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## Preparation of Peripheral Blood Mononuclear Cell Pellets and Plasma from a Single Blood Draw at Clinical Trial Site for Biomarker Analysis

--Manuscript Draft--

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Dear Dr Bajaj,

Please, find below the answers to your comments and some further considerations. Many thanks for taking the time to review and improve our manuscript.

Best regards,

Paola

Line 138: *I have adjusted the highlight to match the title. Please review*.

I have reviewed the highlight and agree in most of the cases, but please find some suggestions herein.

Line 153: *Notes cannot be filmed, so removed highlight:*

That is fine but I was wondering whether we could perhaps have a moment in the film showing all the elements required and also mention or clearly show very briefly the centrifugation units.

If necessary, both steps 1.3 and 3.1.5 could be removed.

Line 202: *Removed the bioprotocol calculator reference as we cannot have commercial terms in the manuscript. If you would like to include the calculator details please include in the table of materials and add here: Use the online calculator (see Table of Materials) for the conversion.*

Thank you for the suggestion. I have added the reference in the table accordingly.

Line 273 (my suggestion): Could we also film this step (3.10) if we process 4 or 5 samples? If that is the case 1 would be for cell pellet, another for cryopreservation and the rest for irradiation. Or if starting filming from section 6 (see comment on note from line 315), only 4 tubes would be required: 1 for cryopreservation the 3 for irradiation would be used for cell pellets and lysis.

Line 280: *If 4.1 is highlighted substeps showing how to perform this need highlighting as well. Done in this case. Please check.*
That is fine, thank you!

Line 315: *Adjusted the highlight to make a cohesive story and to match the title.*

Yes, that is fine, thanks! However, if we film steps 6.2-6.4, should we film them before step 2.3? Or could we not film them and only film how to generate cell lysates and load them for western blot analysis?

Line 379*: Please hide the thermo scientific label from the figure as we cannot have commercial terms in the manuscript.*
The labels are now covered with boxes. Thanks!

Line 390: *Citations?  Else just reword to downstream analysis instead.*
Referring to the citation of obtention of plasma samples for metabolomics or ctDNA sequencing, I have now included 2 citations more. Thanks for this comment.

Line 411: *This study needs a citation. Please include. Also please ensure the citation number following this are adjusted to make it in order.*
I have now cited the study using the WHO guidance on how to cite clinical trials and changed the number of the other references accordingly.

Line 492: *Please include a reprint permission to reuse data from the clinical trial study.*
These data have never been published before, I have generated them and I have asked the study team, which have confirmed I should only cite the study. The citation is included in the references. Thanks! I have now cited the study using the WHO advice on how to cite clinical trials and changed the number of the other references accordingly.

Line 497: *Please expand on the limitation in few sentences.*
Discussion on limitations has been expanded now (lines 558-570).

**TITLE:**

# Preparation of Peripheral Blood Mononuclear Cell Pellets and Plasma from a Single Blood Draw at Clinical Trial Site for Biomarker Analysis

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**KEYWORDS:**

peripheral blood mononuclear cell, PBMC, plasma, clinical trial, biomarker, mononuclear cell preparation tube, pharmacodynamics, translational science, DNA damage response, DDR

**SUMMARY:**

This protocol details clinically implementable preparation of high quality PBMC and plasma biosamples at the clinical trial site that can be used for translational biomarker analysis.

**ABSTRACT:**

Analysis of biomarkers in peripheral blood is becoming increasingly important in clinical trials to establish proof of mechanism to evaluate effects of treatment, help guide dose and schedule setting of therapeutics. From a single blood draw, peripheral blood mononuclear cells can be isolated and processed to analyze and quantify protein markers, and plasma samples can be used for the analysis of circulating tumor DNA, cytokines, and plasma metabolomics. Longitudinal samples from a treatment provide information on the evolution of a given protein marker, the mutational status and immunological landscape of the patient. This can only be achieved if the processing of the peripheral blood is carried out effectively in clinical sites and samples are properly preserved from the bedside to bench. Here, we present an optimized general-purpose protocol that can be implemented at clinical sites for obtaining PBMC pellets and plasma samples in multi-center clinical trials, that will enable clinical professionals in hospital laboratories to

successfully provide high quality samples, regardless of their level of technical expertise. Alternative protocol variations are also presented that are optimized for more specific downstream analytical methods. We apply this protocol for studying protein biomarkers against DNA damage response (DDR) on X-ray irradiated blood to demonstrate the suitability of the approach in oncology settings where DDR drugs and/or radiotherapy have been practiced as well as in preclinical stages where mechanistic hypothesis testing is required.

**INTRODUCTION:**

Drug development aims to deliver new therapeutics addressing unmet medical need and more targeted, personalized medicine. Multiple drug mechanisms are under active investigation including enzymatic inhibition such as kinase[1], protease[2], or PARP inhibitors[3], protein degraders[4], therapeutic antibodies[5], and antibody-conjugated drugs (ADCs)[6], among many others. An example of the efforts to obtain better treatments in oncology is the use of kinase inhibitors with the goal of stopping the signaling cascades that keep cancerous cells proliferating[1,7]. Measuring the levels of substrate phosphorylation specific to those kinases is the best pharmacodynamic biomarker to quantify the mechanism of action of these inhibitors[8]. Other drugs may modulate the expression of a given protein, and in that case being able to quantify the changes in concentration of their target protein longitudinally throughout the course of treatment is paramount. Therefore, independent of the characteristics of a drug or a pathology, evaluation of biomarkers to establish the pharmacokinetics (PK)/ pharmacodynamics (PD) relationship between drug exposure and target modulation is the best practice in early clinical development and enables the determination of a safe and tolerated pharmacologically active dose/schedule[9].

While in oncology clinical development, biomarker analysis in biopsies might be the best setting to establish proof of mechanism of a drug, the number of available biopsies in a trial is usually quite limited[10,11]. Alternatively, peripheral blood samples are highly valuable to clinical trials because they involve a minimally invasive procedure, are quick and easy to obtain facilitating longitudinal analysis, are less expensive than biopsies and provide a vast information for real-time monitoring of the outcome of a treatment. An additional benefit to assessing PD biomarkers in peripheral blood is the capacity to use the biosample to also quantify PK allowing exactness in determining PK/PD quantitative relationships and subsequent PK/PD modeling[12,13]. Peripheral blood mononuclear cells (PBMCs) from whole blood can be isolated to study protein markers, which experience either changes in their expression level or in their post-translational modifications. In addition, PBMCs can be used for immunophenotyping purposes[14,15], immune functionality assays such as assessing antibody-dependent cellular cytotoxicity (ADCC)[16] and epigenetic analysis through RNA isolation. Likewise, plasma from whole blood can be used to quantify cytokines to characterize the immunological response of a patient, to perform metabolic studies, and also to isolate and sequence circulating tumor DNA (ctDNA) for monitoring the clonal evolution of disease under selection from the therapeutic agent, frequently providing a mechanistic basis for treatment resistance[17-19] enabling development of subsequent generations of therapeutics[20]. Finally, isolation of circulating tumor cells (CTCs) from peripheral blood allows for the evaluation of disease progression by longitudinal enumeration, DNA/RNA sequencing and protein-biomarker analysis[21]. Although this isolation is compatible with the protocol described herein[22], the low abundance of CTCs in many cancer types and early stages of the disease makes the use of specialized tubes more suitable by minimizing CTC degradation[23].

In recent years, the use of liquid biopsies has improved the information obtained in clinical trials and PBMC collections have been included in many studies to monitor target engagement and proof of mechanism either directly in tumor cells for some types of hematological malignancies, or on the PBMCs themselves as PD surrogates of tumor cells[24-26]. The preparation of high-quality samples positively impacts determining the safest and most efficacious treatment for a given pathology but in our experience, the quality of PBMC preparations obtained from different clinical sites has been subjected to wide variability in quality resulting in samples that do not fit for the purpose of downstream analysis. This has impacted the amount of PD data that could be collected from those studies.

