ORIGINAL ARTICLE

# Optimisation of Laboratory Procedures for Isolating Human Peripheral Blood Derived Neutrophils

**M Maqbool, (MSc)*, S Vidyadaran, (PhD)*, E George, (MBBS, MD)**, R Ramasamy, (PhD)***

*Immunology Unit, **Haematology Unit, Department of Pathology, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia

**SUMMARY**

**Functional analysis of neutrophils requires isolation of these cells in the laboratory. Current isolation procedures are time consuming and can potentially activate the resting neutrophils. Thus, in this present study, we have optimised an existing laboratory protocol for human neutrophil isolation from peripheral blood. Twenty ml of blood samples were subjected to optimised density gradient separation and dextran sedimentation to obtain a pure population of neutrophils. The efficacy of the optimised manual post isolation of neutrophils was compared with pre isolation count performed by an automated haematology analyzer. The recovery of neutrophils via our optimised methods was 65.5% in comparison with neutrophils counts at pre-isolation. The morphological analysis of isolated neutrophils indicated the purity level more than 95% using Leishman staining. Our optimised laboratory procedures for neutrophils isolation successfully harvested neutrophils with good viability, purity and post recovery yield. This procedure provides an ideal platform to separate neutrophils for *in vitro* studies.**

**KEY WORDS:**
*Neutrophils, Density gradient centrifugation, Dextran sedimentation, Laboratory protocol, Cell viability*

## INTRODUCTION

Neutrophils are the primary mediators of rapid innate immune response against most bacterial and fungal pathogens before the acquired humoral and cell mediated immunity are triggered [1].

Neutrophils, also known as granulocytes are the most abundant white blood cells (WBC), comprising about 50–70% of all WBC [2]. They consist of 2-5 lobed nucleus, connected by fine chromatin filaments with an intact cell membrane and granules in cytoplasm [3]. They are spherical with an average diameter of 8-12µm [4]. In an average adult, approximately ten million of new neutrophils are produced and released into the bloodstream from the bone marrow every minute. However, the number of circulating neutrophils remains constant between 3000-4000/mm$^3$, indicating that an equivalent number of neutrophils must be destroyed continuously to maintain a proper balance of neutrophils in peripheral blood. Neutrophils enter the bloodstream and tissues as terminally differentiated cells, which are unable to proliferate and have the shortest half-life (<1day) of all circulating leukocytes [5, 6]. After leaving the bone marrow, neutrophils are programmed to die within about 24 hours as a result of constitutive and spontaneous apoptosis [7].

Neutrophil responses constitute sequenced phases commencing from cellular activation, migration and effector functions. The effector functions performed by neutrophils are formation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) via respiratory burst activity [2].

Assessment of neutrophil functions at laboratory requires a pure or at least enriched population of neutrophils with good viability. Viability of neutrophil becomes a critical issue when the classical ficoll-dextran methods is utilised due to a long processing time and spontaneous activation of neutrophils [8]. Furthermore, the assessment of neutrophils' vital functions such as chemotaxis, phagocytosis and oxidative burst are required an isolated population of neutrophils [9, 10]. Although, the commercially available assay kit to measure the limited aspects of neutrophil function is useful for unprocessed blood sample with small volume however, measurement of neutrophils mediated effector function is still needs an isolated population of neutrophils. Moreover, the isolation process also warrants the pure population of neutrophils which is free from other polymorphonuclear cells such as basophils and eusionophils. Thus, in this paper, we optimised further the ficoll-dextran methods in order to harvest a pure population of neutrophils without inducing activation and cellular death.

## MATERIALS AND METHODS

Twenty ml of human peripheral blood was obtained from thirty healthy donors ranging from 20-35 years old. Vena puncture was performed by a trained phlebotomist. Whole blood was collected in a vacutainer consisting of NH sodium Heparin as anti coagulant (Greiner bio-one, Australia). Samples were obtained with informed consent and processed immediately within 10 min of collection.

The optimisation of laboratory procedures were performed at four consecutive stages as stated below.

*Stage I: Blood collection*

Whole blood was collected and diluted in endotoxin free 1 x Hank's Balanced Salt Solution (HBSS) (Gibco, UK) buffer without calcium and magnesium ions at 1:1 ratio.

