

# Isolation of WBCs collected from Cell-Free DNA BCT<sup>®</sup> and Nucleic Acid BCT<sup>™</sup>

Jordan LaRue, William Rock, Lisa Bartron, Nicholas George, Ph.D.

Nucleic Acid BCT is For Research Use Only. Not for use in diagnostic procedures.

## Introduction

Cancer is an ever-changing disease characterized and driven by genomic instability and mutations. Pan-cancer liquid biopsy assays, such as the FDA-approved Guardant360<sup>®</sup> CDx test, have revolutionized cancer management and spurred personalized treatment selection based on tumor genetic changes. However, separating circulating tumor DNA (ctDNA) from the larger percentage of background cell-free DNA (cfDNA) remains challenging for certain patients and cancer types. In addition, nucleated blood cells (such as white blood cells, WBC) can breakdown and release fragmented genomic DNA (gDNA) that dilutes the informative ctDNA fraction within the tube. To combat this issue, stabilizing blood collection tubes, such as the Cell-Free DNA BCT and Nucleic Acid BCT, can be introduced into the liquid biopsy workflow. These tubes are specially designed to stabilize blood cells, thus preventing storage time-dependent release of their cellular contents (DNA, RNA, etc.) and keeping plasma analyte concentrations (e.g., ctDNA) reflective of what is observed at draw time.

Further confounding the analysis of tumor-specific genetic changes are those genetic changes that may occur in white blood cells as a result of clonal hematopoiesis. This is especially true for complex blood plasma-based deep sequencing approaches where rare tumor variants must be verified against both the germline sequence and/or new sequences that may arise during clonal hematopoiesis. Therefore, it is useful to isolate both circulating plasma DNA (i.e., ctDNA) and WBCs for downstream genomic nucleic acid isolation. However, the stabilizing reagents present in Cell-Free DNA BCT and Nucleic Acid BCT confound density gradient-based PBMC separation. Here, we outline a workflow for isolation of circulating plasma nucleic acids, WBCs, and genomic nucleic acids from blood collected into Cell-Free DNA BCT or Nucleic Acid BCT.

## Methods and Analysis

### Blood Plasma Processing:

The following protocols are modified from the instructions for use (IFU) for Cell-Free DNA BCT or Nucleic Acid BCT.

Whole blood collected into Cell-Free DNA BCT or Nucleic Acid BCT was centrifuged at draw time or after seven days of ambient storage at 1600 x g for 10 minutes (Cell-Free DNA BCT samples) or 1800 x g for 15 minutes (Nucleic Acid BCT samples) at room temperature. The upper plasma layer was removed carefully, as to not disturb the buffy coat layer, and transferred to a new 15mL conical tube. Isolated plasma was then centrifuged at 16,000 x g for 10 minutes (Cell-Free DNA BCT samples) or 2,800 x g for 15 minutes (Nucleic Acid BCT samples) at room temperature. Resultant supernatant was used immediately for nucleic acid isolation or frozen at -80 °C. Remaining buffy coat and red blood cell layer was reserved for WBC processing.

### WBC and RBC Counts:

Samples collected into Cell-Free DNA BCT or Nucleic Acid BCT were run in "Patient" mode using the CBC-DIFF program on a Sysmex<sup>®</sup> XE5000 analyzer to obtain RBC and WBC counts.

### Analysis of CD45 Expression by Flow Cytometry

Whole blood samples or isolated WBCs were stained with FITC Mouse anti-Human CD45 (BD<sup>®</sup> Biosciences) for 15 minutes at room temperature. Samples were then lysed for 15 minutes at room temperature using 1X BD FACS<sup>®</sup> Lysing Solution (BD<sup>®</sup> Biosciences), washed, and resuspended in a 1X PBS + 0.5% BSA + 0.05% sodium azide buffer. Data were collected on the BD FACSCanto<sup>®</sup> II flow cytometer using the BD FACSDiva<sup>™</sup> software (BD Biosciences) and subsequently analyzed using Kaluza C Analysis Software (Beckman Coulter<sup>®</sup>).

