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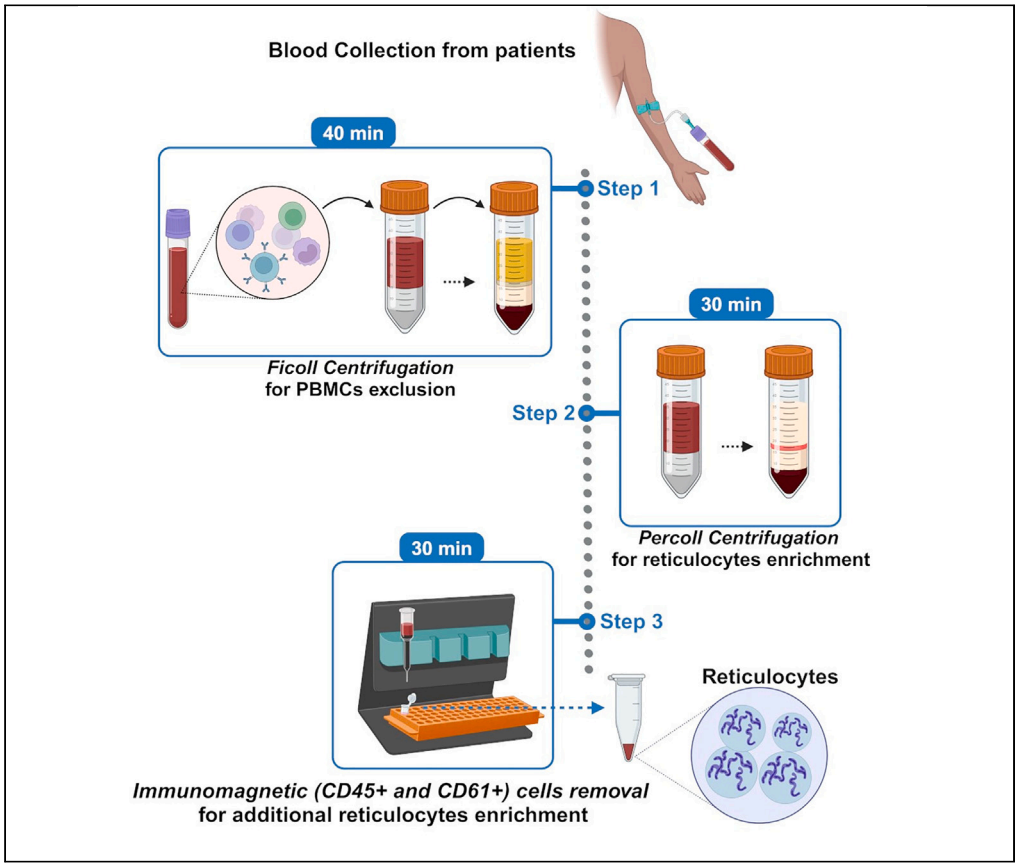
Protocol for reticulocyte enrichment from low-volume human blood samples from alpha-thalassemic and healthy participants



Reticulocyte isolation from peripheral blood is crucial for hematological research. Here, we present a protocol for high-quality reticulocyte enrichment from small blood quantities obtained from alpha-thalassemic and healthy participants. We describe steps for Ficoll and Percoll gradient centrifugation to obtain a reticulocyte-enriched fraction, followed by negative immunomagnetic separation to remove granulocytes and platelets. This technique allows enriched reticulocyte isolation for multi-omics hematological analysis. Additionally, we detail procedures to recover peripheral blood mononuclear cells (PBMCs) and erythrocytes from the original blood sample.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Rapid reticulocyte enrichment from small blood sample

Utilizes Ficoll and Percoll for gradient centrifugation

Immunomagnetic separation for reticulocyte enrichment

Enables comprehensive hematological analysis on reticulocytes

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Protocol for reticulocyte enrichment from low-volume human blood samples from alpha-thalassemic and healthy participants

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SUMMARY

Reticulocyte isolation from peripheral blood is crucial for hematological research. Here, we present a protocol for high-quality reticulocyte enrichment from small blood quantities obtained from alpha-thalassemic and healthy participants. We describe steps for Ficoll and Percoll gradient centrifugation to obtain a reticulocyte-enriched fraction, followed by negative immunomagnetic separation to remove granulocytes and platelets. This technique allows enriched reticulocyte isolation for multi-omics hematological analysis. Additionally, we detail procedures to recover peripheral blood mononuclear cells (PBMCs) and erythrocytes from the original blood sample.

