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Neutrophil (mouse) Isolation Kit

Item No. 601070

www.caymanchem.com
Customer Service 800.364.9897
Technical Support 888.526.5351
1180 E. Ellsworth Rd · Ann Arbor, MI · USA

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GENERAL INFORMATION

Materials Supplied

Kit will arrive packaged as a -20°C kit. For best results, remove components and store as stated below.

Item Number	Item	Quantity/Size	Storage
601071	Sodium Caseinate Solution Assay Reagent (7.5%)	1 vial/5 ml	-20°C
601072	Percoll® Gradient Assay Reagent (63%)	5 vials/5 ml	RT
601077	RBC Lysis Buffer (10X)	1 vial/10 ml	4°C
400086	Bovine Serum Albumin Assay Reagent	1 vial/5 g	4°C
601076	Anti-Mouse Ly6G PE/CD11b FITC Reagent	1 vial/1 ml	4°C

If any of the items listed above are damaged or missing, please contact our Customer Service department at (800) 364-9897 or (734) 971-3335. We cannot accept any returns without prior authorization.

WARNING: THIS PRODUCT IS FOR RESEARCH ONLY - NOT FOR HUMAN OR VETERINARY DIAGNOSTIC OR THERAPEUTIC USE.

Safety Data

This material should be considered hazardous until further information becomes available. Do not ingest, inhale, get in eyes, on skin, or on clothing. Wash thoroughly after handling. Before use, the user must review the complete Safety Data Sheet, which has been sent *via* email to your institution.

Precautions

Please read these instructions carefully before beginning this assay.

If You Have Problems

Technical Service Contact Information

Phone: 888-526-5351 (USA and Canada only) or 734-975-3888

Fax: 734-971-3641

Email: techserv@caymanchem.com

Hours: M-F 8:00 AM to 5:30 PM EST

In order for our staff to assist you quickly and efficiently, please be ready to supply the lot number of the kit (found on the outside of the box).

Storage and Stability

This kit will perform as specified if stored as directed in the **Materials Section** on page 3 and used before the expiration date indicated on the outside of the box.

Materials Needed But Not Supplied

1. Mice - any strain, 6-10 weeks of age
2. 500 ml Balanced Salt Solution (*e.g.*, PBS or HBSS)
3. 70% ethanol
4. 5 ml syringes
5. 21G x 1" hypodermic needles
6. Swinging-bucket tabletop centrifuge (*e.g.*, Sorvall® RT-6)
7. 15 ml conical polypropylene centrifuge tubes
8. 12 x 75 mm polypropylene test tubes
9. Flow cytometer or fluorescence microscope

INTRODUCTION

Background

Neutrophils are a vitally important type of white blood cell (WBC) that is the first line of defense in the innate immune response to many pathogenic organisms. Also known as polymorphonuclear leukocytes (PMN), neutrophils are short-lived inflammatory cells that are highly adept at ingesting and destroying bacteria. They express an impressive array of cell surface receptors that recognize common pathogen-associated molecular patterns (PAMPs). They produce an extensive arsenal of defensive antimicrobial proteins and peptides including lysozyme, myeloperoxidase, neutrophil elastase, cathepsins, and the alpha- and beta-defensins. Neutrophils also form extracellular traps, web-like structures composed of DNA, histones, and various antimicrobial proteins that are extruded from the neutrophil in order to ensnare and kill extracellular pathogens.

In humans, neutrophils comprise ~60% of circulating WBCs. It is commonplace to isolate 1-3 million neutrophils per milliliter of human blood using single-step density centrifugation procedures. Unfortunately, neutrophil isolation is not as easy from mice, in which neutrophils comprise only ~10% of circulating WBCs, and collectable blood volumes are usually limited to ~1 ml per mouse. Therefore, it is usually not possible to isolate even one million neutrophils from a single mouse from peripheral blood. Described below, there are two other options for isolating mouse neutrophils.[1]

Peritoneal Lavage

The first procedure involves injecting an agent into the mouse peritoneal cavity that induces a "sterile" inflammatory response. The inflammatory response is considered "sterile" because the agent that induces it contains no components that are recognized by PAMPs, and therefore does not activate a "danger" signal or antimicrobial response in the neutrophil. The neutrophil responds to this inducing agent by migrating to the injection site, but its antimicrobial defense systems do not become fully active. One of the most common agents that has been used for decades to elicit peritoneal neutrophils is thioglycollate broth, a bacterial growth medium that was commonly found on the lab benches of many early microbiologists.[2] Years later, it was found that the active component of this complex growth medium was the major protein component, casein (sodium caseinate).[3] The injection of a casein solution (7-9%) elicits a sterile inflammatory response that is indistinguishable from the response to thioglycollate broth. Unfortunately, the users of thioglycollate broth would often refer to this simply as "thioglycollate" resulting in much confusion as solutions of pure thioglycolic acid (thioglycollate) are absolutely incapable of eliciting a sterile inflammatory response without adding casein. The use of elicited peritoneal neutrophils is not recommended for all purposes. Despite the use of a "sterile" agent to elicit the response, the neutrophils are slightly more activated than neutrophils isolated from peripheral blood, but far lest activated than neutrophils stimulated with bacterial components such as LPS or fMLP.

