deposit

a uniform layer of cells on the microscope slide. Differential stains (*e.g.*, Diff-quick, or Hemacolor) should be used to accentuate differences between eosinophils, neutrophils, and other cell types.

The most common cellular contaminants of the PMN preparation are eosinophils; however lymphocytes and monocytes may also be present. Eosinophil numbers can vary seasonally and from donor to donor and may comprise a considerable percentage of cells in PMN preparations. Eosinophil contamination can be reduced by magnetic bead sorting using CD16 which is highly expressed on neutrophils but less so on eosinophils. Contaminating monocytes, known to contribute to some biological endpoints (see commentary) can be removed using CD14 selection. CD19 and CD3 can be used to remove B-cells and T-cells, respectively.

Alternate Protocol

Ficoll-Paque Premium as a density gradient for isolation of PMNs

Procedure

1. Collect patient blood in sodium heparin (5–10 U/ml final concentration) or acid citrate dextrose (ACD-A) blood collection tubes
2. Dispense 15 ml HBSS(−) to 50 ml conical centrifuge tube and transfer 15–20 ml blood to tube.
3. Carefully underlay blood mixture with 12 ml Ficoll-Paque Premium.
4. Spin for 30 min at 500 × g, with brake off.
5. Aspirate mononuclear cells, remaining plasma and Ficoll-Paque Premium from the tube, leaving the PMN and erythrocyte-rich pellet.
6. Dilute with HBSS for a final volume of 20 ml.
7. Add 20 ml of 3% dextran. Invert the tube several times to mix. Allow erythrocytes to sediment at 1 × g for 20 min at room temperature.
8. Transfer the PMN-rich supernatant to a new tube and dilute with HBSS(−).
9. Spin at 300 × g for 10 min. Aspirate the supernatant fluid.
10. Lyse the remaining erythrocytes with 10 ml hypotonic lysis buffer (13.6 ml 10× PBS in 600 ml water) and incubate no more than 30 seconds followed by the quick addition of 10 ml re-equilibration buffer (108.1 ml 10× PBS in 500 ml water) to restore the isotonicity. Dilute further with 30 ml HBSS(−).
11. Spin at 300 × g for 10 min, aspirate the supernatant fluid, wash, and repeat process once more. Repeat lysis if there is significant erythrocyte contamination.
12. Resuspend in 1–2 ml HBSS(−) and proceed with step 18 of the primary protocol.

BASIC PROTOCOL 2: Analysis of PMN ROS generation by luminol-enhanced chemiluminescence

Neutrophils possess numerous proteases that can make western blotting for neutrophil proteins a challenge. Diisopropyl fluorophosphate (DFP) is a potent serine protease inhibitor that has been frequently used in PMN studies but, because of toxicity, should only be considered if the following do not work. Serine proteases can be inhibited with AEBSF (Pefabloc) or phenyl methylsulfonylfluoride (PMSF) and a broad-spectrum inhibitor cocktail (e.g., cOmplete Mini tablets) is recommended. Many detergent-based extraction methods can be used but protease inhibitors should be incubated with PMN prior to lysis.

The phagocyte NADPH oxidase is an enzyme complex capable of producing superoxide anion ($O_2^{\bullet -}$). Although $O_2^{\bullet -}$ itself is not thought to be antimicrobial, it is transformed through the actions of proteins with superoxide dismutase (SOD) activity into hydrogen peroxide which is directly antimicrobial. In PMN, an additional enzyme, myeloperoxidase (which may also have SOD activity), utilizes H_2O_2 and halogen ions to generate potently antimicrobial hypohalous acids such as hypochlorite (bleach). Consequently, absence of NADPH oxidase activity, as occurs in the rare genetic immunodeficiency called chronic granulomatous disease (CGD), results in frequent serious infections thus underscoring the importance of the NADPH oxidase in innate immunity and neutrophil function. The steps below describe an enhanced chemiluminescence method used for clinical and research laboratory studies of the respiratory burst. Two Alternate Protocols follow that describe assessment of the respiratory burst using cytochrome c reduction (Alternate Protocol 2) and the dihydrorhodamine (DHR) assay (Alternate Protocol 3).