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*Corresponding Author: Rajesh Ramasamy, Immunology Unit, Department of Pathology, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, 43400 Serdang Selangor Email: r.rajesh@medic.upm.edu.my*

*Stage II: Ficoll-paque density gradient centrifugation*
Diluted blood was then layered over 5 ml of ficoll-paque solution (GE Healthcare, Life Science, Sweden). Each 100 ml of ficoll-paque contains Ficoll PM400 of 5.7 g, Diatrizoate Sodium of 9.0 g, with Edetate Calcium Disodium in purified water. For optimisation purpose, ficoll centrifugation was performed at various speeds (rcf) for the indicated time and temperature. Based on neutrophils yield and viability, centrifugation at 600 rcf for 15 min at room temperature (RT) with no brakes was opted for further isolation. After centrifugation, four distinct layers were observed which consisted of plasma, peripheral blood mononuclear cells (PBMC), ficoll-paque solution and pallet of sedimented red blood cells (RBC) with granulocytes. The first three layers of plasma, PBMC and ficoll-paque were discarded in order to obtain RBC pellet. The RBC pellet was immediately suspended in 5ml of 1 x HBSS buffer for further processing.

*Stage III: Dextran sedimentation*
Dextran sedimentation was performed with 3% and 6% dextran solution, in order to select the optimal dextran concentration to be used in sedimenting neutrophils. Briefly, red cell and granulocyte suspension was immediately mixed with 3% and 6% dextran (Fisher Scientific, NJ, and USA) and optimal RBC sedimentation was finally achieved at 30 min, RT in dark room. After sedimentation, neutrophil rich supernatant at upper layer was collected and centrifuged for 5 min at 600 rcf at RT (centrifugation time and rcf were also optimised).

*Stage IV: Red blood cell (RBC) lysis*
RBC lysis was performed to obtain a pure granulocyte population. After dextran sedimentation, the remnant or trace amount of RBC was lysed using RBC lysis buffer. Ten times (10X) RBC lysis buffer was prepared by adding 8.3g $NH_4Cl$ (Sigma, Germany), 1.0g $KHCO_3$ (Sigma, Germany), 1.8ml of 5% EDTA (Sigma, Germany) in 1000 ml of sterile water. RBC lysis is a sensitive step in neutrophil isolation, hence optimisation of downstream steps were carried out. Based on optimisation, RBC lysis was performed once for 20 sec (time was strictly monitored using a stopwatch). The lysis process was stopped using 1 x HBSS buffer and centrifuged for 10 min at 400 rcf with no brakes. After centrifugation, supernatant was discarded and the white pellet consisting of granulocytes was obtained and re-suspended immediately in RPMI complete medium (Gibco, United Kingdom). After lysis, the morphological examination and trypan blue exclusion test were performed to determine the cell count and purity of the neutrophils.

Full blood count was performed using KX-21 automated haematology analyzer (Sysmex) with 1µl peripheral human blood and an equivalent neutrophils count was derived from Table II. The automated neutrophil counts obtained were used to compare the total yield of neutrophil recovery from the manual isolation.

Leishman staining was performed to confirm the neutrophil morphology. Few drops of neutrophil suspension was spread over on a glass slide and covered with Leishman solution (Merck, Germany) for 1 min. Subsequently the smear was immersed with phosphate buffer solution for 15 min. The slide was rinsed off with tap water, dried and examined under the light microscope at 20x, 40x and 100x magnifications.

## RESULTS

Neutrophils were isolated from whole human blood using ficoll-dextran method, a routine laboratory procedure for granulocyte isolation [11]. Nonetheless, this method was further optimised at four stages namely blood collection, ficoll-paque centrifugation, dextran sedimentation and RBC lysis by using the additional procedures stated below:

*Stage I: Blood Collection*
The freshly withdrawn venous blood was collected in sodium heparin tubes and immediately processed within 10 min of collection. The window period between blood collection and processing is critical whereby overdue storage will potentially activate neutrophils and may cause RBC lysis thus, obscure the isolation process. Besides that the sample was diluted at 1:1 with sterile endotoxin free 1x HBSS buffer instead of PBS because HBSS prevents neutrophil activation due lack of calcium and magnesium ions in the buffer.

*Stage II: Ficoll-pague centrifugation*
Most of the ficoll-paque density gradient separation process consumes a range of 20-30 min of the centrifugation time [8, 10]. In our optimised protocol, the centrifugation time of ficoll-paque was reduced to 15 min in RT at 600 rcf with no brakes (Table I), to avoid stress and long contact period of cells with ficoll due to its toxic nature.

*Stage III: Dextran Sedimentation*
Our optimisation protocol showed that RBC and granulocyte pellet obtained from ficoll-paque separation need to be subjected for 3% and 6% dextran sedimentation immediately for 20 min precisely (Table I). Since both concentrations indicated a similar sedimentation in terms of sedimentation time and the quality of neutrophil obtained, thus 3% dextran was chosen due to economical reason.