BEFORE YOU BEGIN

The following protocol describes the specific steps for isolating reticulocytes (RET) from small amounts of blood. It was used by our team to isolate RET from peripheral blood (PB, 6 mL) obtained from healthy participants (HP) and patients suffering from hematological diseases. The first step in the protocol is to collect the blood sample using EDTA added tubes (Vacumed K3 EDTA type). The protocol requires several preparatory actions before its execution. These include ensuring a sterile work environment by properly cleaning and disinfecting the laboratory bench and equipment. It is crucial to maintain aseptic conditions to prevent contamination and maintain the integrity of the blood sample. Next, it is essential to gather all the necessary materials and reagents required for the protocol. This includes the appropriate volumes of Ficoll, Percoll, immunomagnetic separation beads, and any other specific reagents mentioned in the protocol. Ensuring the availability and proper handling of these materials before starting the procedure is essential for a smooth execution. Additionally, it is crucial to calibrate and set up any equipment or instruments involved in the isolation process. These include centrifuges, pipettes, and any specialized apparatus required for the specific steps reported in the protocol. Furthermore, it is important to have a clear understanding of the protocol steps and the order in which they need to be performed. By following these preparatory actions, the subsequent execution of the isolation protocol of RET from small blood volumes can proceed easily and effectively. RET are transitional cells between nucleated erythroblasts and red blood cells, present in both bone marrow and peripheral blood. They represent the last precursor of circulating erythrocyte, the mature red blood cell (RBC).[1] The RET count, in both percentage and absolute terms per unit volume, is the main indicator of the level of red blood cells production. It therefore provides a current representation of the bone marrow activity of erythropoietin and, if hemoglobin is reduced, expresses the effectiveness of the erythroid system's response to anemia.[2] The

isolation of RET holds immense potential for effective research and diagnosis of hematological diseases, offering valuable insights into the body's responses, treatment mechanisms, and disease progression. Nevertheless, few approaches exist in the literature for isolating RET from PB.[3–5] Nowadays, current methodologies have different drawbacks, including difficulty in obtaining large blood samples, potential loss or damage of RET due to labor-intensive isolation techniques, and potential contamination of samples due to co-purification of different blood cell types. Currently available methods for separating or enriching RET from whole blood include density-gradient based (Ficoll and Percoll),[6–8] immunomagnetic,[9] and stem cell-based methodologies.[10,11] There is currently no conventional procedure or public information accessible for the extraction of these cells from peripheral blood, at least not for a small initial amount of peripheral blood. Considering the workload, cost, and complexity of the procedures, each approach offers advantages and disadvantages. For stem cell-based methods, CD34+ hematopoietic progenitor stem cells must be isolated and cultured for an extended period, increasing the risk of cells contamination.[12] Also, the isolation of CD34+ cells is more difficult since they are even less common in PB than RET.[13] Furthermore, *in vitro* stem cell production has the potential to alter naïve metabolic pathways, hence introducing bias into the molecular data that are obtained. Density gradient centrifugation methods do not require specific equipment, are simple and less expensive. Immunomagnetic methods provide excellent specificity for targeted detection of cell surface markers in small quantities (< 5 mL), but they are more expensive than density-dependent methods and require specialist equipment. Improved techniques are needed to overcome these drawbacks and ensure more effective and reliable separation of RET from PB samples. In the present study, we describe a 3-step procedure for quality RET isolation from a small PB volume. This approach was successfully applied to obtain a reticulocyte-rich population from both HP and α-T participants.

Institutional permissions

This project received ethical approval from the San Luigi Gonzaga University Hospital Clinical Research Ethics Committee (protocol 04–2022). Written informed consent was obtained from the participants. All steps of the study were explained to each participant. The study was carried out from June 2022 to June 2023.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Cytiva Ficoll-Paque PREMIUM Density 1.077 ± 0.00. g/mL	Cytiva	Cat# 17544202
Percoll Density 1.130 g/mL	Merck	Cat# GE17089102
Phosphate-buffered saline (DPBS, 10×) Dulbecco's formula, without calcium, without magnesium	Thermo Fisher Scientific	Cat# J67653K2
Bovine serum albumin (BSA) 0.5%	Merck	Cat# A3311-50G
MS Columns	Miltenyi Biotec	Cat# 130042201
CD61 MicroBeads, human	Miltenyi Biotec	Cat# 130051101
CD45 MicroBeads, human	Miltenyi Biotec	Cat# 130045801
Critical commercial assays		
iScript cDNA Synthesis Kit	Bio-Rad, USA	Cat# 1708890
TaqMan Universal PCR Master Mix	Thermo Fisher Scientific	Cat# 4304437
SsoFast EvaGreen Supermix	Thermo Fisher Scientific	Cat# 1725200
Oligonucleotides		
Assay on Demand (AoD) human ACTB (beta actin) endogenous control (FAM/MGB probe, non-primer limited)	Thermo Fisher Scientific	Cat# 4333762T
Assay on Demand (AoD) human hemoglobin subunit alpha 2 (Hs00361191_g1)	Thermo Fisher Scientific	Cat# 433182

