# FRACTIONATION OF GRANULOCYTES FROM WHOLE HUMAN BLOOD BY CENTRIFUGATION. PRACTICAL HINTS

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*Abstract*. Until now, the two methods used for isolation of white human blood cells are centrifugation and MACS (magnetic antibody cell separation). The first one is most common because of the lower costs and good results; the second one is easier, much faster, but very expensive. Our aim is to present, briefly, the basic principles of gradient centrifugation, the physico-chemical properties of the media we used (Percoll) and some practical hints regarding the granulocytes isolation from whole human blood. We hope to convince the readers that centrifugation/ultracentrifugation is a valuable tool in basic as well as clinically oriented research dealing with neutrophils functions.

*Key words*: neutrophils isolation, centrifugation, density gradients, Percoll, Ficoll.

## INTRODUCTION

Centrifugation can be used for a wide range of application: isolation of viable and pure sperm cells, for in vitro fertilization [4], stem cell isolation for immunotherapy treatment of cancer and chronic infectious diseases [2], sub-cellular fraction. Studies on cells and sub-cellular components (mitochondria, ribosomes, membrane vesicles, nuclei, lysosomes) require not only highly purified suspensions, but, also, highly viability of the cells and the preservation of the biological functions.

The density gradient method of separation has advantages over the normal differential centrifugation; there is no mixture of materials beneath the sample zone, cells and particles of the same size, shape and density sediment as separate zones without convection. For each kind of sample, the density gradient centrifugation may be personalized in order to obtain a complete separation of the components, according to the size (rate zonal centrifugation) or the density (isopycnic centrifugation) of the components, by changing the g-force, sedimentation time or the range of the density gradient interval.

In rate zonal density gradient centrifugation (also termed sedimentation velocity, zone centrifugation) a sample solution containing particles to be fractionated

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is layered on top of the density gradient column. Under centrifugation the particles will start to sediment through the density gradient into separate zones. Each zone consists of particles with the same sedimentation rate. In the rate zonal centrifugation, centrifugation must be terminated before any of the separated zones reaches the bottom of the tube, since the density of some zones may be higher than the highest density area in the density gradient. In isopycnic ("same density") density gradient centrifugation (also termed sedimentation equilibrium centrifugation), the density gradient column encompasses the whole range of densities of sample particles. Each particle will subside only to the position in the gradient where the density in the gradient column equals its own density, and the particle will remain at this position.

In either zonal or isopycnic density gradient centrifugation, a density gradient has to be prepared prior to centrifugation by a hand-layering process or by employing a density gradient device. In the isopycnic method, it is not always convenient to form a gradient artificially and layer the sample on top of the gradient column. It is sometimes necessary to start with a uniformly mixed solution of gradient material and sample. During centrifugation, gradient material redistributes in the tube and forms a linear density gradient. At the same time, sample particles, which are initially distributed throughout the tube, either sediment or float to their isopycnic positions. This type of procedure is termed the self-generating gradient technique.

A number of materials can be used for preparation of the density gradient. They should not alter the cells or particles to be separated and provide a useful density range for separation. The risks are toxicity, osmotic pressure changes and penetration into particles [10].

*Sucrose*. Sucrose is cheap, available in high purity, and yields solutions with a density up to 1.28 g/cm$^3$. Unfortunately, it suffers two major disadvantages: it is very viscous at densities greater than 1.10 g/cm$^3$ and exert very high osmotic effects even at very low concentrations [9] so that cells and sub-cellular particles, which are osmotically sensitive, may band at a different density from their physiological density. The low molecular weight of the sucrose makes it possible to penetrate into cells.

*Polysucrose*. Other substitute compounds have emerged that yield a similar density range, are inert and nonionized and possess low viscosity and osmotic effects. Ficoll is the trade name for a high molecular weight polymer of sucrose and epichlorhydrin produced by Amersham Pharmacia Biotech [1]. However, Ficoll (400,000 molecular weight) gives osmotic gradients with increasing concentrations and this must be compensated by a salt gradient to keep the iso-osmotic condition throughout the centrifuge tube. One variant for blood cells separation is to combine Hystopaque (a mixture of sodium diatrizoate) with Ficoll to form a discontinuous density gradient. Dextran, a natural polymer of glucose, with molecular weights of $10^5$–$10^6$ may be used for cells separation, but the high viscosity makes it difficult to handle. A 40% solution of Ludox (the trade name for colloidal silica produced by Du Pont) is often combined with polysaccharides such as dextran to form gradients that are used to isolate various types of cells.