*Stage IV: RBC Lysis*
Finally RBC lysis was performed using RBC lysis buffer and lysing time was strictly monitored for 20 sec to prevent stress on neutrophils. The lysis was terminated using 1 x HBSS and centrifuged for 10 min at 400 rcf with brakes to obtain a well defined pellet (Table I)

Full blood count was performed in parallel to manual isolation method to compare the efficiency of neutrophil yield after isolation. Using 20 ml of blood, an average of the automated count for neutrophils was 98.8 x $10^6$ cells/ml and a manual count after isolation was 63.8 x $10^6$ cells/ml (Fig. 1). The percentage of neutrophils recovery was 65.5%.

Freshly isolated human neutrophils were stained with Leishman dye and studied for their morphology. Cells were viewed under the light microscope as shown in Fig. 2. The morphological observation revealed that neutrophils with intact cell membrane, having 2-5 lobed nucleus which stained dark blue, joined together with fine strands of chromatins and the cytoplasm stained clear light pink consisting of granules. Based on Leishman staining >95% of the cells isolated were neutrophils.

## DISCUSSION

Neutrophils are short lived and highly active cells where the isolation of neutrophils requires careful steps to yield a good

**Table I: Optimised parameters for neutrophil isolation protocol**

| | Stage II<br>Ficoll-paque centrifugation | Stage III<br>Dextran Sedimentation | Stage IV<br>RBC Lysis |
|---|---|---|---|
| Time | 15 min | 30 min | 20 sec |
| Temperature | RT | RT, in dark room | RT |
| Centrifugation | 600 rcf<br>no brakes | 600 rcf<br>5 min | 400 rcf<br>10 min<br>no brakes |
| Concentration | Ficoll PM 400<br>5.7g | 3% dextran | 0.83 mg/ml Ammonium chloride |

**Table II: Absolute neutrophil counts from automated haematology analyzer compared to post isolation counts from optimised manual count**

| Volume | Exp 1 | Exp 2 | Exp 3 | Exp 4 | Exp 5 | Average |
|---|---|---|---|---|---|---|
| **Automated Counts** | | | | | | |
| 1µl | $4.5x10^3$ | $4.3x10^3$ | $6.6x10^3$ | $4.1x10^3$ | $5.2x10^3$ | $4.9x10^3$ |
| 1000 µl | $4.5x10^6$ | $4.3x10^6$ | $6.6x10^6$ | $4.1x10^6$ | $5.2x10^6$ | $4.9x10^6$ |
| 20ml | $90x10^6$ | $86x10^6$ | $132x10^6$ | $82x10^6$ | $104x10^6$ | $98.8x10^6$ |
| **Manual Counts** | | | | | | |
| 20ml | $66x10^6$ | $52x10^6$ | $75x10^6$ | $61x10^6$ | $65x10^6$ | $63.8x10^6$ |
| **% Recovery** | 73.3% | 60.4% | 56.8% | 74.3% | 62.5% | 65.5% |

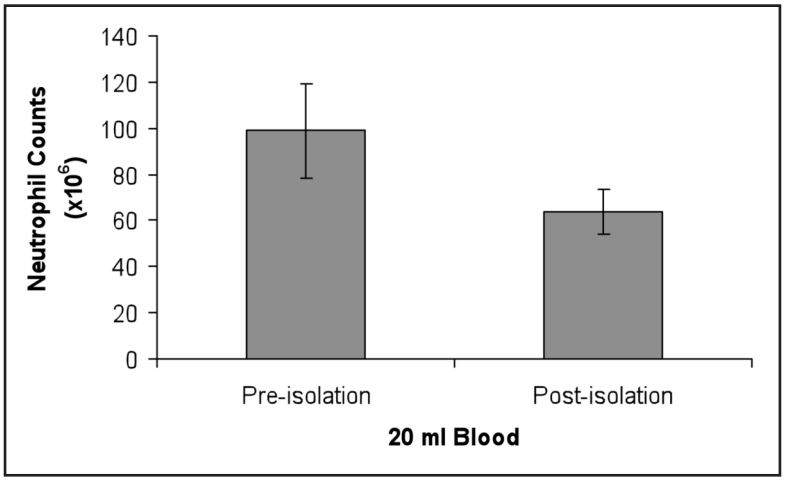


**Fig. 1:** Comparison of neutrophil counts for pre and post isolation. One micro litter (1ul) of peripheral blood was subjected for automated haematology analysis and 20 ml of blood sample was utilised to isolate neutrophil via optimised laboratory procedures. This result is mean ± SD of five repeated individual experiments.