Luminol-enhanced chemiluminescence measures both intracellular and extracellular ROS production, though it may not detect them with equivalent efficiency. Addition of superoxide dismutase significantly reduces both peak height and area under the curve (AUC) after stimulation with PMA, suggesting that at least a portion of the response can be attributed to $O_2^{\bullet -}$. This versatile assay, in addition to its quick and easy set-up, offers the capability to test several different subjects and stimuli on the same plate using reduced amounts of cells, while providing high sensitivity. Results from normal subjects and patients can be assessed simultaneously and monitored kinetically or using AUC. For assays of PMN priming, some cell-concentration dependent effects may occur and such priming tends to be more potent at high cell numbers.

1. Dilute freshly prepared PMN to a concentration of 1.1×10^6/ml in RPMI 1640 with 11 mM HEPES pH 7.2 and 55 μM luminol.

   If microbes are to be used as a stimulus, also add 5–10 % donor autologous serum to provide a source of complement.

2. Add 90 μl per well of a 96-well, flat bottom, white polypropylene plate and incubate plate for 15 min at 37°C.

   The use of 1.1 X master mix (step 1) helps to decrease pipetting errors. Upon addition of the 10 μl stimulus or buffer in the next step, 1X for all

components is achieved. This preincubation allows the cells to warm and adapt to the presence of divalent cations.

3. Add 10 µl of stimulus per well.

   PMA is a convenient and potent activator of the respiratory burst and can be used at concentrations up to 1 µg/ml. fMLF can be used at concentrations from 10^{-9} to 10^{-6} M. Bacteria can be used at ratios of between 1 and 1000 microbes per PMN.

4. Quickly place the plate in a pre-warmed luminometer (37°C) programmed to measure light production for 1 second per well per cycle with gentle agitation between cycles. Depending on the luminometer used, measure each well every 1–5 minutes for 2 hrs.

   The luminescence signal is expressed as relative light units (RLU). Stimuli will display different kinetics that may depend on dose. For example, fMLF induces a rapid respiratory burst that decays quickly unless priming agents (e.g., LPS) have been used. Low doses of PMA (e.g., 1–5 ng/ml are slow activators that may continue to increase luminescence over 2 hrs whereas high doses (20–100 ng/ml) induce peak luminescence within 20–30 minutes that decays thereafter. If only a single stimulus is used, it may be useful to calculate AUC to facilitate comparison of different donors. CGD subjects generally have little to no detectable luminescence in this assay.

Alternate Protocol 2. Quantitative analysis of $O_2^{\bullet -}$ generation using superoxide dismutase-inhibitable ferricytochrome c reduction

Ferricytochrome c is stoichiometrically reduced to ferrocytochrome c in the presence of $O_2^{\bullet -}$ with a resultant increase in absorption at 549.5 nm. The addition of superoxide dismutase (SOD) acts as a blank to validate the specificity of $O_2^{\bullet -}$-dependent ferricytochrome c reduction. Spectrophotometry using an analytical wavelength of 549.5 nm and background wavelengths of 541 nm and 556 nm permits the calculation of the amount of extracellular $O_2^{\bullet -}$ generated as a function of reduced cytochrome c to nanomoles of $O_2^{\bullet -}$ released using the micromolar extinction coefficient of 0.0211.

Procedure

1. Add 200 µl catalase to 10 ml cytochrome c solution.
2. Resuspend PMN at 2×10^6 cells/ml in HBSS(+).
3. Label 6 × 1 ml polypropylene microtubes (#1–6) and prepare tubes as described in the table below. It is important that PMN be added last.

Conditions	Tube No.	Cytochrome c /catalase sol'n	SOD	PMA	PMN
		volume in µl			
Resting	1,2	500			500
Resting-SOD	3	500	10		500
PMA	4,5	500		5	500
PMA-SOD	6	500	10	5	500

4. Add reagents to tubes and rotate end-over-end at 12 rpm at 37°C for either 10 min or 60 min.

   For a 60 min incubation with PMN from normal subjects or CGD carriers with >20% normal cells, tubes # 4–6 should be diluted 1:5.

5. After incubation, immediately place tubes on ice to quickly chill then centrifuge at 500 × g for 15 min at 4°C.

6. Transfer supernatants to cuvettes and read on spectrophotometer at 549.5, 541, and 556 nm.

   549.5 nm is the analytic wavelength and 541 and 556 nm are isosbestic points (parts of the spectrum that are not responsive to oxidation/reduction.). The background wavelengths are determined by spectral scans of oxidized and reduced cytochrome c, the latter formed by addition of a few grains of sodium dithionite.