*Colloidal silica*. The use of colloidal silica was first reported in 1959 [3]. It seems to fulfill the criteria for a performing density gradient media. The result is a rapid growth in the use of Percoll, especially in cell separation. Further we describe two methods for granulocytes separation using density gradient (discontinues and continuous gradient) Percoll centrifugation, developed in our laboratory.

## PHYSOCO-CHEMICAL PROPERTIS OF PERCOLL

The silica in Percoll is a sodium-stabilized colloid, which is polydisperse with particle diameters between 10 nm and 30 nm [7]. Pure silica is toxic to cells and causes hemolysis of red blood cells [11]. The particles are coated with polyvinylpyrrolidone (PVP) to avoid the toxic effect. The molecular weight is $6 \cdot 10^6$ and the osmolality is < 20 mOsm at a density of 1.13 g/ml. Physiological salts can be added to Percoll to keep the osmolarity constant at the density intervals used for cells and particles [9]. The charges on the silica-surface, covered by PVP, allow the particle to act as a polyelectrolyte. In water the PVP is expanded by the counter ions to silica; at higher ionic strength the PVP is closely attached to the silica particles. The viscosity of Percoll depends on the ionic strength, becoming lower in saline solutions compared to water [6]. This is why the self-forming gradients are created much more rapidly in 0.15 M NaCl than in iso-osmotic sucrose.

The gradient of Percoll is formed by the sedimentation of Percoll particles and become progressively steeper with g-force and centrifugation time. Excessive centrifugation leads to the sedimentation of whole silica particles at the bottom of the tube, as a gel. After the gradient formation by high-speed centrifugation, at the bottom of the tube the Percoll is enriched in aggregates and larger particles than at the top, where the particles are smaller in size and diluted. This is the same result as is obtain during ordinary differential centrifugation.

## MATERIALS AND METHODS

### PREPARING THE GRADIENT MEDIUM

The osmolality is a critical variable in the isopycnic separation of granulocytes from whole human blood. Percoll from the bottle must be adjusted with saline or cell culture medium to make it isotonic with the biological material to be separate. To obtain a stock solution of isotonic Percoll, we added 1 part of 10 X concentrated PBS to 11 parts of Percoll. Alternatively, add 0.8 g NaCl per 100 ml Percoll to obtain an osmolality of about 300 mOsm. Osmolality, density and pH of the solutions should be checked routinely.

### SAMPLE LOADING

Sample can be applied on Percoll gradients in three ways (mixing of the sample with gradient medium, sample layered on top of a density gradient or underlayering of sample), depending on the properties of the sample. In our case the sample is whole human blood; we selected a swinging-out rotor centrifuge and layered the sample on the top of perform gradient. The advantage is the reduction of the contamination by cells debrids. Different cells from whole blood subside to their buoyant density, according to their size. The buoyant densities of the cells from whole human blood are very closely (trombocytes 1.04–1.06, lymphocytes 1.06–1.08. granulocytes 1.08–1.09 and erythrocytes 1.09–1.1 g/cm$^3$), so that the density gradient must have a high resolution.

### GRADIENT FORMATION

We tested two different systems to form the gradients: step density gradient and pre-formed continuous gradient.

Step density (discontinuous) gradient centrifugation supposes that two layers of different concentrations (and densities) of Percoll are added in the tube: at the bottom the 72% Percoll solution (72 ml Percoll + 8 ml 10 × PBS + 20 ml 1 × PBS) and above the 63% Percoll solution (63 ml Percoll + 7 ml 10 × PBS + 30 ml 1 × PBS). We added 2.5 ml whole human blood and, after centrifugation (25 minutes, 1500 rpm), the granulocytes separated into a monolayer (Fig. 1).

Continuous gradient of Percoll can be prepared employing a gradient-mixing device or under high forces of gravity, when the Percoll particles sediment. We prepared a pre-forming gradient by ultracentrifugation (Beckman, 20,000 g, 20 minutes at 20 ºC) [5]. After the gradient is formed, the whole blood (2 or 2.5 ml) was added attentively (to avoid the mixture of the blood and Percoll gradient) on the top of the tube and the cells are isolated by centrifugation (1000 g, 20 minutes, 5 ºC) (Fig. 2).