Here we describe in detail an easy to follow protocol that shows how to efficiently isolate both PBMCs and plasma samples from a single blood draw in a clinical setting. The protocol is based on the instructions provided by the manufacturer of the mononuclear cell preparation tubes, which incorporates modifications where real-world experience has highlighted difficulties in protocol execution as reported by clinical sites, such as centrifugation issues, processing delays and sample transfer to cryovials. There are alternative commercially available methods to the use of mononuclear cell preparation tubes based on the density gradient separation using polysaccharide solutions with or without a barrier that separates the solution from the blood[27]. If the relevant clinical site is already well-experienced in these alternative methodologies, this protocol can be acceptably substituted with these. In such cases, two factors can be considered: some alternative methods require a transfer of whole blood from a collection tube to a separate preparation tube where an additional transfer of human primary biological material may present a slightly increased safety risk, and the success of methods without barriers separating the blood from the polysaccharide solution relies on critical steps such as layering the blood sample on the density gradient medium requiring development of a refined level of technical expertise not always found in a hospital laboratory setting. The above points notwithstanding, the overall viability and cell recovery are comparable between these techniques[15,28]. Choice of the methodology is, therefore, somewhat dependent upon prior technical experience, but in our hands, mononuclear cell preparation tubes can be used successfully in a broad clinical context and are our, otherwise, recommendation.

While one endpoint of this protocol is to produce PBMC pellets for further processing into lysates, other final applications of the PBMC collection could be implemented, such as isolation of nucleic acids, or producing PBMC smears or PBMC blocks suitable for immunohistochemistry (IHC) methods. Importantly, since each biosample taken from patients represents an invasive procedure on at least some level, this protocol maximizes the useful material from each sample by also isolating plasma which can be used for cytokine analyses, metabolomic studies or ctDNA sequencing.

The analysis of peripheral biomarkers in oncology trials is one of the many applications of PBMC lysates. One example is the evaluation of the DNA damage response (DDR) in treatments such as chemotherapy, radiotherapy or the use of inhibitors of enzymes involved in the DDR such as the phosphatidylinositol-3 kinase-related kinases (PIKKs)[7] and PARP[3,13]. The aim of these treatments is to increase DNA damage in proliferating cells, which generates high toxicity in cells with impaired DDR mechanisms and cell cycle checkpoints, such as cancer cells. Here we present an example on the study of DDR biomarkers in peripheral blood subjected to X-rays.

**PROTOCOL:**

Informed patient consent and full compliance with relevant national ethics requirements in each jurisdiction e.g., the Human Tissues Act (HTA – United Kingdom, 2004) and the Health Insurance Portability and Accountability Act (HIPAA – United States, 1996) are mandatory. Be certain to have fully documented ethics approval before beginning any work on human-derived materials. Blood used in the optimization of this protocol was provided with appropriate consent from the Volunteers Advancing Medicine Panel (VAMP) (ethics reference 16/EE/0459, study CRF494, sub study 001) run by the NIHR Cambridge Clinical Research Facility, Cambridge, United Kingdom, under a Human Biological Samples supply agreement with the National Institute for Health Research (NIHR) Cambridge Biomedical Research Centre. The AstraZeneca Biobank in the UK is licensed by the Human Tissue Authority (License No. 12109) and has National Research Ethics Service Committee (NREC) Approval as a Research Tissue Bank (RTB) (REC No 17/NW/0207).

**1. General preparation guidance**

NOTE: All work with unfixed human material such as the blood, plasma and PBMCs in this protocol must operate under the assumption that these materials may carry potentially infectious agents and so must be performed under suitable biosafety precautions. For patients that have been tested as negative for known pathogens, do not ensure samples are non-infectious and so suitable safety precautions must still be applied. Waste products generated from these materials should be treated with the same biosafety precautions and disposed according to local rules.

1.1 Choose between the two type of mononuclear cell preparation tubes that utilize either sodium citrate or sodium heparin as anticoagulants. For many downstream applications these two anticoagulants can be used interchangeably but both anti-coagulants should be tested before selecting one for the trial.

1.2 Label one 8 mL mononuclear cell preparation tube to be used for the blood collection with the patient’s coded ID. Keep the tubes at room temperature (18-25 ˚C).

1.3 Store 1x PBS at room temperature (18-25 ˚C). Use 30 mL per preparation.

1.4 Make sure tubes for separate plasma samples and the cryovial for the PBMC sample are accurately labeled with unique identifiers, as specified in the appropriate section of the lab manual.

1.5 If PBMCs are going to be used to monitor phosphorylated proteins, prepare PBS supplemented with phosphatase inhibitors: mix 5 mL of PBS with 50 µL of phosphatase inhibitor cocktail 2 + 50 µL of phosphatase inhibitor cocktail 3. Prepare fresh and keep on ice until use.

1.6 If PBMCs are going to be cryopreserved, prepare 1 mL freezing mixture per sample by mixing 90% FBS + 10% DMSO.

**2. PBMC collection (Figure 1A)**

2.1. Draw 8 mL blood into the mononuclear cell preparation tube using the standard technique described by the manufacturer. Invert the tube gently 8 to 10 times to mix the anticoagulant additive with blood. Do not shake to avoid hemolysis. Record the time at which the blood was drawn.

2.2. After collection, store the tube upright at room temperature until centrifugation. Process the samples as soon as possible, ideally within one hour of this blood collection but not later than 4 h post collection. Record the timing when starting the processing of the blood.

2.3. Immediately prior to the centrifugation remix the blood sample by gently inverting the tube 8 to 10 more times.

2.4. Centrifuge the tube/blood sample tubes in a horizontal rotor (swing-out head) at 1,500 – 1,800 *x g* for 30 min at room temperature (18-25 ˚C). Ensure that all the tubes are balanced properly.

[Place **Figure 1** here].

NOTE: *x g* (also referred to as RCF, relative centrifugal units) and RPM (revolutions per minute) are different units. Be sure to set the centrifuge for *x g* (RCF). Use the online calculator (see **Table of Materials**) for the conversion. If only fixed-angle rotor is available, perform this step for 10 min at 1,500 – 1,800 *x g*.

2.5. After centrifugation, check for the presence of a dark red layer under the barrier (containing mostly red blood cells), and 2 layers above the barrier. The top layer is the plasma (straw-colored) and the whitish layer underneath is the buffy coat containing the PBMCs. These layers can be easily distinguished when viewing the tube against a dark/black background.

[Place **Figure 2** here].

NOTE: If these layers are not visible, this probably indicates an error in setting up the centrifugation units. Ensure the right centrifuge adapter is used and the correct "x *g*" are set. Repeat step 2.4.

2.6. Immediately following centrifugation, use a serological pipette to transfer approximately half of the plasma into a labeled 15 mL size conical centrifuge tube with cap, while being careful to not disturb the PBMC layer (approximately 4-5 mL). Temporarily store this tube with plasma on wet ice to be used later in the step 4. Set aside for now.

2.7. Collect the entire PBMC layer with a Pasteur pipette by placing the pipette within the layer of cells and transfer to a different 15 mL size conical centrifuge tube with cap. It is acceptable to also take a small amount of plasma, if necessary, to completely get all the PBMC layer. The volume is usually 1-2 mL. Immediately continue with the step 3 below.

**3. PBMC washing steps**

NOTE: The purpose of the wash steps is to dilute out and remove residual platelets and plasma from the PBMC pellet. All centrifugation steps should be performed at room temperature (18-25 ˚C).

3.1. Add room temperature PBS to the PBMC tube to bring the volume to 15 mL. Cap the tube. Mix cells by gently inverting the tube 5 times.

3.2. Centrifuge for 15 min at 300 *x g*. Note that this is a much gentler centrifugation than the initial centrifugation.

3.3. Visualize the pellet by viewing the tube against a dark/black background. Remove the supernatant by vacuum aspiration or with a pipette (**Figure 3A**). Discard the supernatant leaving a volume of approximately 500 µL above the whitish colored PBMC pellet.

[Place **Figure 3** here].

3.4. Resuspend the cell pellet by gently pipetting up and down in the residual supernatant.

3.5. Add additional 1x PBS to bring the volume to 10 mL. Cap the tube. Mix the cells by inverting the tube gently 5x. If cell counting is required in the study protocol go to step 3.5.1, if not required move directly to step 3.6.

3.5.1. Take a 40 µL aliquot of the re-suspended cells and mix with 40 µL of trypan blue. Count the viable cells using a hemocytometer or an automatic cell counter.

3.6. Centrifuge the suspension from step 3.5 for 10 min at 300 *x g*. Remove as much supernatant as is reasonably possible without disturbing the cell pellet.