7. The net absorbance of each sample is determined using the following formula:

   $$A_{549.5} - (((A_{556.0} - A_{541.0}) * (8.5/15)) + A_{541.0\ nm})$$

8. The basal production of $O_2^{\bullet -}$ generated (nmoles/10^6 cells) is calculated by subtracting the net absorbance of the SOD-containing sample (tube # 3) from the mean of net absorbance of tubes #1 and 2, and dividing the difference by 0.0211, the micromolar extinction coefficient for reduced cytochrome c. Repeat the calculations for tubes #4–6. For 60 min samples with reduced cell numbers (2 × 10^5), multiply by 5 to normalize to 10^6 cells.

   An estimate of normal $O_2^{\bullet -}$ generated in 60 min can be obtained by reducing the number of PMN in the assay to 2 × 10^5 cells. PMN isolated from normal subjects produce 0.4 ± 0.3 nmol/10^6 PMN/10 min and 2.2 ± 1.6 nmol/10^6 PMN/60 min under basal conditions. Treatment of normal PMN with PMA results in 43.5 ± 12.6 nmol/10^6 PMN/10 min and 235.4 ± 55.7 nmol/10^6 PMN/60 min. The $O_2^{\bullet -}$ generated by PMN isolated from CGD patients ranges from 0.50 to as much as 60 nmol/10^6 PMN/60 min. Although NOX2 function is impaired in all patients with CGD, there is variability (0.1–27% of normal) in residual ROS production by patient PMN and also in CGD carriers. Reference ranges should be established in each laboratory using normal subjects.

Alternate Protocol 3. Analysis of PMN H_2O_2 production by flow cytometry of dihydrorhodamine 123 staining

NADPH oxidase activity of individual cells can be measured by flow cytometry using dihydrorhodamine 123 (DHR). DHR is only weakly fluorescent and can be passively loaded into PMN. Upon PMN activation, $O_2^{\bullet -}$ produced by NOX2 is transformed by superoxide dismutase (and possibly myeloperoxidase) into hydrogen peroxide that oxidizes DHR into the strongly fluorescent molecule, rhodamine 123.

1. Collect blood using K_2EDTA as an anticoagulant.
2. Add 12 ml lysis buffer (pre-warmed to 37°C) to a conical 15 ml polypropylene centrifuge tube.
3. Add 1.2 ml blood to the 12 ml lysis buffer. Cap the tube and invert to mix.
4. Incubate at 37°C for 5 min.
5. Spin at 600 × g for 5 min and aspirate supernatant. Wash cells with 12 ml of flow buffer and repeat spin. If there are red blood cells remaining, repeat lysis.
6. Resuspend the cells in flow buffer to 2×10^6 cells/ml and transfer 400 μl of cells into each of two polypropylene tubes labeled “Basal” and “PMA-stimulated.”
7. Add 2 μl of 25 mM DHR and 2.5 μl of 10 mg/ml catalase per tube and vortex gently.

   Catalase is added to the medium to prevent cell-to-cell diffusion of H_2O_2.
8. Incubate at 37°C in a shaking water bath for 5 min.
9. Add PMA or flow buffer to appropriate tubes and incubate for 15 min at 37°C in shaking water bath.
10. Analyze the samples on a flow cytometer within 25 min of adding DHR. Use forward and side scatter to gate on the PMN population and collect at least 5000 events in the PMN gate. If (for example in CGD patients) the expected number of DHR(+) cells ≤1% then count 200,000 events in the PMN gate for a more accurate measurement.

BASIC PROTOCOL 3: Assessment of internalization of microbes by PMNs

Isolated PMN are incubated with unlabeled or pHrodo-labeled microbes on cover glasses for chosen time points up to an hour to allow phagocytosis to occur. For experiments with unlabeled bacteria, cells on cover glasses are subjected to differential staining and internalized microbes are scored using light microscopy. For experiments with pHrodo-labeled microbes, cover glasses are washed, fixed, stained with DAPI, mounted, imaged via fluorescence microscopy, and scored for internalized bacteria.