Bone Marrow

The second source of neutrophils in the mouse is the bone marrow. Bone marrow in mice contains a relatively high number of neutrophil-like cells. Some are fully mature neutrophils that are poised to migrate to sites of inflammation.[4] The remaining cells are immature neutrophils that are still in the process of development and differentiation. A typical bone marrow preparation from two mouse femurs and tibias might produce 30 million cells of which ~20-40% might have the characteristics of neutrophils. Care must be used when using bone marrow as a source of mature neutrophils as it is easy to isolate the immature neutrophils along with the mature when using negative-selection or density centrifugation procedures.[5]

Identification of Neutrophils

Another source of error or confusion with isolating mouse neutrophils is the method of identification of the neutrophils after performing the isolation procedure. Diff-Quick staining of cells pelleted onto microscope slides, air dried, and fixed is an appropriate method for neutrophil identification. However, this requires the appropriate equipment, reagents, and ability to distinguish a neutrophil from other leukocyte types by microscopic examination. Alternatively, basic two-color flow cytometric analysis will do the job, if the correct antibodies are used. Many early flow cytometric analyses of mouse neutrophils used a Gr-1-specific antibody RB6-8C5 to identify neutrophils. Later, it was discovered that a monocyte subset in mice also expressed Gr-1 and was recognized by RB6-8C5, allowing the misidentification of these monocytes as neutrophils. It was then determined that the Gr-1 antigen was actually a shared epiotope on two distinct cell surface proteins, Ly6G and Ly6C. The Ly6G receptor is expressed almost exclusively on neutrophils, while the Ly6C receptor is more widely distributed. 1A8, an antibody specific for Ly6G has been found to be more selective for staining neutrophils than the Gr-1 antibody RB6-8C5.[6] A combination of Ly6G, CD11b, and high 90° light scatter (side scatter) is often used in combination for definitive neutrophil analysis by flow cytometry.

About This Assay

Cayman's Neutrophil (mouse) Isolation Kit provides the reagents necessary for inducing a sterile inflammatory response in the peritoneal cavities of up to five mice, the density separation medium required to separate the neutrophils from other peritoneal leukocytes, and the fluorochrome-labeled antibodies required to assess the success of the isolation procedure by flow cytometry. The density separation medium, red blood cell lysis buffer, and fluorochrome-labeled antibodies can also be used to enrich and assess mature neutrophils from mouse bone marrow. However, bone marrow contains high-density lymphocytes that will pellet with the mature neutrophils in the density separation medium. Thus, for bone marrow preparations, neutrophil purity will rarely exceed 70%.

PRE-ASSAY PREPARATION

Reagent Preparation

1. **7.5% Casein Solution**

   This vial of Sodium Caseinate Solution Assay Reagent (7.5%) (Item No. 601071) contains a sterile solution of 7.5% (w/v) sodium caseinate in isotonic saline. Open and use under sterile conditions. Warm to 37°C prior to injection. Enough casein is provided to perform five 1 ml injections, sufficient for five mouse preparations. Any unused casein can be stored at -20°C for use at a later time for up to six months.

2. **63% Percoll® Gradient**

   Each vial of Percoll® Gradient Assay Reagent (63%) (Item No. 601072) contains a sterile solution of 63% Percoll®. Open and use under sterile conditions. Warm to room temperature prior to use. Five gradients are provided, sufficient for five mouse preparations.

3. **Red blood Cell Lysis Buffer**

   On the day of use, combine 1 ml of RBC Lysis Buffer (10x) (Item No. 601077) with 9 ml distilled water. Warm to room temperature prior to use. Discard the combined reagent after 48 hours. The remaining unused RBC Lysis Buffer (10x) can be stored at 4°C for one year.

4. **Neutrophil Isolation Medium**

   To prepare the Neutrophil Isolation Medium, add Bovine Serum Albumin Assay Reagent (Item No. 400086) to 500 ml Balanced Salt Solution (PBS or HBSS). Store and use this reagent at 4°C. Sterile filter if storing this reagent for more than two days.