3.7. Carefully remove and discard any remaining supernatant above the cell pellet by pipetting using a fine tip Pasteur pipette or a micropipette without disturbing the cell pellet. Leave little to no liquid above the pellet after completing this step. If the endpoint of this protocol is a PBMC pellet move to step 3.8; if the endpoint is cryopreserved PBMCs go to step 3.10.

3.8. Add 50 µL of new PBS (or the supplemented PBS prepared in step 1.5 if applicable to your endpoint) to the pellet and pipette up and down gently using a micropipette to homogenously resuspend all the cells. Transfer the entire cell suspension to a labeled 1.5 mL cryovial and immediately place on wet ice until freezing the samples.

3.9. Freeze the labeled samples by placing them in liquid nitrogen or directly in dry ice. Cells can also be frozen at -80 ˚C freezer using cell freezing boxes. In this case, prechill the boxes completely in – 80 ˚C before adding the cell tubes. Record the time at which cell pellets were frozen. Once cells freeze, store at -80 ˚C, ship frozen on dry ice.

3.10. Optionally, to cryopreserve the PBMCs re-suspend the pellet in 1 mL of the freezing mixture (step 1.6) and transfer to a labeled cryovial. Continue as described in step 3.9, but, in this case, it is only acceptable to use cell freezing boxes to freeze down the samples. Transfer to liquid nitrogen for shipping and storage after being in the freezing box for a minimum of 24 h.

**4. Plasma preparation steps (Figure 1A)**

4.1. Following -80 ˚C storage of the PBMC pellets, now prepare the plasma aliquot that was temporarily stored on wet ice in step 2.6. Perform the following centrifugation steps to clarify the plasma if the purpose of the sample is ctDNA analysis, otherwise move directly to step 4.2.

4.1.1 Centrifuge the plasma for 10 min at 1,600-2,000 *x g* at 4 – 8 °C in a fixed-angle rotor (or 15 min in a swing-out rotor).

4.1.2. Carefully pipette off the plasma supernatant, taking care not to disturb any pellet and transfer to a new 15 mL tube. Discard the tube containing the pelleted material.

4.1.3 Centrifuge the plasma for 10 min at 1,600- 2,000 *x g* at 4 – 8 °C in a fixed-angle rotor (or 15 min in a swing-out rotor).

4.1.4. Carefully transfer the plasma supernatant to a new 15 mL tube, being sure not to disturb any pelleted material. Discard the tube containing the pelleted material.

NOTE 5: If a refrigerated centrifuge is not available, keep cells on ice for 5 min between each spin, or centrifuge samples in a cold room.

4.2. Transfer plasma in 1 mL aliquots into 5 fresh 2 mL microtubes. Use fewer than 5 vials if there is not enough plasma to fill 5 vials with 1 mL aliquots and expected that the last vial will contain less than 1 mL plasma. If there is more than 5 mL plasma, discard the remainder. Record the total volume of plasma in each tube.

4.3. Immediately freeze the plasma aliquots upright, by storing them at -80 °C.

## 5. Sample shipment

5.1. Ship both PBMC pellets and plasma samples on dry ice.

5.2. Ensure that the sample is not thawed before and during shipment. Pack sufficient dry ice with the samples to ensure they remain frozen for the entirety of the shipping process, considering possible delays that may occur in transit.

## 6. Whole blood irradiation and western blot analysis of DDR biomarkers (Figure 4A)

NOTE: This is an ex vivo treatment that will not be required in most cases in the clinic, but these experiments are a valuable exploratory strategy to find suitable clinical biomarkers. Blood samples should be treated as soon as possible upon collection to ensure best results.

6.1. Warm up the X-ray cabinet. Label three 15 mL tubes as 0, 0.2 and 7 Gy, respectively.

6.2. Take three mononuclear cell preparation tubes containing freshly drawn blood from a single individual and after gently inverting the tubes 8 to 10 times transfer the bloods to the three 15 mL tubes.

6.3. Place 0.2 Gy tube in the x-ray cabinet, close the door and apply 0.2 Gy dose to the tube by selecting the shelf number and dose. Apply 7 Gy radiation dose to the 7 Gy tube by adjusting the dose selected in the cabinet.

6.4. Incubate the three tubes at 37 ˚C for one hour.

6.5. Transfer the blood back to the mononuclear cell preparation tubes and carry out the PBMC preparation protocol as for a clinical setting from step 2.4 to step 3.8.

6.6. Add a volume of lysis buffer supplemented with protease and phosphatase inhibitors equal to the cell pellet volume (in this case 70 µL of RIPA buffer) to each of the cell pellets, pipette up and down and incubate on ice for 10 min. Sonicate the samples if a sonicator is available (3 cycles, 30 s ON/30 s OFF, 4 ˚C), alternatively syringe the samples to break the nucleic acids to eliminate viscosity in the sample.

6.7. Centrifuge the samples for 10 min at ≥ 15,000 *x g*, at 4 ˚C. Transfer each supernatant to a new 1.5 mL tube, qualitatively assess the level of hemolysis (visual inspection) and measure the protein concentration by any preferred method.

6.8. Mix 40 µg of total lysate with sample loading buffer containing SDS and sample reducing agent. Boil samples for 5 min in a heat block.

6.9. Load 20 μg of each sample per lane in duplicate in a 4-12% bis-tris protein gel and run an SDS-PAGE.

6.10. After separation, transfer the proteins to a nitrocellulose membrane using a commercially available system (20 V, 10 min) and block with 5 % milk in TBST.

6.11. Cut the membrane at the relevant molecular sizes and incubate with the primary antibodies overnight at 4 ˚C (see **Table of Materials** for antibodies and dilutions).

6.12. Remove the primary antibodies and wash the membranes 3 times with TBST for 5 minutes at room temperature. Incubate with the HRP-conjugated secondary antibodies for 45 minutes at room temperature.

6.13. Wash 3 times with TBST for 5 min.

6.14. Apply the ECL reagent and analyze the images obtained relative to HRP signal.

**REPRESENTATIVE RESULTS:**

To improve the quality of PBMC preparations in our clinical trials, we have generated a protocol with concise, clear steps that can be followed by hospital laboratory professionals, independent of their molecular biology background and laboratory skills. We have adapted the manufacturer's protocol incorporating modifications on those steps where execution issues have been identified or reported from clinical sites involved in various multi-center clinical trials. However, the protocol can be further optimized to meet specific requirements, such as time constraints in the clinical site or type of downstream analyses (see **Supplementary File**). We demonstrate that the DDR can be analyzed in PBMCs by looking at specific biomarkers upon DNA damage generated by radiation.

The most common queries we have received from clinical sites relate to the centrifugation steps, which directly impact being able to successfully isolate PBMCs and obtain the final PBMC pellet. Using the appropriate type of swing-out head rotor centrifuge (**Figure 1B,C**) is the key to the success of the protocol. However, when only a fixed-angle rotor centrifuge is available at the clinical site, we suggest carrying out step 2.4 in a fixed-angle rotor at the same RCF as for a swing-out head rotor but for only 10 minutes. This ensures that a PBMC layer is separated from the plasma, (see **Figure 2B,C**). While layering blood on a density gradient separation medium requires a brake-off centrifugation, blood in mononuclear cell preparation tubes can be centrifuged with brakes on due to the presence of a gel barrier in the tube that ensures the preservation of the PBMC layer separated from the denser blood components[27,28].

At least 4 mL of high quality, non-diluted plasma were obtained from the centrifugation of blood in the 8 mL tube format, which could be further clarified for its use in specialized analyses such as ctDNA sequencing or metabolomics studies[29,30] (optional steps 4.1.1-4.1.4). The amount of isolated PBMCs, size of the pellets and hemolysis or red blood cell contamination have been other concerns coming from clinical sites and experience analyzing samples. The number of cells obtained from 8 mL blood was variable depending on the patient and disease setting, but in general the pellet obtained is small in size, and of a transparent/white coloration (**Figure 3A,B**). Due to these characteristics, it is important to visualize the pellet against a dark background to avoid its accidental aspiration during the wash steps (2.5 and 3.3). Sometimes pellets can have some red coloration due to red blood cell contamination (**Figure 3C**), and this has a negative effect on the quality of the preparation. To avoid losing small pellets such as those shown in **Figure 3**, transferring the PBMC pellet to a cryovial is facilitated by step 3.7 where the PBMC pellet is resuspended in 50 µL of PBS.