Materials

Round cover glasses (12mm diameter, No. 1 thickness)

70% ethanol

24-well sterile tissue culture plates

Serum (fresh autologous)

Unlabeled or pHrodo-labeled bacteria (labeled as per manufacturer's instructions)

Refrigerated centrifuge that holds tissue culture plates

Forceps

HARLECO® Hemacolor® Stain Set (Methanol fixative, eosin stain, methylene blue stain)

Hard set mounting media (Vectashield® or ProLong® Antifade)

Phosphate buffered saline without calcium or magnesium (PBS(−)

4% paraformaldehyde

DAPI (4',6-diamidino-2-phenylindole)

Perform internalization assay

1 Clean cover glasses with 70% ethanol and air dry in 24-well plates. Coat cover glasses with 10% serum in HBSS(−) for 1 hour at 37°C and wash three times with HBSS(−) immediately before use.

Precoating cover glasses with serum reduces PMN activation by the glass.

2 Add 1×10^6 PMN to cover glasses in RPMI 1640 containing 10% autologous serum in a total volume of 0.5 ml per well. Incubate for 30 minutes at 37°C to allow PMN adherence to cover glasses.

3 Add 1×10^7 unlabeled or pHrodo-labeled bacteria to PMN.

The multiplicity of infection is 10 microbes per PMN. However, other ratios can be used.

4 Centrifuge plate at 362 × g for 8 minutes at 4°C to synchronize phagocytosis.

5 Incubate at 37°C for phagocytosis.

Depending on the microbe and the mode of internalization, time points between 5–60 min or longer may be required for optimum uptake.

6 Follow differential staining protocol for internalization of unlabeled bacteria (steps 7–10) and fluorescence microscopy protocol for internalization of pHrodo-labeled bacteria (steps 11–17).

Perform differential staining & light microscopy

7 Use forceps to transfer cover glasses to unused 24-well plate containing methanol fixative.

8 Fix in methanol for 1 min, aspirate, add eosin stain for 1 min, aspirate, add methylene blue stain for 1 min, aspirate, and add distilled water.

Gently rotate the plate manually in a circular motion during incubations with methanol, eosin, and methylene blue.

9 Mount cover glasses cell side down on ethanol-cleaned microscope slides using hard set mounting media. When hardened, place slides in dark at 4°C until ready to image.

10 Image with a light microscope at 1000× within 1–2 days. Collect images of 20–25 cells and score the number of bacteria associated with each cell.

This method provides information about bacterial association with PMN, but cannot differentiate between external bacteria on the surface of the cell versus bacteria that has been internalized.

Perform fluorescence microscopy of pHrodo-labeled bacteria

11 Aspirate media from 24-well plate.

12 Wash cover glasses twice with 0.5 ml PBS(−) per well per wash.

13 Fix cells with 0.5ml of 4% paraformaldehyde per well for 5 minutes at room temperature (RT) in the dark.

14 Wash once with 0.5ml PBS(−) per well.

15 Incubate with 0.5ml of 1μg DAPI/ml PBS(−) per well for 5 minutes, RT in the dark.

16 Mount cover glasses cell side down on ethanol-cleaned microscope slides using hard set mounting media. When hardened, place slides in dark at 4°C until ready to image.

17 Image within a few days with an epifluorescence or confocal microscope. Collect images of 100 cells and score for the presence of internalized pHrodo-labeled bacteria.

The pHrodo dye is expected to fluoresce at lower pH (e.g. the phagosome), so positive fluorescence signal should indicate that the bacteria have been internalized. Confocal microscopy can also be used to confirm internalization. However, epifluorescence microscopy should be considered for quantification since confocal microscopy would require imaging and scoring multiple sections of a single cell.

BASIC PROTOCOL 4: Assessment of killing of microbes by PMN

The antimicrobial activity of PMN is essential for normal human health, however there is little consensus on assays to measure this important function. Outcomes of killing assays are dependent upon internalization (see method above) as well as differences between microbes in their resistance to the many different antimicrobial factors of the PMN. The phase of

growth (*e.g.*, mid-log, stationary) of the microbe to be tested is also an important variable and must be standardized to reduce variation.

In this method, *Staphyloccocus aureus* are mixed with neutrophils in a physiologically balanced salt solution containing 10% serum to promote internalization. After incubation of the bacteria with the PMN at 37°C with end-over-end mixing, aliquots are removed at specific time intervals and added to pH 11 distilled water to lyse the PMN. Colony forming units (CFU) are counted on pour plates.