## RESULTS AND DISCUSSIONS

Figure 1 presents the result of separation using the step density Percoll gradient. A step gradient often gives a good separation of the cells: the cells are concentrated at different interfaces as visible bands. One drawback with this variant is that contaminating cells often are trapped at the wrong interfaces and the purity of the desired cell-population (granulocytes in our case) is low. It happens that even is the intention was to use discontinuous gradient, this turns into a continuous one. The reason is: if a large volume of whole blood is layered on top of Percoll and centrifuged, the cells pull serum through the Percoll layers and form a continuous gradient [8].

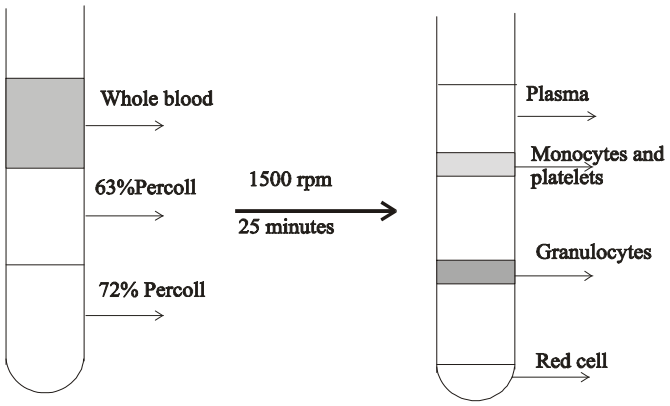


Fig. 1. Step Percoll gradient centrifugation (zonal centrifugation). The granulocytes are separated in a distinct band after centrifugation in a swing-out rotor.

Figure 2 presents the result of separation using pre-formed continuous Percoll gradient. We appreciate this method as being more convenient than the first one (step Percoll gradient centrifugation) because is much faster and easier. Unfortunately, the method is extremely sensitive. The time and speed to be used to forming the density gradient are determined by the length and volume of the tube, the type of rotor, and the density of the initial Percoll solution [6]. One problem with this method may be a bad separation of the lymphocytes band and granulocytes band. In such case, we have to check the properties of the density gradient, especially osmolality and resolution.

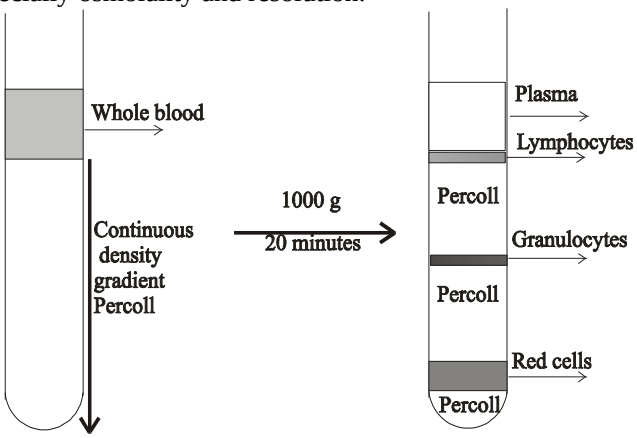


Fig. 2. Continuous Percoll gradient centrifugation (isopycnic centrifugation). The granulocytes are separated in a distinct band after centrifugation in a swing-out rotor.

Both methods for granulocytes separation from whole blood cells are superior to Hypaque-Ficoll gradient centrifugation because avoid hypotonic lysis of red blood cells and dextran sedimentation, which may activate granulocytes. If a bad separation appears between lymphocytes and granulocytes bands with

continuous Percoll gradient, one may perform first a Hypaque-Ficoll gradient centrifugation, to eliminate the lymphocytes. The sediment, compose of granulocytes and red cell may be, further, the subject of continuous Percoll gradient centrifugation.

The purity and the viability of the granulocyte population were the same, using continuous either step density Percoll gradient; we appreciate that the use of the continuous density Percoll gradient as being much faster and we recommend it. The purity of the granulocyte population was checked by hematoxilin-eosine stain (over 98%) and the viability by trypan blue stain (over 95%).

## CONCLUSIONS

In our work we presented two faster and sensitive methods for granulocytes separation from whole human blood. Histopaque-Ficoll centrifugation is the most used method in the majority of the papers dealing with granulocytes research, in the present literature; we hope to convince the readers that Percoll gradient centrifugation is a good alternative. The trick to get a good separation (distinctive bands, high purity and viability of the desired cells, preservation of the functions, short time to work) is to respect the condition of a proper gradient, namely the osmolality, density and resolution.

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