The typical yield when using these tubes and blood from healthy individuals ranges between 7 to 21 x $10^6$ cells for 8 mL, and a cell recovery between 70 and 80% as it is our experience and it has previously shown[27,28]. This depends on both the individual cell counts and the operator and it is comparable to the cell numbers and cell recovery values obtained by other methods using density gradient (including systems utilizing tubes with a separation barrier)[15,27,28]. An illustrative example of the variation on number of PBMCs isolated with this method depending on disease setting is the analysis of PBMC markers in chronic lymphocytic leukemia (CLL) patients. The number of cells recovered from an 8 mL mononuclear cell preparation tube when applying this protocol varied from 1.62 x $10^4$ to 1.99 x $10^9$ in 45 samples obtained from 7 patients in study NCT03328273[31] (**Table 1**). A related parameter is the protein concentration of the PBMC lysates obtained by this method, and this depends on the number of cells isolated and the efficiency of the protein extraction. The cell pellets lysed in section 6 were generated with RIPA buffer and sonication (step 6.6). The resuspension of the cell pellets in their same volume of lysis buffer usually results in a range of concentrations from 3 to 10 mg/mL, and in this particular example the lysate concentrations were 6.8, 8.3 and 8.6 mg/mL for 0, 0.2 and 7 Gy, respectively. However, this is subjected to a high variability when receiving samples from clinical sites that have not been optimally prepared, patient's disease, and the presence of hemoglobin from red blood cell contamination. For example, very small pellets need to be resuspended in a volume of lysis buffer larger than the cell pellet volume to allow for both protein concentration measure and downstream biomarker analysis, resulting in more diluted samples. In such case, if a sample concentration is below 1 mg/mL this can pose a challenge to perform assays like western blot due to this high dilution factor. In contrast, in the CLL samples previously mentioned, the volume of lysis buffer added to resuspend the cell pellets varied from 50 to 500 µL, and protein concentration spanned from 1.62 to 19.77 mg/mL (**Table 1**).

When samples present red blood cell contamination or hemolysis (**Figure 3C**), the protein concentration of the PBMC lysate becomes overestimated due to the inclusion of hemoglobin from the erythrocytes. This is the reason why one should do a visual inspection and annotation of such samples, as described in step 6.7 of the protocol. Loading sample in excess can compensate for the presence of hemoglobin when performing biomarker analysis as far as a loading control is included in the assay. Other more quantitative methods to measure hemolysis could be implemented, such as measuring absorbance at 414 nm[32].

The DDR was analyzed in PBMCs obtained following this clinical protocol. To illustrate a situation that mimics DNA damaging clinical treatments such as radiotherapy or chemotherapy, whole blood from healthy volunteers was ex vivo subjected to X-ray radiation (**Figure 4A**). Ionizing radiation (IR) such as X-rays induces different types of DNA damage, including DNA double-strand breaks (DSBs). DNA damage sensing of these lesions activates PIKKs such as ataxia-telangiectasia mutated (ATM), ATM and Rad3-related (ATR) and DNA-dependent protein kinase (DNA-PK), which engage DNA repair mechanisms like homologous recombination (HR) or non-homologous end joining (NHEJ). Activation of ATM by the presence of DSBs occurs by its recruitment to sites of damage by the MRN (MRE11-RAD50-NBS1) complex, causing ATM autophosphorylation at several residues including Ser 1981. In turn, activated ATM phosphorylates the components of the MRN complex and other proteins such as histone variant H2AX on Ser 139 (where pSer139-H2AX is also known as γH2AX) to promote a structural change in the chromatin spanning from the DSB which facilitates the recruitment of other DDR factors[33]. PBMCs are responsive to ionizing radiation despite their low proliferation rate and mass spectrometry methods have allowed quantification of the upregulation of phosphorylated Ser 635 on RAD50 by ATM. This phosphorylation is reduced in the presence of ATM inhibitors and RAD50 pS635 has been further validated as a pharmacodynamic biomarker for clinical ATM inhibitor treatments in tumors by immunohistochemistry[8]. To evaluate the response of PBMCs to radiation, blood from healthy volunteers was subjected to different IR doses and samples were collected after a 1 h incubation at 37 ˚C (**Figure 4A**). For this purpose, bloods were transferred to plastic tubes to avoid reducing the yield of the PBMC isolation due to high temperature (step 6.2). We analyzed how ATM was activated not only by looking at the previously reported RAD50 pS635 but also ATM pS1981 and γH2AX. In the three cases examined an increase in these post-translational modifications was observed at higher IR doses (**Figure 4B**). Interestingly, the phosphorylation of ATM and RAD50 was substantial at the low dose of 0.2 Gy, which suggests these post-translational modifications may be feasibly interrogated as PD biomarkers for treatments involving the generation of DNA DSBs with a good dynamic range, not only in tumor samples but in peripheral blood. This allows the monitoring of the PD response to the treatment by acquiring longitudinal samples through the course of treatment. The timing from the blood draw to the processing of the samples is critical to ensure these signaling cascades are still active as delays in processing will impact on the kinetics of such cascades and one could miss the phosphorylation events used as phosphomarkers.

**FIGURE AND TABLE LEGENDS:**

**Figure 1: General overview of the protocol and representative images of the centrifuge**. (**A**) Schematic overview of the protocol for the preparation of PBMCs and plasma. *If there are time constraints, step 2.4 can be shortened to 20 min and steps 3.3 to 3.6 can be removed. ** Take an aliquot to count cells, if required. *** 2 extra centrifugation steps required for ctDNA analysis/metabolomics (**B**) image of a swing-out head rotor centrifuge set for step 2.4. (**C**) image of the rotor, which includes two buckets containing the adaptors to spin CPT tubes.

**Figure 2: Tubes to isolate PBMCs.** (**A**) Image of the tubes before centrifugation (step 2.4), either empty (left) or containing blood (right). (**B**) Successful separation of the PBMC layer after centrifugation (step 2.5). (**C**) Image of the PBMC layer after centrifugation and a bit of plasma left to ensure all PBMCs are collected (step 2.7).

**Figure 3: PBMC pellets.** (**A**) Pellet obtained in the first wash step 3.3. (**B**) Pellet isolated in the second wash (step 3.7). (**C**) Pellet with high level of hemolysis obtained from blood processed later than 4 h from the blood draw isolated in the second wash (step 3.6).

**Figure 4: PBMCs isolated from ex vivo irradiated blood following the present clinical protocol display biomarkers to inform on the extent of the DNA damage caused by the treatment.** (**A**) Schematic overview of the protocol for the preparation of PBMCs and analysis of the DDR upon whole-blood irradiation. 2 extra centrifugation steps required for ctDNA analysis/metabolomics. (**B**) Western blot showing dose-dependent upregulation of DDR phospho-biomarkers in PBMCs.

**Table 1: Cell number and protein concentration of PBMC pellets from chronic lymphocytic leukemia patients (CLL)**. The data presented in this table correspond to 45 PBMC samples from 7 CLL patients participating in study NCT03328273 collected in mononuclear cell preparation tubes. These are original data generated using samples from the cited study[31].

**Supplementary File: Alternative protocol options.**

**DISCUSSION:**

High-quality preparations of PBMCs and plasma that can be robustly and reproducibly prepared at clinical trial sites are invaluable to inform clinical trial peripheral predictive and pharmacodynamic translational biomarker endpoints. Here we have provided a short, clear protocol that addresses the typically problematic steps that have been heretofore vulnerable to execution errors in a clinical trial setting. However, the protocol can be further optimized to meet specific requirements, such as time constraints in the clinical site or type of downstream analyses (see **Supplementary File**).