5. **Anti-mouse Ly6G PE/CD11b FITC Reagent**

   Anti-Mouse Ly6G PE/CD11b FITC Reagent (Item No. 601076) is ready to use as supplied at 4°C. Enough antibody solution has been provided to stain 10 samples for flow cytometry.

ASSAY PROTOCOL

Performing the Assay

Elicit and Collect Peritoneal Cells

1. Using a 5 ml syringe fitted with a 21G x 1” hypodermic needle, fill with 7.5% Casein Solution and inject 1 ml into the peritoneal cavity of each mouse. Neutrophils will infiltrate the peritoneal cavity by four hours after injection, peaking approximately 24 hours after injection. After 24 hours, neutrophils will gradually be replaced by inflammatory macrophages in the peritoneal cavity. For best results, we recommend harvesting at 24 hours after casein injection.
2. Euthanize the mouse using an institutionally-approved method.
3. Immobilize the mouse on a dissecting board or other suitable work surface and clean the abdominal fur with 70% ethanol.
4. *Optional*: Make a shallow ventral midline incision through the abdominal skin with scissors (point up) taking care to avoid puncturing the transparent peritoneal wall below. Retract the abdominal skin to either side revealing the intact peritoneum.
5. Using a 5 ml syringe fitted with a 21G x 1” hypodermic needle, fill with 5 ml Neutrophil Isolation Medium. Inject the entire 5 ml into the peritoneal cavity with the bevel of the needle facing up. Take care to avoid puncturing the intestines.
6. Gently massage the peritoneal cavity to dislodge adherent neutrophils. Rotate the syringe 180 degrees so that the bevel faces down and gently withdraw the fluid from the peritoneum. Avoid aspirating abdominal fat or other organs. Move the needle to a new location if it catches a piece of fat or tissue. It should be possible to recover more than 4 ml of the injected 5 ml volume.

Isolate Neutrophils on the Percoll® Gradient

1. Layer the peritoneal lavage fluid slowly and gently on top of the 63% Percoll®, taking care not to mix the fluid layers at the interface. Alternatively, add the peritoneal lavage fluid to a clean 15 ml conical polypropylene centrifuge tube. Add a glass Pasteur pipette to the tube, with the tip resting freely on the bottom. Slowly add the contents of one 5 ml aliquot of 63% Percoll® to the top of the Pasteur pipette, thereby gradually under-laying the peritoneal lavage fluid with the Percoll®. Using a moistened finger, cover the top of the Pasteur pipette so that no air can enter, then withdraw the pipette from the tube and discard.
2. Carefully transfer the tube(s) to a centrifuge with a swinging bucket rotor, taking care not to mix the layers. Centrifuge for 20 minutes at room temperature and no brake at 1,000 x g.
3. Remove from centrifuge. Collect the non-neutrophil cells at the interface between the lavage fluid layer and the Percoll® layer. Save these for flow cytometric analysis or discard as desired.
4. Aspirate remaining Percoll® slowly from the top until lowermost 1 ml remains. Add 9 ml Neutrophil Isolation Medium to this final 1 ml of Percoll®, mix by inversion, and centrifuge again for 10 minutes at room temperature at 500 x g.
5. Aspirate fluid, taking care not to disturb the loose cell pellet. Add 5 ml Red Blood Cell Lysis Buffer and incubate at room temperature for 10 minutes. Centrifuge for 10 minutes at room temperature at 500 x g.
6. Aspirate fluid and resuspend the cell pellet in 1 ml Neutrophil Isolation Medium. This suspension should contain approximately 2-10 million neutrophils (85-95% pure) from each mouse that was used.

Flow Cytometric Analysis of Isolated Neutrophils

1. In a clean polypropylene test tube, add 100 µl of the neutrophil cell suspension and 100 µl of the Anti-mouse Ly6G PE/CD11b FITC Reagent (Item No. 601076). Incubate on ice for 20 minutes.
2. Add 3-5 ml of cold Neutrophil Isolation Buffer and centrifuge at 4°C for five minutes at 500 x g.
3. Aspirate fluid taking care not to disturb the cell pellet. Add 500 µl cold Neutrophil Isolation Buffer and mix to make a cell suspension.
4. Analyze by flow cytometry. The neutrophils will stain brightly positive for both Ly6G and CD11b. Contaminating macrophages, if present, will stain positive for CD11b, but will not stain with the Ly6G antibody. Contaminating lymphocytes, if present, will be negative for both antibodies.