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Assay on Demand (AoD) human hemoglobin subunit beta (Hs00758889_s1)	Thermo Fisher Scientific	Cat# 4331182
CD45 Forward 5′-GACATCATCACCTAGCAGTTC-3′ Reverse 5′-GCCCTGTCACAAATACTTCTG-3′	Invitrogen	N/A
GAPDH Forward 5′-ACCACAGTCCATGCCATCAC-3′ Reverse 5′-TCCACCACCCTGTTGCTGTAG-3′	Sigma	N/A

MATERIALS AND EQUIPMENT

Buffer PBS/EDTA		
Reagent	Final concentration	Amount
EDTA	2 mM	4 mL Stock solution 0.5 M
PBS	1×	100 mL PBS 10×
ddH_2O	N/A	896 mL
Total	**N/A**	**1 L**
Store at 4°C for up to 3 months.		

Buffer PBS/EDTA/FBS		
Reagent	Final concentration	Amount
EDTA	2 mM	4 mL Stock solution 0.5 M
PBS	1×	991 mL
FBS	N/A	5 mL
Total	**N/A**	**1 L**
Store at 4°C for up to 3 months.		

Buffer Percoll 100%		
Reagent	Final concentration	Amount
Percoll	1.135 g/mL	35.6 mL Percoll Stock solution
PBS	10×	4 mL
HEPES	10 mM	400 µL Stock solution 1 M
Total	**N/A**	**40 mL**
Store at 4°C for up to 3 months.		

Buffer Percoll 70%		
Reagent	Final concentration	Amount
Percoll	70%	42 mL Buffer Percoll 100%
PBS	1×	18 mL
Total	**N/A**	**60 mL**
Store at 4°C for up to 3 months.		

STEP-BY-STEP METHOD DETAILS

Ficoll-Paque centrifugation

Timing: 40 min along with brakes

This section provides detailed instructions for the exclusion of PBMCs from PB. The PBMCs-deprived PB obtained in this step will be used for the next step.

1. Collect 6 mL PB in an EDTA tube.
   a. Keep the tube at 4°C until processing.

⚠ CRITICAL: It is important to process the blood sample within 2 h.

⚠ CRITICAL: It is important to keep all reagents at 4°C.

Optional: You can collect 10–20 μL for one blood smear slide.

2. Transfer blood to a 50 mL tube.
3. Dilute blood 1:1 v/v with PBS-EDTA pH 8 and gently mix with a serological pipette.

Note: Store PBS-EDTA at 4°C for up to 3 months.

4. Add 6 mL Ficoll in a new 50 mL tube.
5. Stratify the diluted blood (Step 3) on the surface of the Ficoll by using a serological pipette.
   a. Tilt the tube and place the pipette on the wall of the tube and slowly stratify the blood.

⚠ CRITICAL: It is important to use the uniform control-speed function of the pipettor.

6. Centrifuge at 400 × *g* for 40 min with reduced acceleration rate (F.i. 7 of 19), and without brake.

Note: Sample will stratify in 3 phases: an upper plasma phase, PBMCs phase ring and, a lower phase which mainly includes RBC and RET and Granulocytes.

7. Aspirate and discard the plasma upper phase above the ring by using a p100-p1000 μL range pipette.

Optional: You can collect the PBMCs ring for further research use.

   a. Remove the remaining supernatant, leaving only the bottom containing the red blood cells and granulocytes.
   b. Add 10 mL of PBS-EDTA pH 8 and centrifuge at 400 × *g* for 8 min at 4°C with reduced acceleration, and without brake.
      i. Remove the supernatant leaving 5 mL of it on top of the pellet and gently and accurately resuspend the pellet.

Percoll centrifugation

Timing: 30 min along with brakes

This section provides detailed instructions for the first step of RET enrichment. The RET phase collected in this step will be used in the next step for further RET enrichment.

8. Add 6 mL of 70% PERCOLL in a new 50 mL tube (prepared fresh and kept at 4°C).

Note: Store Percoll fresh at 4°C for up to 3 months.

9. Stratify the diluted resuspended pellet (Step 7.b.i.) on the surface of the Percoll by using a serological pipette.
   a. Tilt the tube and place the pipette on the wall of the tube and slowly stratify the blood.

⚠ CRITICAL: It is important to use the uniform control-speed function of the pipettor.

10. Centrifuge at 250 × *g* for 30 min with reduced acceleration, and without brake.

Note: Sample will stratify in 3 phases: an upper acellular aqueous phase, a reddish ring that includes RET and Granulocytes and a lower phase which mainly includes RBC.