To this aim, we have shown how to isolate both PBMCs and plasma from whole blood using mononuclear cell preparation tubes to produce frozen PBMC pellets and frozen plasma suitable for a variety of downstream analyses. We have called attention to particularly critical protocol steps involving centrifugation and identification of the PBMC layer in step 2.5, and PBMC pellets in steps 3.3 and 3.6. Historically, where clinical sites have often gone wrong is in setting the centrifuge to the correct units (confusing an RCF or x *g* value with an RPM value), delaying the processing of blood samples, temperature and the presence of large volumes of PBS above the frozen cell pellet. In most centrifuge rotors erroneously entering a *x g* value as an RPM setting will result in significant under-centrifugation with a resulting poorly defined or absent PBMC layer, and potential inadvertent PBMC discarding during wash steps due to inefficient cell pelleting. However, there is a possibility that a PBMC layer is not visible despite using the right centrifugation settings and rotor adaptor if the patient has developed leukopenia. This condition can affect patients enrolled in oncology trials because of chemotherapy or radiation therapy and should be considered. Another critical point that has been made clear in the protocol is that samples must be processed within 1-2 h from the blood draw to decrease the possibility of hemolysis negatively impacting the protocol. Furthermore, aiming to process the samples during the first hour of the blood draw reduces ex vivo variability, which can have a great impact in pharmacokinetics readouts and on biomarkers affected by blood preservation or active signaling pathways, such as the case shown in **Figure 4**. Delays in sample processing can also have a detrimental effect in cell viability if cells are going to be cryopreserved[34]. Another factor that can affect both the yield and red blood cell contamination is the storage and centrifugation temperature, which should be kept at room temperature (18-25 ˚C). Lower temperatures increase the density of the density gradient medium, which results in a higher degree of red blood cell and granulocyte contamination as these cells do not aggregate as well. On the other hand, higher temperatures lead to PBMCs trapped between aggregated erythrocytes, hence reducing the yield of the preparation[15,27,28]. And finally, it is crucial that no more than 50-100 µL of liquid are present with the cell pellet in the cryovial, as this negatively impacts the concentration of any protein lysates obtained in downstream processing of these PBMC preparations. An excess of liquid will overdilute samples, leading to lysates with very low protein concentration not suitable for biomarker analysis. In addition, preservation of any post-translational modifications will be impaired, and the efficiency of the lysis will be also greatly reduced.

Mononuclear cell preparation tubes were chosen as they offer the most straightforward way to isolate both PBMCs and plasma in a single blood draw for clinical trials with, in our experience, excellent reproducibility. The blood processing does not require highly trained operators, and the use of a single tube removes the need of diluting the blood and its transfer to a different tube, lowering the hazard risk; shortens the protocol due to performing the centrifugation steps with brakes on; and all reagents are in the tube, which reduces variability. In our experience, these benefits outweigh the higher cost of these tubes when compared to other classical methods comprising only the use of a density gradient separation medium[27,28] (£ 410 per 60 units while lymphoprep medium for 66 50-mL preparations is £ 215). They are available in two types of anticoagulants, heparin and citrate, both of which are comparable at maintaining functionality of the isolated PBMCs[35], therefore, the choice of one anticoagulant over the other will be based on possible influence of heparin or citrate in the downstream biomarker studies. While it has been shown that EDTA tubes provide the highest PBMC isolation yield compared to heparin or citrate[13], the benefit of ease of use of the one-tube-only manipulation counterbalances this consideration. If cytokines are going to be analyzed anti-coagulants can have an effect of the levels detected in plasma, hence both anticoagulants should be tested before selecting one for the clinical trial[36]. If the plasma is going to be used for metabolomics studies, using heparin as anticoagulant would be preferred[37]. Therefore, the only point left to the end user or clinical trial translational scientist is whether citrate or heparin will be more appropriate for their purposes once costs have been assessed.

While the benefits of using cell preparation tubes are numerous compared to the limitations they pose (higher cost and availability of a restricted range of anticoagulants), the main limitation of the use of PBMCs or plasma to obtain PD biomarkers in clinical trials, especially in oncology, can be unrelated to the isolation method. Except for hematological cancers, where tumor is directly sampled from peripheral blood, for other cancer indications plasma and PBMCs are surrogate tissues which do not necessarily mimic the primary tumor. Peripheral tissue may not share the genome and epigenome with the primary tumor, therefore, the peripheral analysis of biomarkers dependent on a specific tumor mutation is mainly limited to ctDNA analysis (from plasma) or

CTCs (by subsequent sorting of the PBMC layer). In addition, signaling cascades driving or contributing to the tumor proliferation may not be as active in peripheral blood. This challenge can be overcome by applying biomarker discovery approaches targeting blood[8] to identify alternative biomarkers or coupling ex vivo treatments to the isolation of plasma[38] and PBMC preparations[26].

In the current protocol frozen PBMC pellets can be easily processed off the clinical site to give protein lysates which can be evaluated by western blotting or ELISA techniques. Alternatives methods to use PBMCs to enable IHC methods have also been presented (**Supplementary File**). In addition, we have also detailed the possibility of cryopreserving PBMCs (see **Supplementary File**) for immune cell monitoring, a relevant application in oncology, with immune checkpoint inhibitors and ADCs increasingly tested in clinical trials. The assessment of immune functions such as ADCC[16] and immunophenotyping are applications compatible with cryopreserved PBMCs isolated from mononuclear cell preparation tubes[15]. There is a caveat on cryopreservation, as it can promote down-regulation of certain surface and internal markers and might impair certain cell functions, however PBMC cryopreservation is the only feasible way to perform these assays due to time constrains when handling samples from multiple clinical sites to the processing in external labs[14,15], and these detrimental effects can be greatly overcome by good thawing methods and resting periods[39].

In conclusion, the protocol provided here will allow the dependable preparation of PBMCs and plasma samples in any clinical institution with common equipment and materials so that translational endpoints from peripheral blood can be robustly enabled in global clinical trials.

Finally, we demonstrate how the analysis of PBMC lysates can mechanistically inform the response to DNA-damaging agents by showing a dose-dependent post-translational modification of key DDR factors, which can be used to help shape clinical development. Forward-looking, implementation of methods that are more quantitative than western blotting (e.g., mass spectrometry[40]) and require less input material (such as capillary western blotting and ELISA) would help to move these preclinical results towards a more robust, systematic evaluation of PBMC patient samples.

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**DISCLOSURES:**
All the authors are employees and stake holders of AstraZeneca. VM is an employee of Acerta Pharma, owns stock in AstraZeneca and Gilead Sciences.

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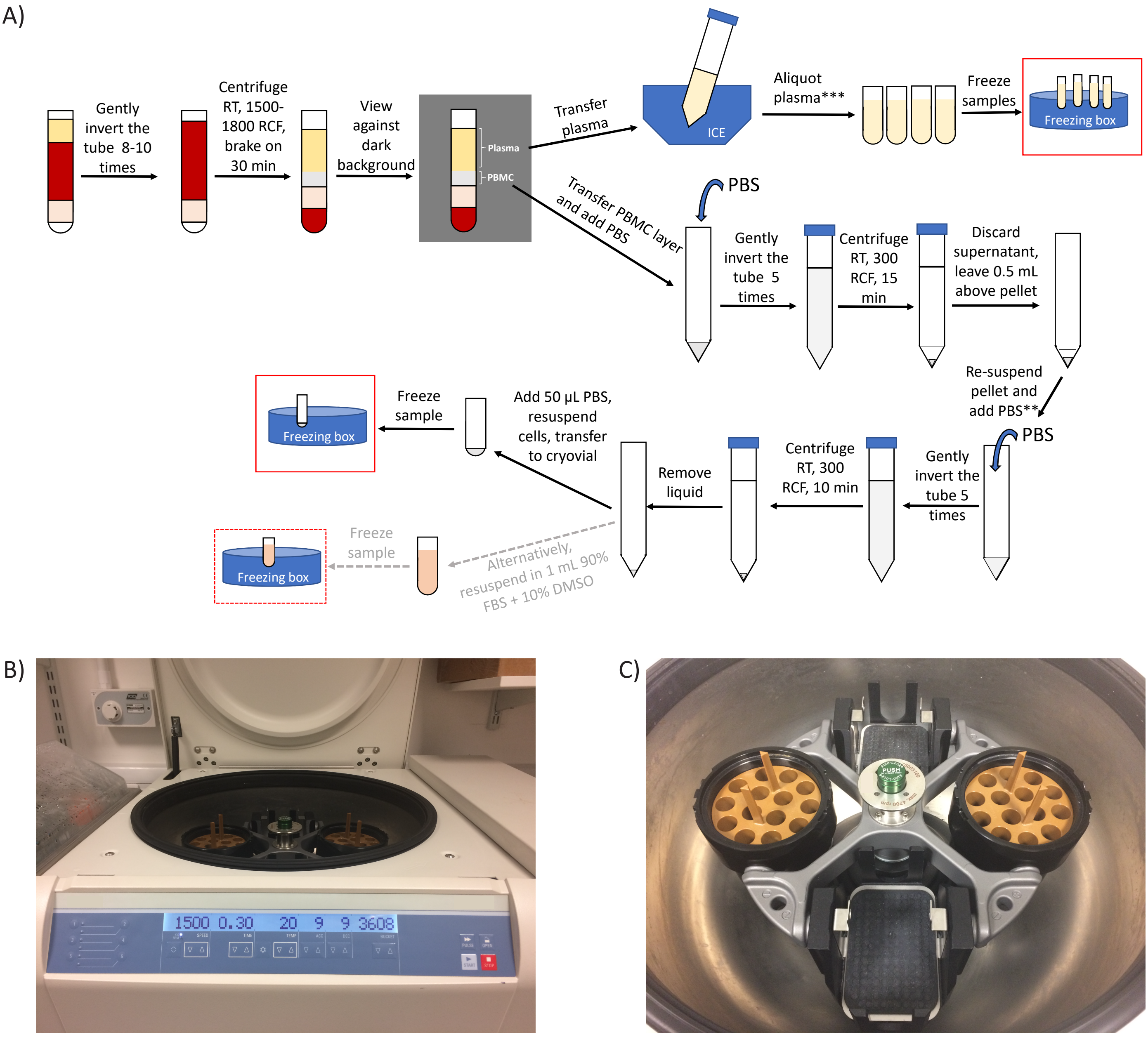