Method

1. Transfer the contents of one vial *S. aureus* (see reagents) using a 1 ml aerosol barrier tip into 50 ml TSB in a 100 ml flask with lid and incubate culture at 37°C for 4 h, monitoring the $O.D._{650nm}$ until the bacteria reach early stationary phase of growth.
2. Transfer *S. aureus* culture into a 50 ml centrifuge tube and centrifuge for 5 minutes at 3000 × g at 4°C.
3. Safely discard supernatant and wash bacteria twice in 50 ml HBSS(−).
4. Resuspend the bacteria in 10 ml of HBSS(+).
5. Transfer and aliquot to a cuvette and read the $O.D._{650nm}$ and adjust the absorbance to the equivalent of 10^8 bacteria/ml.

   Conversion factors should be established for each microbe and spectrophotometer by plating and back-calculating $CFU/O.D._{650nm}$.
6. Prepare two bottles of 375 ml TSA (one bottle serves as a backup) and place in 50°C water bath to prevent the agar from solidifying.
7. Sterilize the 10ml dispenser (*e.g.*, Dispensette III), and 75 × 100 mm disposable glass tubes with metal caps.
8. Label 75 × 150 mm glass tubes and corresponding 60×15mm tissue culture dishes in triplicate. Dispense 10 ml of sterile distilled water, pH 11, into each 75×150 glass tube using the sterile dispenser.

Setup of incubation tubes for bactericidal assay. Label 2 ml conical tubes in triplicate for the inoculum control, normal PMN and patient(s) PMN for each effector:target ratio. As shown below, add PMN, human AB serum, HBSS(+) and *S. aureus* to tubes, adding bacteria last.

	Inoculum control (E:T=1:2)	**Inoculum control (E:T=1:8)**	**NL or PT PMN (E:T=1:2)**	**NL or PT PMN (E:T=1:8)**
	Volume (ml)			
PMN (1×10^7/ml)	--	--	0.5	0.5
AB serum	0.1	0.1	0.1	0.1

	Inoculum control (E:T=1:2)	Inoculum control (E:T=1:8)	NL or PT PMN (E:T=1:2)	NL or PT PMN (E:T=1:8)
	Volume (ml)			
HBSS (+)	0.8	0.5	0.3	--
S. aureus	0.1	0.4	0.1	0.4

10 Place the tubes containing PMN from the normal subject and patients on an end-over-end rotator in a 37°C incubator. Remove a 10μl aliquot (t=0) from each inoculum control tube and add to a 75×150 mm glass tube containing 10 ml of sterile water, pH 11. Vortex vigorously to dissociate cell clumps and ensure uniform distribution. Then place the tubes containing inoculum controls on the rotator in the 37°C incubator.

11 At 20, 45 and 90 min, remove a 10 l aliquot from each tube and add to a 75×150 mm glass tube containing 10 ml of sterile water, pH 11. Vortex vigorously to disrupt cells and ensure uniform distribution. After the aliquot is removed, immediately return the incubation tube to the rotator to continue the incubation.

> For other microbes that may not tolerate pH 11 water, an alternative method of releasing phagocytosed microbes from PMN is to add saponin to a final concentration of 0.5 % and incubate for 10 min on ice before passing the suspension at least 10 times through a 28-1/2-gauge tuberculin syringe, and diluting as appropriate. Stronger detergents may also work if tolerated by control microbes.

12 Transfer an aliquot (see tables below) of the bacterial suspension to the center of a 60×15mm tissue culture dish. These aliquots ensure that the number of colonies/plate fall within a suitable range for counting of bacterial colonies either manually or electronically. For a negative control, transfer 75 μl of distilled water from a 75×150 mm glass tube onto the tissue culture plate.

Plating volumes for 1:2 Effector:Target Ratio—

	Sampling time (min)			
	0	20	45	90
	Aliquot plated (μl)			
Inoculum control	25	--	25	25
Normal PMN	--	25	75	75
Patient(s) PMN	--	25	75	75

Plating volumes for 1:8 Effector:Target Ratio—

	Sampling time (min)			
	0	20	45	90
	Aliquot plated (μl)			
Inoculum control	10	--	10	10
Normal PMN	--	10	25	25
Patient(s) PMN	--	10	25	25

13 Pour 5–6 ml warm agar (50°C) into a 60×15mm tissue culture dish and swirl 8× clockwise and counterclockwise to ensure homogeneous distribution of the aliquot of bacteria suspension in the warm agar before it solidifies.

Bacteria can also be plated on solid media by spreading with sterile glass beads or using an automated spiral plater.

14 Place the plates into a 37°C incubator for 24–72 h or until colonies are large enough to count either manually or using an automated colony counter.