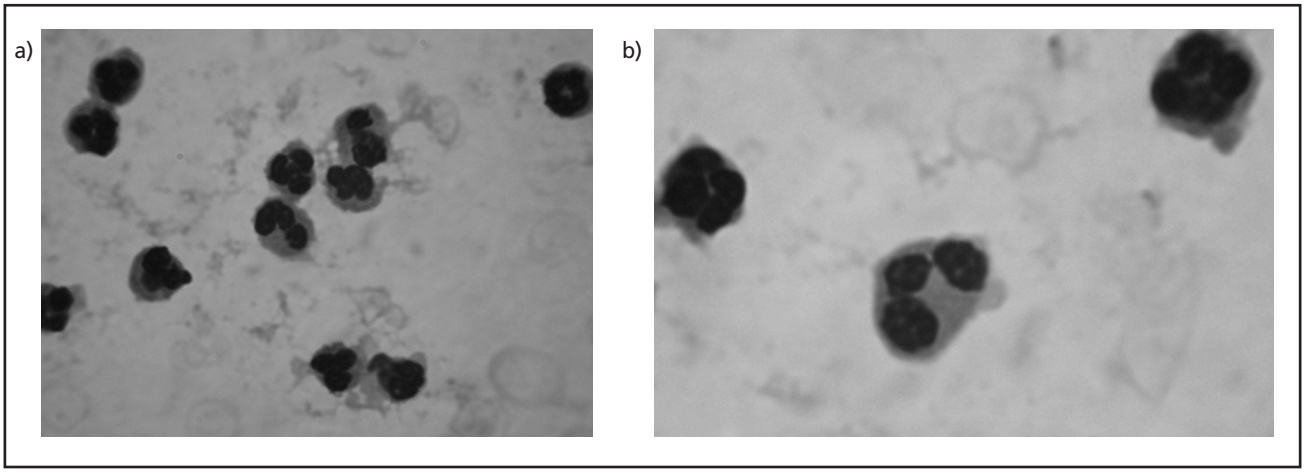


**Fig. 2:** Morphological analysis of neutrophils stained with Leishman staining. (A) Purity of neutrophils at 40x magnification indicates no contamination from other granulocytes (B) More than three lobulated neutrophils at 100x magnification. All microphotographs were taken under the light microscope.

amount of cells within a shorter period of time. To characterize the specific functions of neutrophils, a high purity, fast and reliable method of separating them from other blood cells is desirable for *in vitro* studies. The earlier research work by Ferrante and Thong, 1980 reported that PMN could be isolated from whole human blood through ficoll density gradient separation at concentration of 8.2% and a density of 1.114 g/ml [12]. However, recently Oh and colleagues demonstrated a good yield of neutrophils using commercially available separation media that is a mixture of sodium metrizoate and Dextran 500 [13]. In this study we have optimised neutrophil isolation protocol using the conventional ficoll-dextran method from human blood at four different stages of procedures namely blood collection, ficoll-paque centrifugation, dextran sedimentation and RBC lysis.

Numerous papers have reported that the periods between blood collection and processing time is an important factor as prolong storage of blood sample might triggers the activation of neutrophils which would affect the assessment of functional properties of neutrophils [12, 14]. Our optimised conventional ficoll-dextran procedures minimised the time periods for centrifugation and sedimentation process by optimising the right centrifugal speed, centrifugal dis-acceleration (no break) and temperatures (Table I).

Employing the optimised protocol, we found that the morphology and purity of neutrophils using Leishman staining was >95% which is similar to other study [8]. Although this protocol does not differentiate neutrophils from other granulocytes, Leishman staining clearly revealed that 95% of PMN were neutrophils that exhibit more than 3 nuclear lobules (Fig 2). Apart from the morphological analysis by Leishman staining, post isolation neutrophil counts also were determined by both trypan blue cell counts and compared with pre isolation absolute neutrophil counts which derived from a KX-21 automated haematology analyser. The post isolation yield of neutrophil after the manual isolation showed a recovery rate of 65% in comparison pre isolation absolute counts. This clearly indicated that the optimised neutrophil isolation method is reliable because of the purity (>95%) and recovery (>50%). Our optimised manual neutrophil isolation protocol also was comparable with other commercially available isolation kit. Oh and colleagues have isolated human neutrophils from whole blood using commercially available separation media and showed that their sample yield was >95% neutrophil with >95% viability [13]. Furthermore, this is a reliable and economical procedure and can be used for isolate a pure population of neutrophil for laboratory assessment of neutrophil functions.

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