Figure 1.

Figure 2 Click here to access/download;Figure;Figure2_July2020.pdf

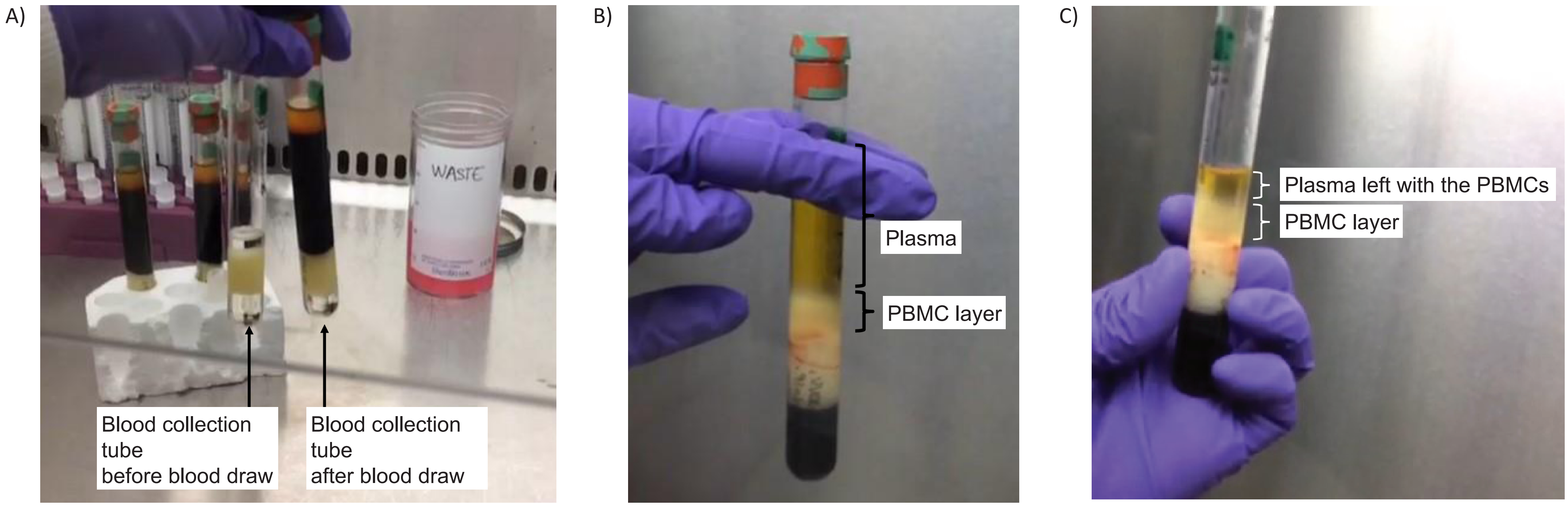


Figure 2.