REAGENTS AND SOLUTIONS

All media, reagents, and water should be pyrogen-free.

Hank's Balanced Salt Solution without divalent cations, without phenol red, HBSS(−)

Hank's Balanced Salt Solution with calcium and magnesium, without phenol red, HBSS(+)

Phosphate buffered saline without calcium and magnesium, PBS(−)

RPMI 1640 with 2 mM Glutamine, without phenol red, supplemented with 10mM–25mM HEPES pH 7.4

3% Dextran

Dissolve 15 g pyrogen-free dextran (250,000 to 500,000 MW) in 500 ml HBSS(−) or PBS and sterilize with a 0.2 μm filter. Store at 4°C and pre-warm to room temperature prior to use.

90 %Percoll

Add 1 volume of 10× PBS(−) to 9 volumes of Percoll-Plus (low-endotoxin), mix and store at 4°C.

Luminol

Prepare 50 mM stock solution in DMSO, store in single-use aliquots at ≤−80°C and shield from light.

Lysis buffer

Add 0.83 g NH_4Cl, 0.2 ml of 0.5 M EDTA pH 8.0, and 0.2 g $KHCO_3$ to 100 ml water and sterilize through a 0.2 μm filter.

Flow buffer

Add 0.5 g albumin (human serum, fraction V) and 1 ml of 0.5 M EDTA, pH 8.0 to 500 ml HBSS(−) and sterilize through a 0.2 μm filter.

Catalase (10 mg/ml)

Dissolve 100 mg catalase in 10 ml PBS. Store at ≤−80°C in 100 μl aliquots.

Dihydrorhodamine 123 (DHR)

Dissolve 10 mg DHR in 1.156 ml DMSO (25 mM). Store at ≤−80°C in 25 μl aliquots and shield from light.

Cytochrome c (200 μM)

Dissolve 26 mg cytochrome c in 10 ml 10 mM HEPES pH 7.4 in HBSS(+).

Superoxide dismutase (SOD, 10 mg/ml)

Dissolve 20 mg SOD in 2.0 ml PBS. Store at ≤−80°C in 100 l aliquots.

Phorbol myristate acetate (PMA)

Dissolve PMA in DMSO at 1 mg/ml. Store in single use aliquots at ≤−80°C.

***Staphylococcus aureus* 502A**

Staphylococcus aureus 502A can be purchased from the American Type Culture Collection (ATCC) *S. aureus*, is an opportunistic pathogen and BSL-2 level precautions should be employed during these protocols. Personal protective devices such as safety eyewear, gloves, and lab coat should be worn and a biosafety cabinet is recommended. Dispose of all materials that have come in contact with *S. aureus* by sterilizing in an autoclave or exposure to 10% bleach for at least 30 min.

To standardize growth conditions, inoculate trypticase soy broth (TSB) and measure $O.D._{650}$ at beginning of incubation (time zero). Grow bacteria at 37°C with shaking and measure $O.D._{650}$ every 0.5 h and plot the log $O.D._{650}$ on the y-axis with time on the x-axis. Once bacteria reach early stationary phase, add an equal volume of 20% sterile glycerol. Prepare single use 1 ml aliquots in cryovials and store indefinitely in the vapor phase of a liquid nitrogen freezer.

Pooled AB serum

Commercially sourced AB sera frequently have poor complement activity. Fresh, patient autologous serum can be used for all experiments or, if AB donors can be identified, pools of such serum can be prepared from several donors and flash frozen in single-use aliquots.

Trypticase Soy Broth (TSB)

Dissolve 30 g of trypticase soy medium per liter of water and autoclave 30 minutes and store at room temperature.

Trypticase Soy Agar (TSA)

Add 40 g trypticase soy medium per liter of water and autoclave 30 minutes. Gently swirl after cooling to about 60°C and store at room temperature or cool to 50°C for use in pour plates.

COMMENTARY

Background information

Neutrophil Isolation—The first two protocols provide methods to separate neutrophils from other blood cells using dextran sedimentation and density gradient fractionation. For some endpoints, such as RNA analysis, the presence of small amounts of contaminating cells can contribute significantly to the results. Furthermore, some PMN responses to LPS can be significantly altered by small numbers of contaminating monocytes present in typical PMN preparations (Sabroe et al., 2002; Sabroe et al., 2003) and small amounts of contaminating pyrogen (Haslett et al., 1985). For these reasons, in certain experiments it will be essential to test highly purified PMN depleted of other cell types with magnetic bead sorting or other methods (Parker et al., 2009).