11. Aspirate and discard the upper aqueous phase above the ring using a p100-p1000 μL range pipette.
12. Aspirate and transfer the RET ring (reddish ring) into a new 15 mL tube.

Note: To collect the whole reddish ring normally you must collect approximately 2–3 mL of volume from the number 10 step tube.

a. Wash the RET ring twice with 1 mL of PBS-EDTA pH 8 and centrifuge, at 550 × *g* for 8 min at 4°C with reduced acceleration, and reduced brake.

⚠ CRITICAL: It is important to gently discard the supernatant after each wash because the pellet is not well adhered to the bottom of the tube.

13. Resuspend in 2 mL PBS-EDTA pH 8.
14. Transfer the suspension into a new 2 mL tube.

Optional: You can collect 20–30 μL for cell counting.

Note: The expected number of cells is detailed in the expected outcomes' section.

15. Centrifuge the suspension (Step 13) in PBS-EDTA pH 8 at 550 × *g* for 8 min with reduced acceleration, and reduced brake and remove the supernatant.

Immunomagnetic CD45+/CD61+ removal for RET isolation

Timing: 30 min

This section provides detailed instructions for immunomagnetic removal of CD45+/CD61+ for RET isolation. The previously collected RET phase is further purified and enriched in this last step.

16. Resuspend the pellet in 80 μL of PBS EDTA-BSA pH 8.8.
    a. Add 20 μL of anti-CD45 and 5 μL of anti-CD61 beads.
    b. Incubate for 15 min at 4°C on a rocking laboratory shaker.

⚠ CRITICAL: It is important to check the MS column Miltenyi protocol to adjust the PBS-EDTA-FBS/CD45/CD61 beads ratio.

17. Prepare Immunomagnetic system using the MS column holder and the columns (one column per sample).
    a. Wash/hydrate the columns with the PBS-EDTA-FBS buffer.

⚠ CRITICAL: It is important that the column is always hydrated until the cell suspension is used, use approximately 500 μL per wash.

Note: The columns are mounted on the holder to fit a 2 mL tube rack below.

Note: Store PBS-EDTA-FBS at 4°C for up to 3 months.

18. After 15 min of incubation at 4°, add 1 mL of PBS EDTA-FBS buffer to labeled cells (Step 16).
19. Centrifuge at 300 × *g* for 10 min at 4°C.

Table 1. Detailed cell count data at the different steps of reticulocyte isolation procedure on samples obtained from two healthy participants and two alpha-thalassemia participants

		HP1	HP2	α-T1	α-T2
Participants' samples		Healthy	Healthy	Alpha-T	Alpha-T
Whole blood	RBC (10^6/μL)[a]	4.49	5.24	4.82	5.41
	RET%[a]	1.3%	1.7%	5.4%	5.9%
	NEUT (10^3/μL)[a]	2.15	2.96	4.16	2.45
	LINF (10^3/μL)[a]	1.16	3.37	1.37	1.30
	MONO (10^3/μL)[a]	0,48	0.41	0.25	0.17
	EOS (10^3/μL)[a]	0.19	0.29	0.13	0.03
	BASO (10^3/μL)[a]	0.05	0.06	0.06	0.01
	PT (10^3/μL)[a]	232	255	113	169
PRE-immunomagnetic separation	Cell Count (N°)[b]	$4.6*10^6$	$1.1*10^6$	$5.7*10^6$	$1.8*10^6$
	RET%[c]	34.1%	24.7%	42.1%	40.8%
	RBC%[c]	63.7%	66.4	57.9%	59.2%
	NEUT%[c]	2.2%	8.9%	0.5%	0.4%
	PT[c]	present	present	present	present
POST-immunomagnetic separation	Cell Count (N°)[b]	$3.5*10^6$	$865*10^3$	$4.1*10^6$	$1.7*10^6$
	RET%[c]	30.3%	28.9%	43.8%	42.5%
	RBC%[c]	69.7%	71.2%	56.2%	57.5%
	NEUT%[c]	0%	0%	0%	0%
	PT[c]	absent	absent	absent	absent

HP, healthy participants; α-T, alpha-thalassemia participants; RBC, Red blood cells; RET, RET; NEUT, Neutrophils; LINF, lymphocytes; MONO, Monocytes; EOS, Eosinophils; BASO, Basophils; PT, platelets.
[a]Blood count analysis with automatic hemocytometer.
[b]Trypan Blue dye.
[c]Cytospin stained with New Methylene Blue (NMB) dye.

20. Discard the supernatant and resuspend the pellet in 500 μL PBS- EDTA-FBS buffer.
21. Prepare a 2 mL tube under each column to collect the flow through.
22. Load the sample onto the column.
    a. After collecting the flow through, wash the columns by adding 500 μL of PBS-EDTA-FBS buffer (3 times).