# Isolation and expanded use of WBCs collected from Cell-Free DNA BCT® and Nucleic Acid BCT™

## WBC Isolation:

WBCs were isolated from samples collected into EDTA, Cell-Free DNA BCT, or Nucleic Acid BCT at draw time or after seven days of ambient temperature storage by ammonium chloride-based RBC lysis or magnetic bead-based separation. Ammonium-chloride based WBC isolation was carried out using an adapted workflow from the QIAGEN® Buffer EL instructions for use (See WBC Isolation Using Ammonium Chloride-Based RBC Lysis with QIAGEN Buffer EL for detailed protocol). Magnetic bead-based isolation was carried out using a workflow adapted from the STEMCELL™ Technologies EasySep™ Direct Human PBMC Isolation Kit instructions for use (See WBC Isolation Using Magnetic Bead-Based Cellular Depletion with EasySep Direct Human PBMC Isolation Kit for detailed protocol).

## Nucleic Acid Isolation:

gDNA or cellular RNA (cRNA) were extracted from  $5 \times 10^6$  isolated WBCs with a workflow adapted from the DNeasy® Blood and Tissue Kit "Tissue" protocol (QIAGEN; See gDNA Isolation with DNeasy® Blood and Tissue Kit for detailed protocol) or using the Quick-RNA™ FFPE Miniprep Kit (Zymo; See cRNA Extraction with -RNA™ FFPE Miniprep Kit for detailed protocol).

## Results

### WBCs are successfully isolated from Cell-Free DNA BCT or Nucleic Acid BCT using both ammonium chloride-based and magnetic bead-based isolation techniques

While RBCs are efficiently removed by targeted lysis, both after blood draw and following seven days ambient blood storage, WBCs are retained in Streck Cell-Free DNA BCT and Nucleic Acid BCT, suggesting that ammonium chloride-based RBC lysis still occurs in the presence of the stabilizing reagents found in the Streck tubes (Figure 1). When reactivity for the pan-leukocyte marker CD45 was used to gauge concentrations of each white blood cell type following RBC lysis, we observed that relative concentrations of neutrophils, monocytes, and lymphocytes remained relatively constant independent of blood collection tube and storage time. These data indicate that ammonium chloride-based RBC lysis effectively isolated WBCs that can be utilized for downstream flow cytometric or genetic-based assays.