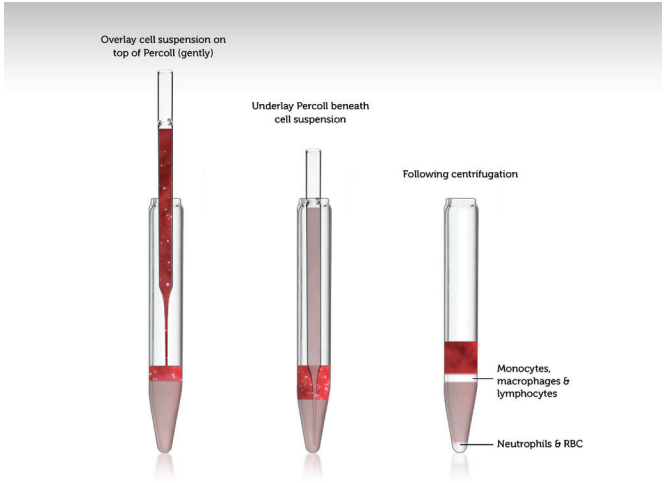

Figure 1. Example of gradient overlay and underlay. The cell suspension can be carefully layered atop the Percoll® solution (left). Alternatively, the Percoll® solution can be underlaid below the cell suspension (center). Either method should produce a layered effect after centrifugation (right).

Performance Characteristics

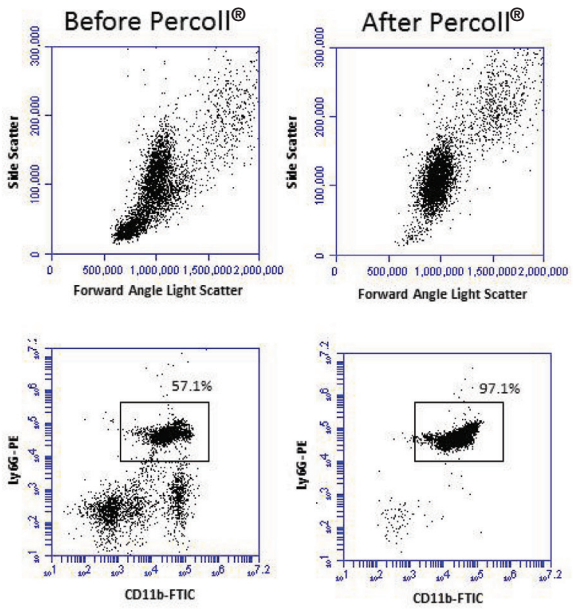

Figure 2. Flow cytometric analysis of peritoneal exudate neutrophils. Casein-elicited peritoneal cells were subjected to Percoll® density separation. Prior to separation, 57.1% of the peritoneal cells were $CD11b^{+}Ly6G^{+}$ neutrophils. After Percoll®, 97.1% of the cells in the pellet were neutrophils. The Percoll®-purified cells also demonstrated forward angle light scatter and side scatter properties consistent with neutrophils.

Troubleshooting

Problem	Possible Causes	Recommended Solutions
Peritoneal lavage cloudy or full of particulate matter	Intestines were punctured during injection or lavage	Discard and treat another mouse
Poor RBC lysis	Insufficient time or temperature for lysis to occur	Transfer the tube to a 37°C water bath for 10 minutes

References

1. Luo, Y. and Dorf, M.E. Isolation of mouse neutrophils. *Curr. Protoc. Immunol.* 3 (Unit 3.20), 1-6 (2001).
2. Baron, E.J. and Proctor, R.A. Elicitation of peritoneal polymorphonuclear neutrophils from mice. *J. Immunol. Methods* **49(3)**, 305-313 (1982).
3. Brummer, E., McEwen, J.G., and Stevens, D.A. Fungicidal activity of murine inflammatory polymorphonuclear neutrophils: Comparison with murine peripheral blood PMN. *Clin. Exp. Immunol.* **66(3)**, 681-690 (1986).
4. Boxio, R., Bossenmeyer-Pourié, C., Steinckwich, N., *et al.* Mouse bone marrow contains large numbers of functionally competent neutrophils. *J. Leukoc. Biol.* **75(4)**, 604-611 (2004).
5. Hasenberg, M., Köhler, A., Bonifatius, S. , *et al.* Rapid immunomagnetic negative enrichment of neutrophil granulocytes from murine bone marrow for functional studies *in vitro* and *in vivo*. *PLoS One* **6(2)**, e17314 (2011).
6. Daley, J.M., Thomay, A.A., Connolly, M.D., *et al.* Use of Ly6G-specific monoclonal antibody to deplete neutrophils in mice. *J. Leukoc. Biol.* **83(1)**, 64-70 (2008).

NOTES

Warranty and Limitation of Remedy

Buyer agrees to purchase the material subject to Cayman's Terms and Conditions. Complete Terms and Conditions including Warranty and Limitation of Liability information can be found on our website.