Functional analyses—The methods briefly described here provide techniques for measuring basic functions of neutrophils. The recently updated book Neutrophil Methods and Protocols (Quinn and Deleo) is an excellent compendium of a variety of additional methods covering additional functions of PMN.

Respiratory burst—Activation of the phagocyte NADPH oxidase (NOX2) significantly increases the rate of oxygen consumption by PMN - the so-called “respiratory burst”. Originally, the respiratory burst was measured using a Warburg apparatus to directly measure O_2 consumption. It was later discovered that mutations in the NADPH oxidase lead to chronic granulomatous disease (CGD), a rare primary immunodeficiency resulting in frequent serious infections in CGD patients. Electrochemical probes are available to directly detect O_2, superoxide anion ($O_2^{\bullet -}$) and other O_2-metabolites, however, these methods are generally limited to only a few samples at a time. Currently, most assays of respiratory burst activity rely on indirect detection of reactive oxygen species (ROS) such as $O_2^{\bullet -}$ and H_2O_2 using chemical probes. The nitroblue tetrazolium (NBT) test (described in the previous version of this chapter) remains a useful and low-cost technique although interpretation of results can be difficult and non-quantitative. A wide variety of fluorometric probes are also available to assess ROS production. Unless the experimenter is interested in the specific types of reactive oxygen species produced by PMN (e.g., $O_2^{\bullet -}$, H_2O_2, $^{\bullet}HO$, etc), the assays described here can generally be used interchangeably. However, each measures slightly different aspects of NADPH oxidase activation. For example, cytochrome c measures only extracellular superoxide production, luminol enhanced chemiluminescence measures both intra- and extra-cellular ROS, mostly superoxide, while DHR measures only intracellular production of H_2O_2. While stimuli such as PMA tend to result in both intra- and extra-cellular ROS, particulate stimuli such as zymosan at some concentrations can tend to favor intracellular ROS.

Killing of microbes by PMN—Depending on the microbes tested, it may be desirable to use a differential centrifugation step or an antibiotic such as gentamycin to remove extracellular microbes. In such cases, however, it is essential to perform control experiments to show that under the conditions used, there is no effect of the antibiotic on microbes internalized by permissive PMN (e.g., CGD cells do not kill certain microbes).

Critical Parameters and Troubleshooting

It cannot be stressed enough that rapid isolation of PMN and analysis of their functions as soon as possible after purification are essential for successful studies. Sterile technique helps to reduce contamination of PMN preparations with pyrogens that are known to alter their responsiveness in downstream assays. Avoid vortexing or vigorous mixing of leukocytes as this tends to damage cells and may promote release of granules. The use of HBSS(−) or PBS supplemented with glucose is recommended for short term-storage. Calcium and magnesium should be present at physiologic levels in functional assays but should be avoided during purification to reduce clumping and activation of PMN.

During purification of PMN, hypotonic lysis buffers are generally preferred compared to isotonic NH_4Cl/potassium (ACK) buffers due to the effect of ammonium on phagosomal pH. However, ACK buffers are useful in the DHR assay and in other flow cytometry studies.

Anticipated Results

The yield of PMN from blood can vary considerably, especially in patients with ongoing clinical problems. In general, between $1–2 \times 10^6$ PMN can be obtained per ml of blood. As mentioned above, contamination with eosinophils can also vary considerably with season and donor. In some cases, up to 20–30% of recovered polymorphonuclear leukocytes can be eosinophils. Differential staining should therefore be used to accurately assess purity.

Flow cytometry: Using forward light scattering (FS) and right angle light scattering (SS), PMN can be easily segregated from other leukocyte populations. Stimulation of PMN with PMA results in a decrease in FS with a reduction in the variance. In the DHR assay, PMA stimulation of normal PMN results in a ~two-log increase in the mean fluorescence intensity (MFI) in more than 90% of the PMN.

Time Considerations

Neutrophil enrichment from whole blood can be completed in about 2–3 hrs. Respiratory burst assays require about 30 minutes of hands-on time and 2 hrs for data collection in the luminometer. Internalization kinetics need to be determined for each particle but the assay can be completed in about 2 hrs. Killing kinetics are highly variable and multiple time points should be tested.

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