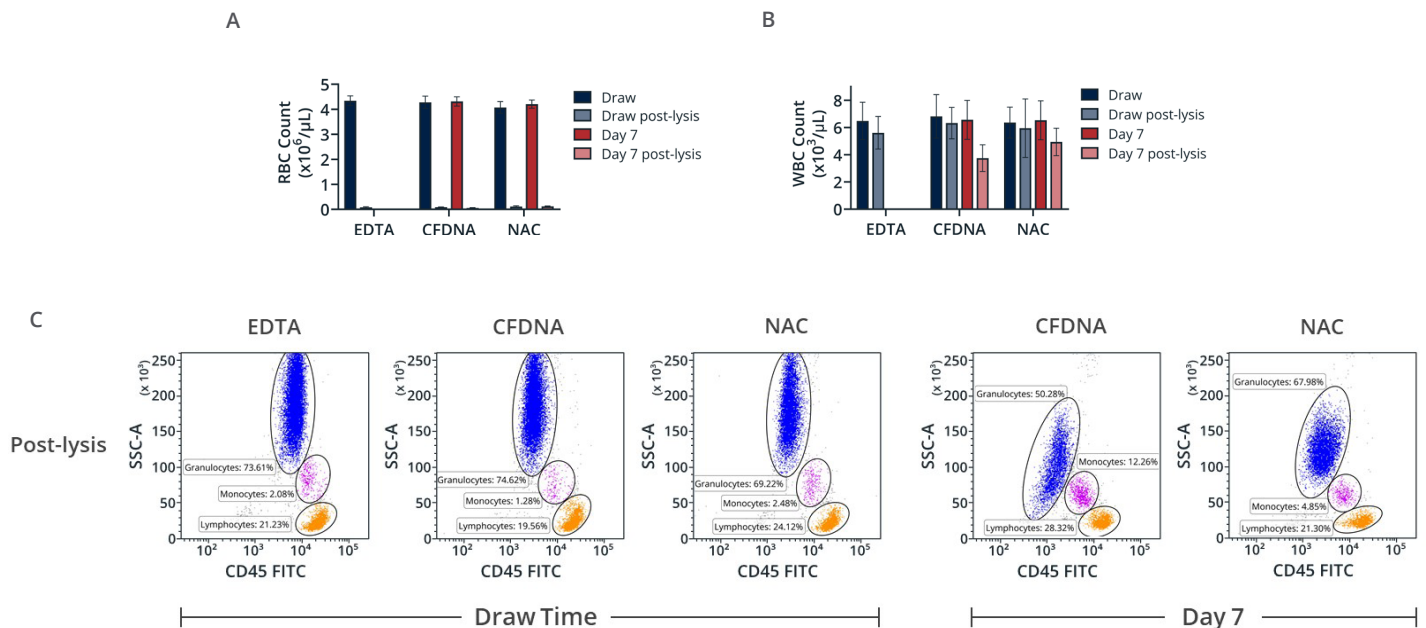
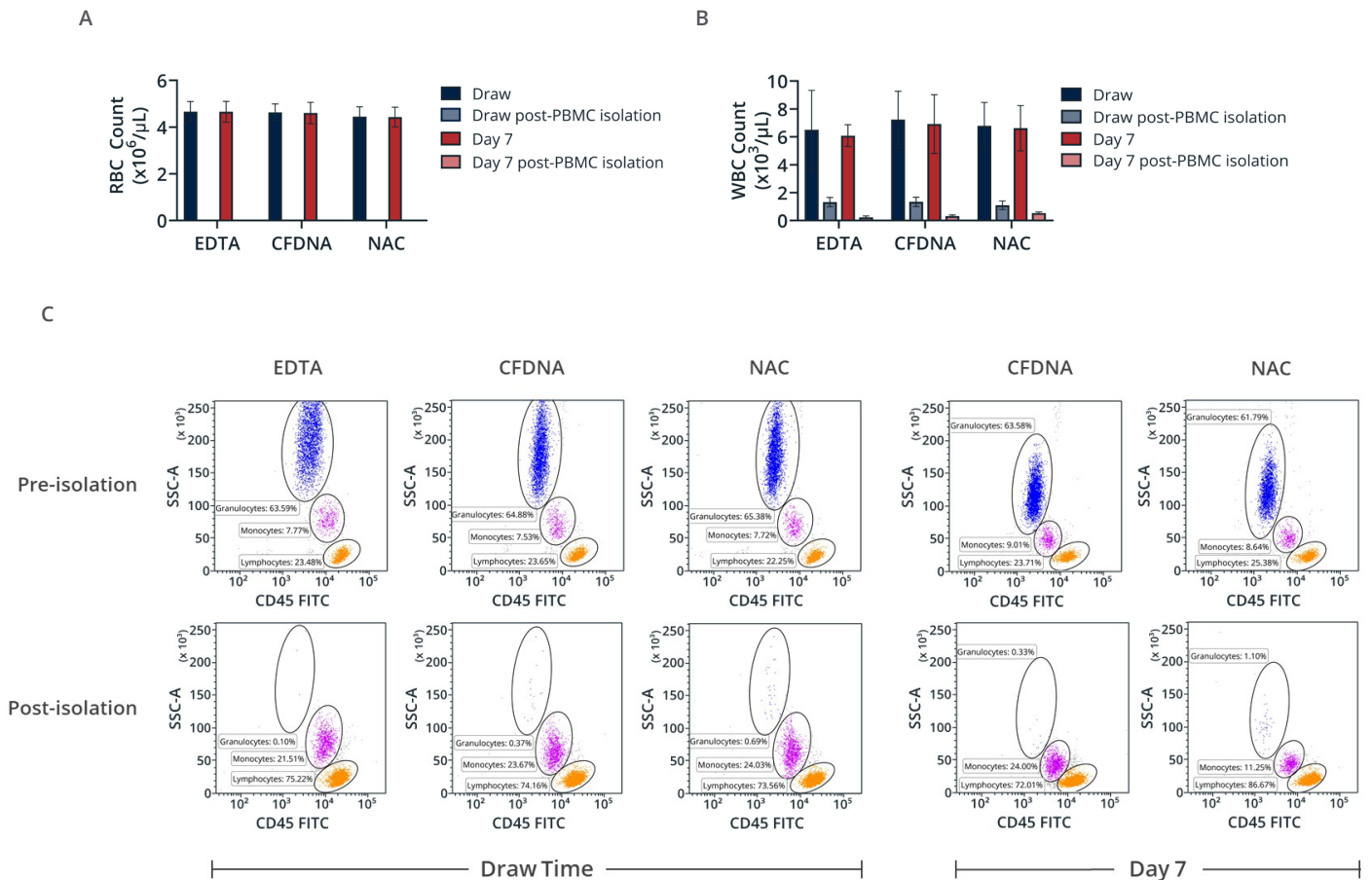