Optional: You can collect 20–30 μL for cell counting.

Note: The expected number of cells is detailed in the expected outcomes' section.

23. Centrifuge the suspension at 300 × *g* for 10 min at 4°C.
24. Store the pellets at −80°C.

EXPECTED OUTCOMES

The reticulocyte isolation protocol described in this study is expected to yield an enriched RET pure population from small-volume PB samples. In our study the described procedure was applied to PB samples from HP and α-T patients. To assess and verify the quality and purity of the enriched reticulocyte population, two approaches were used.

First, a morphological and quantitative characterization was performed by using a specific supravital staining for RET (new methylene blue dye) to verify the enrichment of RET population at different steps of the procedures. The results obtained are described and included in Table 1 and in Figure 1.

Second, a molecular analysis was performed, by evaluating reticulocyte RNA yield and purity and the RNA expression levels of some specific genes. Reagents and steps for reverse transcription (RT) and

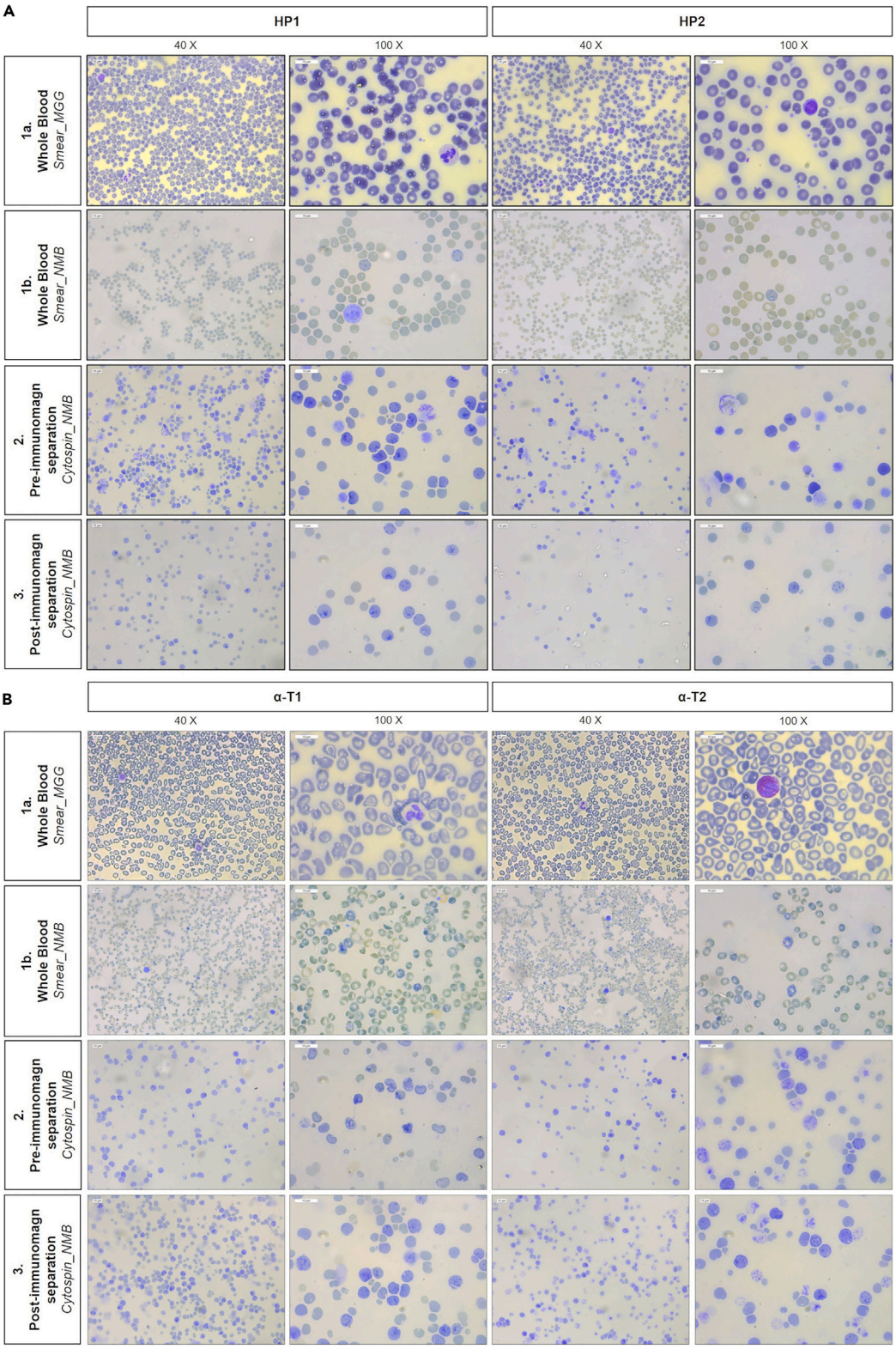

Figure 1. Reticulocyte images at the different steps of enrichment procedure from PB of two HP and two α-T participants