Figure 3



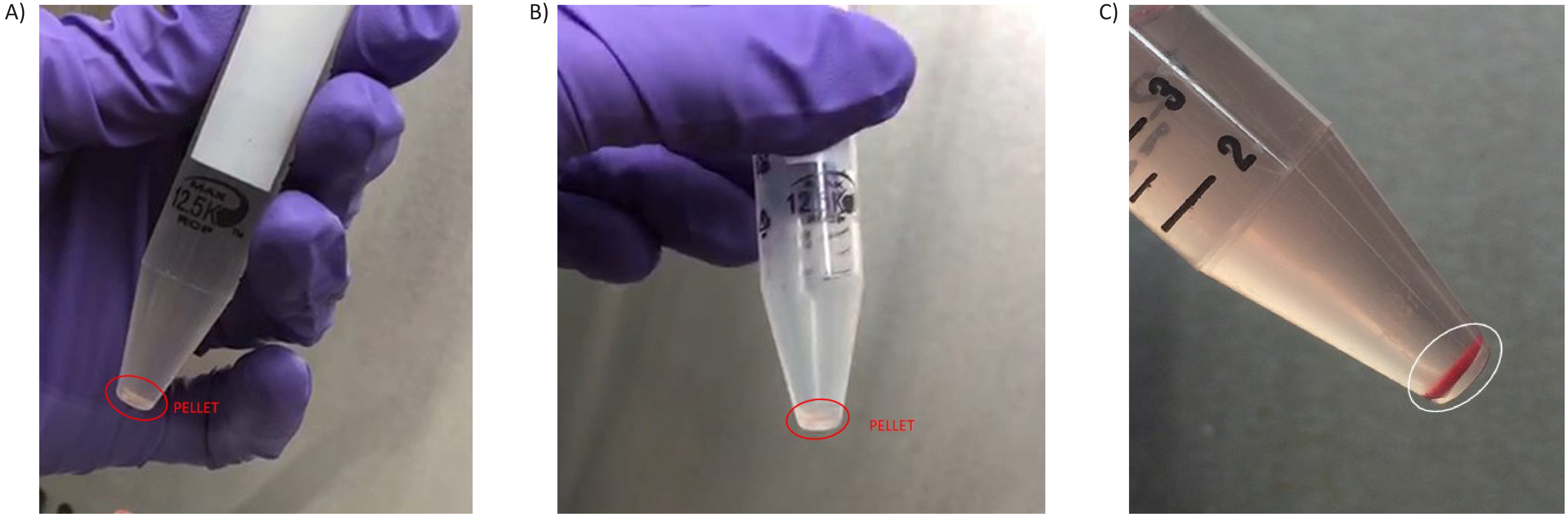

Figure 4



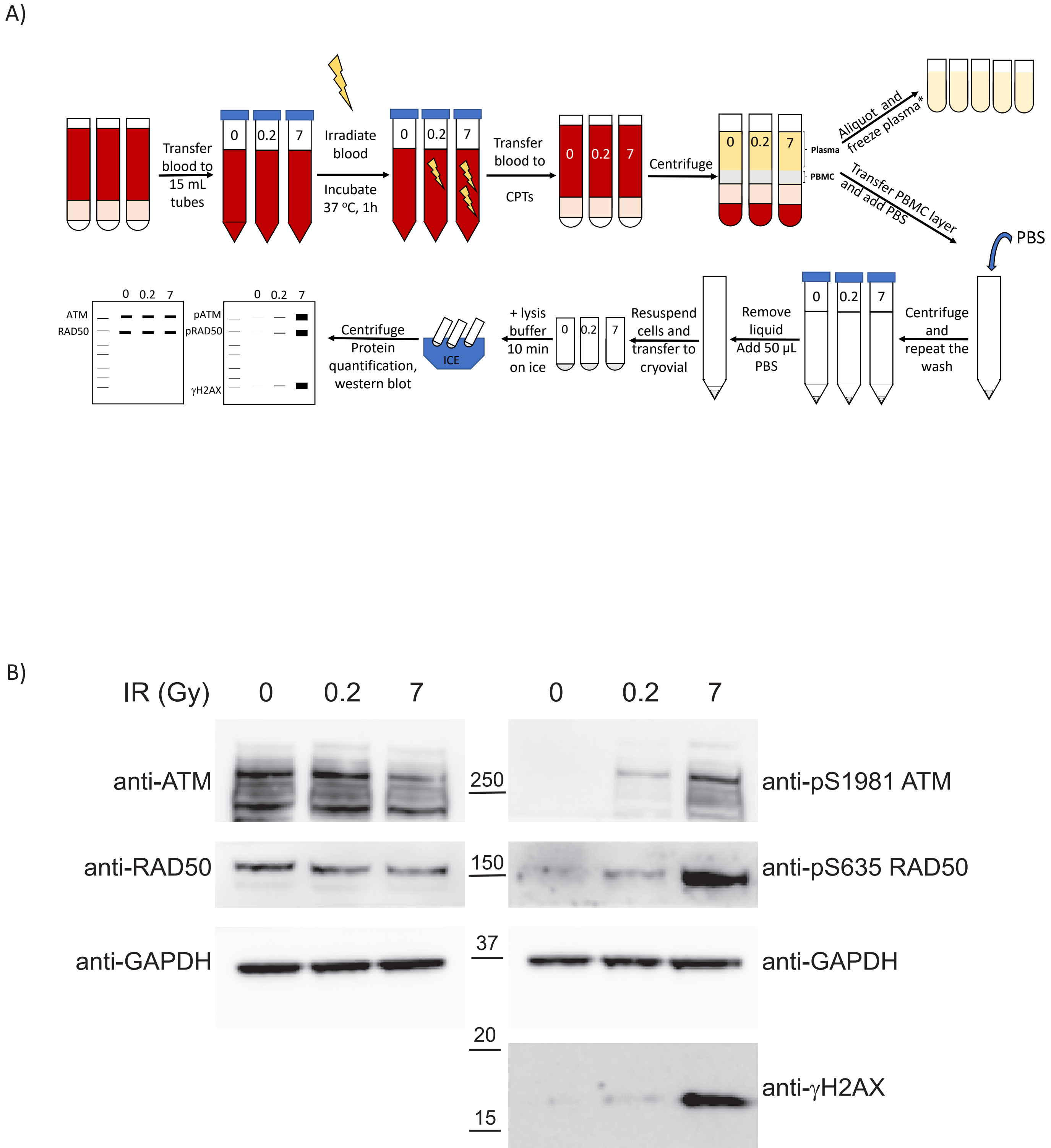


Figure 4.

Table 1

Click here to access/download;Table;Table 1.xlsx

| Sample | Number of PBMCs/ 8 mL blood | Supplemented RIPA buffer volume (µL) | Final concentration (mg/mL) |
|---|---|---|---|
| A.1 | 3.91E+07 | 70 | 12.16 |
| A.2 | 9.74E+07 | 100 | 4.54 |
| A.3 | 2.33E+08 | 150 | 7.63 |
| A.4 | 3.16E+08 | 150 | 16.87 |
| A.5 | 3.87E+08 | 150 | 12.60 |
| A.6 | 4.59E+08 | 150 | 12.71 |
| A.7 | 4.14E+08 | 200 | 15.67 |
| A.8 | 2.53E+08 | 150 | 14.56 |
| A.9 | 5.09E+08 | 300 | 10.67 |
| B.1 | 1.52E+07 | 70 | 2.96 |
| B.2 | 1.05E+07 | 50 | 4.59 |
| B.3 | 1.32E+07 | 50 | 3.99 |
| B.4 | 3.48E+07 | 100 | 10.41 |
| B.5 | 1.62E+06 | 70 | 7.11 |
| B.6 | 7.02E+07 | 100 | 9.26 |
| B.7 | 6.54E+07 | 100 | 12.10 |
| B.8 | 9.11E+07 | 150 | 11.82 |
| C.1 | 6.33E+06 | 70 | 4.04 |
| C.2 | 4.40E+06 | 150 | 19.77 |
| C.3 | 6.80E+07 | 100 | 8.96 |
| C.4 | 3.51E+07 | 50 | 9.30 |
| C.5 | 3.54E+07 | 70 | 10.55 |
| C.6 | 9.92E+07 | 100 | 16.19 |
| D.1 | 4.02E+08 | 70 | 7.23 |
| D.2 | 8.26E+08 | 300 | 16.95 |
| D.3 | 1.99E+09 | 300 | 14.87 |
| D.4 | 1.00E+09 | 300 | 18.34 |
| D.5 | 1.16E+09 | 400 | 16.13 |
| D.6 | 8.06E+08 | 400 | 19.40 |
| E.1 | 3.02E+08 | 300 | 13.86 |
| E.2 | 9.90E+08 | 500 | 19.04 |
| E.3 | 1.20E+09 | 400 | 17.13 |
| F.1 | 4.01E+06 | 50 | 1.62 |
| F.2 | 5.17E+06 | 50 | 2.84 |
| F.3 | 2.81E+06 | 50 | 3.69 |
| F.4 | 3.70E+06 | 75 | 3.62 |
| F.5 | 3.46E+06 | 70 | 4.03 |
| F.6 | 7.06E+06 | 50 | 3.32 |
| G.1 | 6.07E+07 | 70 | 6.57 |
| G.2 | 8.21E+07 | 150 | 7.78 |
| G.3 | 3.05E+07 | 70 | 8.28 |
| G.4 | 1.34E+08 | 100 | 15.14 |
| G.5 | 9.19E+07 | 100 | 8.61 |
| G.6 | 3.72E+08 | 150 | 15.88 |
| G.7 | 5.74E+08 | 200 | 15.01 |

Longitudinal PBMC samples corresponding to 7 patients

 

| Name of Material/ Equipment | Company | Catalog Number |
|---|---|---|
| 1.5 mL cryovial | Nalgene, ThermoFisher | 5000-1020 |
| 1.5 mL microcentrifuge tubes | VWR | 525-0990 |
| 15 mL conical sterile propylene centrifuge tube | Nunc, ThermoFisher | 339651 |
| 2 mL screw cap tube sterile, with attached cap | ThermoFisher | 3463 |
| 20X TBS Buffer | ThermoFisher Scientific | 28358 |
| 20X TBS Tween 20 Buffer | ThermoFisher Scientific | 28360 |
| Automated cell counter or haemocytometer | ThermoScientific | AMQAX1000 |
| Adjustable micropipette allowing 50 µL measurements | | |
| BD Vacutainer CPT mononuclear cell preparation tube (Na-citrate or Na-heparin) 8 mL | BD | 362761, 362753 |
| Cell-freezing box | ThermoFisher Scientific | 5100-0001 |
| Centrifugation unit converter | LabTools | |
| DMSO | Sigma-Aldrich, MERK | D2438 |
| ECL horseradish peroxidase substrate | ThermoFisher | 34075 |
| Faxitron MultiFocus X-ray cabinet | Faxitron Bioptics | |
| Fetal Bovine Serum (FBS), heat inactivated | ThermoScientific | 102706 |
| Fine tip, sterile 1.5 mL Pasteur pipettes | VWR | 414004-018 |
| Fixed-angle rotor centrifuge | | |
| Gel doc imaging system | SYNGENE | |
| Heat block | | |
| Horizontal rotor (swing-out head) centrifuge | Thermoscientific | Heraeus Megafuge 40R |
| Liquid nitrogen/dry ice | | |
| Marvel dried skimmed milk | Premier Foods | |
| Micropipette tips for range 1-200 µL | | |
| NuPAGE 4-12% Bis-Tris protein gel, 1 mm, 10 wells | ThermoFisher Scientific | NP0321BOX |
| NuPAGE LDS Sample Buffer (4X) | ThermoFisher Scientific | NP0007 |
| NuPAGE Sample Reducing Agent (10X) | ThermoFisher Scientific | NP0009 |

| | | |
|---|---|---|
| PBS, no calcium, no magnesium | Gibco, ThermoFisher | 14190-144 |
| Phosphatase inhibitor cocktail 2 | Sigma-Aldrich, MERK | P5726-1ML |
| Phosphatase inhibitor cocktail 3 | Sigma-Aldrich, MERK | P0044-1ML |
| Rabbit anti GAPDH | Cell Signaling Technology | CST 2128 |
| Rabbit anti $\gamma$H2AX | Cell Signaling Technology | CST 2577, lot 11 |
| Rabbit anti pS1981 ATM | Abcam | ab81292 |
| Rabbit anti pS635 RAD50 | Cell Signaling Technology | CST 14223 |
| Rabbit anti total ATM | Abcam | ab32420 |
| Rabbit anti total RAD50 | Cell Signaling Technology | CST 3427, lot 2 |
| RIPA buffer | Sigma-Aldrich, MERK | R0278-50ML |
| Sonicator | Diagenode | B01060010 |
| Sterile 1.7 mL Pasteur pipettes | VWR | 414004-030 |
| Sterile serological pipettes (5 and 10 mL volume) | Costar | 4101, 4051 |
| Trypan blue | ThermoScientific | T10282 |
| Wet ice | | |