Figure 1. Cell-Free DNA BCT and Nucleic Acid BCT are compatible with commercially available ammonium chloride-based WBC isolation methods. WBCs from whole blood samples collected into EDTA, Cell-Free DNA BCT (CFDNA), or Nucleic Acid BCT (NAC) were isolated using ammonium chloride-based RBC lysis at draw time or after seven days of ambient temperature storage. (A-B) Average pre- and post-lysis RBC (A) and WBC (B) counts at draw time or day 7. Mean  $\pm$  SE is shown (n=5 blood donors). (C) CD45 expression of isolated WBCs following RBC lysis at draw time or day seven. Data from a single representative donor is shown.

## Isolation and expanded use of WBCs collected from Cell-Free DNA BCT® and Nucleic Acid BCT™

Following magnetic bead-based WBC isolation, analysis of the RBC and WBC counts on a Sysmex XE 5000 revealed that all RBCs were removed from the sample regardless of collection tube and storage time. Further, CD45 reactivity analysis demonstrated that the WBCs isolated were primarily lymphocytes and monocytes, suggesting that the kit specifically enriched for PBMCs. It should be noted that the overall decrease in WBC counts observed following isolation is due to the removal of the granulocyte population. This is not of concern in this application, as lymphocytes are primarily used for the downstream genomic nucleic acid techniques focused on in this note.



**Figure 2.** PBMCs are effectively isolated from Cell-Free DNA BCT and Nucleic Acid BCT using magnetic bead-based methods. WBCs from whole blood samples collected into EDTA, Cell-Free DNA BCT (CFDNA), or Nucleic Acid BCT (NAC) were isolated using the STEMCELL™ Technologies EasySep™ Direct Human PBMC Isolation Kit at draw time or after seven days of ambient temperature storage. (A-C) Average pre- and post-isolation RBC (A) and WBC (B) counts and CD45 expression (C) at draw time or day 7. Mean ± SE is shown (n=5 blood donors). Flow cytometric data from a single representative donor is shown.

### Cellular nucleic acids are effectively extracted from the WBCs isolated from Cell-Free DNA BCT or Nucleic Acid BCT

When nucleic acids were extracted from isolated WBCs, we found that the concentration of extracted gDNA or cRNA did not depend on collection tube type or storage time was within the range required for downstream analyses such as next generation sequencing assays.