Reticulocyte images at the different steps of enrichment procedure from PB of two HP (A) and two α-T participants (B). Microscope images were acquired at the main different steps of reticulocyte enrichment procedure: 1) Whole blood smears were stained with both classical May-Grünwald

Figure 1. *Continued*

Giemsa dye (1a) and New Methylene Blue dye (1b), a supravital dye which stains the reticulo-filamentous RNA material, differentiating RET from erythrocytes; all PB cell population are detected (erythrocytes, leucocytes, platelets and RET) at different percentages. 2) Pre-immunomagnetic separation: enriched reticulocyte cells obtained after Ficoll and Percoll centrifugation steps were stained with NBM and cytospins were prepared (200000 cells, 700 rpm × 3 min, low speed); though erythrocytes and RET are the most abundant cell populations, platelets and neutrophils are still present (as described in Table 1). 3) Post-immunomagnetic separation: enriched purified reticulocyte population obtained after CD45+ and CD61+ immunomagnetic separation were stained with NBM and cytospins were prepared; RET and erythrocytes are present, while platelets and neutrophils were removed (Table 1). Scale bars are shown in each panel. Magnification 40× and 100×. HP, Healthy Participants; α-T, alpha-thalassemia participants; MGG, May Grünwald-Giemsa; NMB, New methylene Blue.

real-time PCR analysis are described in the quantification and statistical analysis section. Results are shown in Figure 2.

Table 1 shows an assessment of several parameters describing the main cell population types at the different stages of reticulocyte isolation procedure. The table shows data obtained from two HP and alpha thalassemia patients. The reticulocyte count percentage in whole blood (blood count analysis) were 1.3% and 1.7% in HP, while higher RET were observed in two α-T participants, equal to 5.4% and 5.9% respectively (Figures 1A and 1B, row 1a and 1b show whole blood staining). The first and second steps of the protocol, represented by Ficoll and Percoll gradient centrifugation, allow the enrichment of RET restricted to whole blood. In particular, 34.1% and 24.7% of RET were obtained from HP and 42.1% and 40.8% were isolated from α-T people (Figures 1A and 1B, row 2 show immunomagnetic pre-separation staining). Notably, in this step, RBC, WBC, and platelets were still present in the sample, contaminating the reticulocyte population. A further purification step using immunomagnetic separation to remove granulocytes and platelets significantly improved the purity of RET population. At the end of the procedure, a final reticulocyte-rich suspension containing 30.3% and 28.9% reticulocytes in HP and 43.8% and 42.5% in α-T participants was obtained (Figures 1A and 1B, row 3 show post-immunomagnetic separation staining). Notably, we

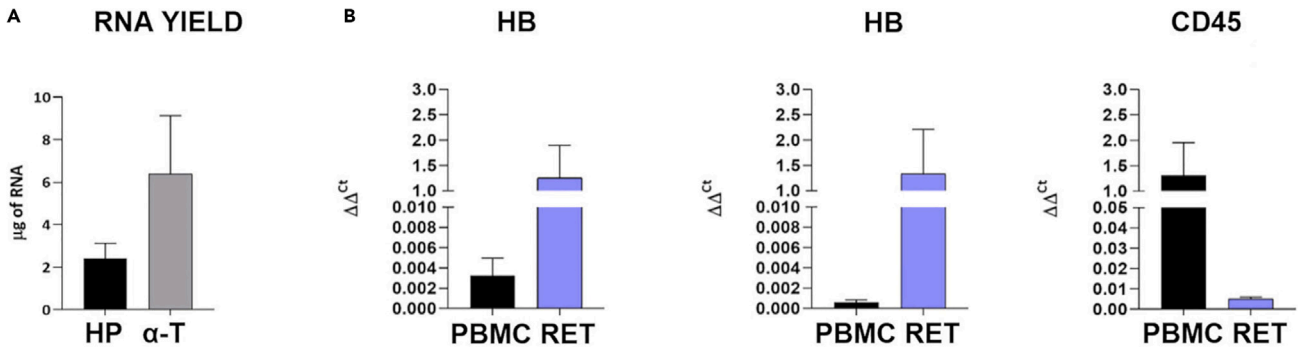

Figure 2. RNA yield and gene expression analysis

RNA yield (A) and gene expression analysis (B).