| Comments/Description |
|---|
| To store PBMC pellets and re-suspended PBMCs |
| This is an example, use your preferred provider |
| Other brands can be used |
| For plasma aliquoting |
| Final is 25 mM Tris, 0,15 M NaCl; pH 7,5. This is an example, you can prepare your own stock or use a different provider |
| Or supplement TBS with 0.05 % to prepare TBST buffer |
| We use  Countess device and slides but could be other methods. |
| To handle small volumes (i.e. western blot, transfer PBMC pellets to 1.5 mL tubes) |
| There are 4 mL tubes but if possible 8 mL tubes are recommended to obtain more PBMCs from a single blood draw |
| This is an example, use your preferred provider. |
| http://www.labtools.us/centrifugation-speed-rpm-to-g-conversion/ |
| Use your preferred provider. Ued for PBMC cryopreservation |
| Use your preferred reagent according to the sensitivity required to detect your biomarker by western blot. Other systems can be used such as IRDye secondary antibodies with imaging systems. |
| To irradiate blood. Other models/makers are available |
| Use your preferred provider. Ued for PBMC cryopreservation |
| Optional |
| Optional for preparation of plasma for ctDNA/metabolomics |
| For imaging HRP developed membranes |
| To denature lysates prior to run them in western blot, any maker equipped with suitable tube adaptors |
| This is an example |
| To flash-freeze samples |
| This is an example, use your preferred provider |
| To handle small volumes (i.e. western blot, transfer PBMC pellets to 1.5 mL tubes) |
| This is an example, cast your own or use your preferred provider |
| For imaging HRP developed membranes |
| This is an example. |

| |
|---|
| This is usually provided in the clinical kit. |
| Optional for step 3.7 |
| Optional for step 3.7 |
| 1:1000 dilution in 5% milk TBST |
| 1:2000 dilution in 5 % milk TBST |
| 1:2000 dilution in 5 % milk TBST |
| 1:1000 dilution in 5 % milk TBST |
| 1:1000 dilution in 5 % milk TBST |
| 1:1000 dilution in 5 % milk TBST |
| For cell lysis. This is an example, use your preferred provider |
| Used for 3 cycles at 30 s on/ 30 s off, 4 $^{o}$C. If using a different instrument, adjust number of cycles and intensity according to your sonicator. |
| This is an example, use your preferred provider |
| This is an example, use your preferred provider |
| This is for the automated cell counter listed above. |
| To keep plasma samples and lysates cold |

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25th May 2020

To: Dr. Vineeta Bajaj

Dear Dr. Vineeta Bajaj and other JoVE editors,

Thank you for your message of May 12th, 2020 about our manuscript "**Clinical trial site preparation of peripheral blood mononuclear cell (PBMC) pellets and plasma from patients for biomarker analysis**" (JoVE60776R1). We are pleased to let you know that we have now submitted a revised version of our manuscript that addresses most of the points raised by the reviewers and the editorial board, together with a detailed reply to all of their comments. Here we would like to describe briefly the additional changes that we have performed in the revised manuscript.

We have made all format and style changes requested to adhere to the editorial line of JoVE, including spacing, indentations, margins, and removal of any trademark and registered symbols. In addition, we have created a supplementary file containing information about modifications on the protocol focusing on alternative applications.

In response to the request from several reviewers of adding some quantitative data, we have included data on cell number and PBMC lysate concentrations obtained from a clinical trial. This is commented in the text and a table with the data has been added to the manuscript. In addition, a more detailed explanation of the benefits of using the cell preparation tubes over other methods has been included, as requested by most reviewers.

As suggested by reviewer 1, a new panel in figure 1 (figure 1A) includes a scheme of the overview of parts 2, 3, and 4 of the protocol, which we hope adds visual clarity for the readers of our manuscript.

In response to reviewers 1 and 2, we have made clear that the use of mononuclear cell preparation tubes is compatible with isolation of CTCs but there are other more broadly used methods.

As suggested by reviewers 2, 3, and 4, we have included an alternative protocol to cryopreserve PBMCs, and have added additional references to demonstrate that cryopreservation of PBMCs can be extremely valuable for immunophenotyping and monitoring immune functions such as antibody-dependent cellular cytotoxicity.

Other areas that we have further strengthen have been the discussion of critical points such as how the timing in processing samples and the temperature affect the quality and quantity of the isolated PBMCs and have provided a more structured explanation of the importance of analysis of DDR biomarkers assay.

We trust that you will find our revised manuscript satisfactory and we look forward to hearing from you.

Yours sincerely,

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Supplementary file

**ALTERNATIVE PROTOCOL OPTIONS**

The protocol describes the shortest protocol to provide plasma samples adequate for analysis of cytokines. If the user intends to isolate ctDNA from these plasma samples further centrifugation steps are recommended to remove any residual cellular debris that could reduce the purity of small fragment ctDNA isolation (steps 4.1.1 to 4.1.4). Alternatively, the user could consider the use of blood collection tubes that stabilize white blood cells to minimize release of genomic DNA, resulting in contamination of ctDNA preparations. However, if samples are processed within 4 hours of the blood draw, blood collection tubes such as those used for PBMC collection, containing citrate or heparin as anticoagulants, provide samples of comparable quality to those obtained with EDTA tubes or tubes that stabilize white blood cells[41,42]. This protocol can also be used for metabolomics studies[29,37].

Regarding PBMCs, our main endpoint in this protocol was to obtain PBMC pellets for protein lysate preparation. An alternative that can be included is to prepare resuspended PBMCs from step 3.8 as smears. For this, 5 µL drops of PBMC suspension can be smeared on charged glass slides, and when the liquid has dried out, they can be fixed in 10% neutral buffered formalin (NBF) and embedded in paraffin. Similarly, another option for downstream analysis of biomarkers is to produce PBMC blocks. PBMC pellets from step 3.7 can be directly fixed with NBF, gel processed, and paraffin embedded. The goal of the last two alternatives would be to study biomarkers by IHC methods. While there seems to be a preference for collection tubes that better preserve cell integrity[23] or EDTA tubes, cells from step 3.7 can also be used for downstream isolation of CTCs[22]. Finally, one other possibility is the cryopreservation of PBMCs by gently re-suspending the PBMC pellet generated in step 3.7 in 1 mL of FBS supplemented with 10% DMSO and freezing them in pre-chilled cell-freezing boxes and storing them in liquid nitrogen (see step 3.10). This preparation results useful for evaluation of the immune function and immunophenotyping[14,15,16] as far as the freezing process has been followed according to the guidelines and samples are stored in liquid nitrogen. Failing to store cells in liquid nitrogen will result in loss of viability over time.

If clinical sites report time constraints to perform the present protocol, changes can be implemented to improve these situations: centrifugation step 2.4 can be shortened to 20 minutes, and steps 3.3 to 3.6 could be removed. This could impact in the number of PBMCs recovered and a higher number of contaminating cells could be expected, however advantages and disadvantages should be carefully considered to implement these changes in the clinical protocol.

Another option presented in the protocol aims to improve the preservation of phosphorylated biomarkers if this is important for the endpoint analyzed. In this case, addition of phosphatase inhibitors to the PBS (step 1.5) in step 3.8 is highly recommended.

Another alternative method to ensure the small pellets are collected can be followed when a microcentrifuge is available at the clinical site. PBMC pellets obtained in step 3.7 can be directly transferred to the final labelled 1.5 mL cryovial by resuspending the cells in 1 mL PBS. Samples are centrifuged for 10 min at 300 *x g*, and all the supernatant above the cell pellet needs to be removed, without disturbing the pellet, using a micropipette or fine tip Pasteur pipette. The cryovial should be flash-frozen at this point. The benefit of this modification relies on the fact that a small cell pellet can be better visualized in a smaller tube.