(A) The mean amount of extracted RNA from reticulocytes samples was 2.420 µg ± 1.004 from HP and 6.385 µg ± 3.868 from α-T participants. SEM error bars are shown in the graph.

(B) After cDNA synthesis, the RNA transcripts of Hemoglobin Alpha (HBA), Hemoglobin Beta (HBB) and CD45 and the housekeeping genes Beta Actin (ACTB) and Glyceraldehyde-3-phosphate Dehydrogenase (GAPDH) were assessed to verify the purity of the reticulocyte population; the same transcript levels were assessed in PBMCs, obtained during Ficoll centrifugation, as control. In particular, HBA and HBB were used as marker of reticulocyte population, while CD45 as leucocyte marker. The real time PCR results demonstrate the expression of HBA and HBB transcripts in RET, while as expected, no expression was observed in PBMCs. Conversely, CD45 RNA was highly expressed in PBMCs, while low levels were detected in RET. SEM error bars are shown in each graph. These data demonstrate that the enriched reticulocyte populations (post-immunomagnetic separation) are not contaminated by leukocyte RNA, confirming the findings of hematological staining. Real-time PCR reactions for each gene were performed in duplicate and were carried out on the CFX96 real-time system c1000 touch-thermocycler (Bio-Rad, USA). A negative control of amplification was run in each amplification plate. Mean Ct values were analyzed by the comparative Ct-method. Undetermined values were empirically set to 45 for Delta-Ct analysis. The technique described allows to obtain a population enriched in RET, making it ideal for studying these cells in hematological and therapeutic experimental settings. Furthermore, this procedure allows to recover other cell populations (e.g., PBMCs and erythrocytes). By effectively isolating RET from whole blood, the described protocol enables to obtain sufficient biological material (cells, RNA, protein, etc.) for different subsequent downstream molecular, metabolomics, proteomics applications. This allows comprehensive in-depth investigations of biological markers associated with reticulocyte function or disease.

observed that the purification of RET from alpha thalassemia patients' samples is relatively simpler due to the higher reticulocyte counts compared to HP.

QUANTIFICATION AND STATISTICAL ANALYSIS

Reverse transcription

Reverse transcription (RT) was performed to synthesize cDNA from reticulocytes and PBMCs. Reagents and reaction protocol are detailed in the following tables.

RT reaction master mix

Reagent	Amount
RNA template	2 μg
iScript Reverse transcriptase	2 μL
5× iScript Reaction Mix	8 μL
Nuclease-free water	variable
Totale volume	**40 μL**

Reaction Protocol		
Steps	Temperature	Time
Priming	5°C	5 min
Reverse transcription	46°C	20 min
RT inactivation	95°C	1 min
Optional step	4°C	Hold

Real-time PCR analysis

After cDNA synthesis, the RNA transcripts of Hemoglobin Alpha (HBA), Hemoglobin Beta (HBB) and CD45 and the housekeeping genes Beta Actin (ACTB) and Glyceraldehyde-3-phosphate Dehydrogenase (GAPDH) were assessed. Primer sequences are listed in the key resources table section. Real-time PCR reactions for each gene were performed in duplicate and were carried out on the CFX96 real-time system c1000 touch-thermocycler (BioRad, USA).

HBA, HBB and B-Actin were assessed by using Taqman gene expression assays; Real-Time reagents and protocol are described in the following tables.

PCR reaction master mix for each sample	
Reagent	Amount
TaqMan Universal PCR Master Mix	5 μL
AoD (HBA, HBB or β-ACTIN)	0.5 μL
Nuclease-free water	variable
cDNA template	3 μL
Total volume	**10 μL**

PCR cycling conditions			
Steps	Temperature	Time	Cycles
UNG incubation	50°C	2 min	1
Polymerase activation	95°C	10 min	
Denature	95°C	15 s	40
Anneal/extend	60°C	1 min	

CD45 and GAPDH were assessed by using SYBR-green assays; reagents and protocol are described in the following tables.

PCR reaction master mix for each sample	
Reagent	Amount
SsoFast EvaGreen Supermix	5 μL
Forward primer (CD45 or GAPDH)	0.5 μL (500 nM)
Reverse primer (CD45 or GAPDH)	0.5 μL (500 nM)
Nuclease-free water	variable
cDNA template	3 μL
Total volume	**10 μL**

PCR cycling conditions			
Steps	Temperature	Time	Cycles
Enzyme activation	95°C	30 s	1
Denaturation	95°C	5 s	
Anneal/extend	60°C	20 s	35

LIMITATIONS

This methodological paper describes a protocol for RET enrichment from 6 mL of PB using Ficoll and Percoll density gradient centrifugation as well as magnetic beads conjugated with antibodies to exclude granulocytes and platelets. Although the protocol offers a useful method, it is crucial to take into account a few restrictions, such as mechanical and environmental limits, that might have an impact on the success and validity of the results. Another factor that should be taken in consideration is the diversity in circulating Reticulocyte population: RET are a heterogeneous population in terms of maturation stages and cellular properties. Researchers should acknowledge of this potential variability and consider its influence on the interpretation of their results. Lastly, it should be taken in consideration that the present protocol succeeds in enriching RET population, although RBCs could remain in the final pellet.

Environmental factors

Temperature: The temperature at which the protocol is conducted can impact cell viability and the efficiency of the isolation process. Variations in reagent temperature during sample processing, centrifugation, or magnetic separation steps may affect cell integrity and the purity of the isolated RET.

Mechanical constraints

1. **Centrifugation Speed and Time:** The centrifugation steps using Ficoll and Percoll gradients are critical for separating diverse cells based on the density. However, deviations from the suggested centrifugation speed or duration may lead to imperfect separation or merging of cell layers.
2. **Immunomagnetic Separation Efficiency:** The effectiveness of immunomagnetic separation and the binding capacity of the magnetic beads conjugated with antibodies can be affected by following factors such as the strength of the magnetic field, incubation duration, and sample amount. Insufficient magnetic force and/or incubation may hinder the elimination of granulocytes and platelets, influencing the purity of the isolated RET.

It is essential to consider the described limitations which may introduce variability and possible causes of error in the isolation procedure. In summary, the protocol provides a systematic method for RET isolation, environmental factors such as temperature and, mechanical constraints related to centrifugation and immunomagnetic separation steps, should be stringently considered. By

following these limitations, we confirm the reproducibility and success of the procedure in achieving enriched RET populations for downstream analyses.

TROUBLESHOOTING

Problem 1

Blood Sample Processing Delay.

Potential solution

If the blood sample is not processed within 2h window period, the cell viability can decrease. Ensure timely processing to preserve cell integrity.

Problem 2

Temperature Control.

Potential solution

It is essential to store all reagents and samples at 4°C. Deviations can impact cell separation and viability. Preserve the sample by storing it at the appropriate temperature until use.

Problem 3

Dilution and Mixing: Insufficient dilution or mixing may result in improper layering.

Potential solution

Ensure a 1:1 v/v dilution with PBS-EDTA pH 8 and mix gently but thoroughly.

Problem 4

Layering Technique: Imperfect layering of blood over Ficoll can disturb the interface.

Potential solution

Tilt the tube and use a serological pipette for controlled layering the blood against the tube wall.

Problem 5

Centrifugation Speed and Time: Centrifuging at different speeds or time may lead to insufficient separation.

Potential solution

Follow to the specified 400 × g for 40 min without brake for Ficoll, and 250 × g for 30 min without brake for Percoll.

Problem 6

Plasma Phase Aspiration: Aspirating too much or too little of the plasma phase can contaminate the PBMC layer or result in loss of cells.

Potential solution

Use a precise pipette and carefully remove the plasma without disturbing the PBMC ring.

Problem 7

Percoll Preparation.

Potential solution

Ensure that the 70% Percoll is prepared fresh and kept at 4°C before use.

Problem 8

Reticulocyte Ring Handling: the reticulocyte ring is delicate.

Potential solution

Wash and centrifuge gently to avoid losing cells.

Problem 9

Immunomagnetic Separation.

Potential solution

Follow the Miltenyi protocol precisely for bead-to-cell ratio and ensure the columns are hydrated properly before use.

Problem 10

Column Washing: Inadequate washing can lead to nonspecific binding.

Potential solution

Wash the columns three times with PBS-EDTA-FBS buffer after elution.

Problem 11

Sample Storage.

Potential solution

Store the final pellets at −80°C promptly to prevent degradation.

Remember to follow steps crucially and maintain uniform control-speed function of the pipettor to ensure the integrity of the cell layers. If the problems persist, consider the possibility of repeating the protocol from the beginning, ensuring first that the patient can repeat the peripheral blood sampling, you can consult us for any further clarifications.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Stefano Comità (stefano.comita@unito.it).

Technical contact

Technical questions on executing this protocol should be directed to and will be answered by the technical contacts, Stefano Comità (stefano.comita@unito.it) and Paola Valentino (paola.valentino@unito.it).

Materials availability

This study did not generate new reagents.

Data and code availability

This study did not generate/analyze datasets/code.

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AUTHOR CONTRIBUTIONS

Conceptualization, A.R.; investigation, A.R., S.C., and P.V.; funding acquisition, A.R. and G.B.F.; supervision, A.R. and G.B.F.; writing – original draft, S.C., P.V., and N.K.; review and editing, S.C., P.V., N.K., T.C., H.K., G.B.F., and A.R.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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