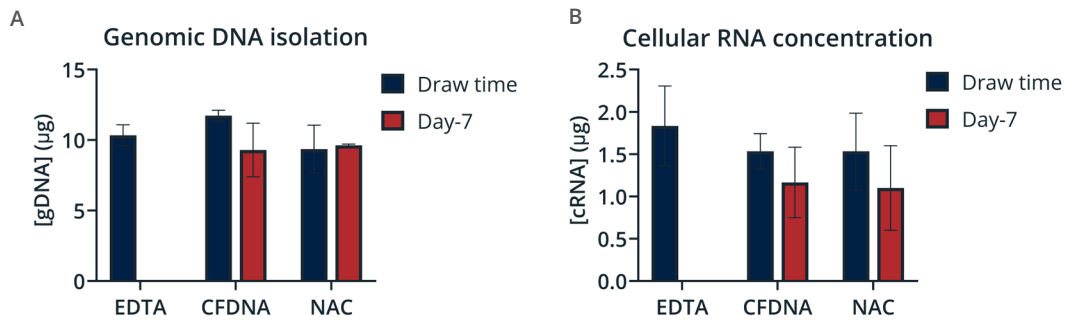


Figure 3. Cell-Free DNA BCT and Nucleic Acid BCT are compatible with commercially available genomic DNA and cellular RNA isolation kits. Concentration of gDNA (A) or cRNA (B) extracted from WBCs isolated from blood collected into EDTA, Cell-Free DNA BCT (CFDNA), or Nucleic Acid BCT (NAC) at draw time or after seven days of ambient temperature storage. Mean  $\pm$  SE is shown (n=5 blood donors).

### Conclusion:

These data demonstrate that Cell-Free DNA BCT and Nucleic Acid BCT are compatible with either ammonium chloride-based RBC lysis or magnetic bead-based separation WBC isolation methods. Further, gDNA and cRNA can be extracted from these isolated WBCs for downstream analysis. Collectively, the workflows provided in this technical note allow for successful isolation of circulating plasma nucleic acids, WBCs, and genomic nucleic acids from blood collected into Cell-Free DNA BCT or Nucleic Acid BCT.

## WBC Isolation Using Ammonium Chloride-Based RBC Lysis with Buffer EL

Adapted from Buffer EL instructions for use (QIAGEN®), Cat. No. 79217.

1. Isolate and remove plasma per the plasma processing protocol and resuspend remaining cells in a volume of saline (0.9% NaCl solution) equal to the plasma volume removed.
  - a. Recap the blood collection tube and mix by end-over-end inversion  $\geq 10$  times.
2. In a 15 mL conical tube, combine 1 volume of resuspended blood cells with 5 volumes of Buffer EL.
  - a. Cap and mix thoroughly by end-over-end inversion  $\geq 10$  times.
3. Incubate for 10-15 minutes on ice. Mix end-over-end briefly halfway through the incubation.
4. Centrifuge at 400 x g for 10 minutes (4 °C).
5. Remove and discard supernatant using a pipette, being careful not to disturb WBC pellet.
6. Add 2 volumes of Buffer EL to the pellet and resuspend using end-over-end inversion.
7. Centrifuge at 400 x g for 10 minutes (4 °C).
8. Remove and discard supernatant.
9. Wash cells by resuspending in 1 volume of PBS.
10. Centrifuge at 400 x g for 5 min (4 °C).
11. Remove supernatant and use the WBCs immediately for surface staining studies (flow cytometry) or freeze at -80 °C for downstream RNA or DNA isolation.

## WBC Isolation Using Magnetic Bead-Based Cellular Depletion with EasySep™ Direct Human PBMC Isolation Kit

Adapted from EasySep Direct Human PBMC Isolation Kit instructions (STEMCELL™ Technologies), Cat. No. 19654.

1. Isolate plasma as per above and resuspend remaining cells in a volume of saline (0.9% NaCl solution) equal to the plasma volume removed. Recap the blood collection tube and mix by end-over-end inversion  $\geq 10$  times.
2. Add 2 mL of resuspended blood to a 14 mL round-bottom tube.
  - a. For Nucleic Acid BCT only: add 25  $\mu$ L of 0.5M Disodium EDTA.
3. Add 100  $\mu$ L of isolation cocktail to each sample. Mix by 1 mL pipette.
4. Incubate at room temperature for 5 minutes.
5. Add 1.9 mL of PBS + 2% FBS to the sample and mix.
6. Vortex RapidSpheres for 30 seconds prior to use.
7. Add 100  $\mu$ L of RapidSpheres to the sample, mix by 1 mL pipette, then immediately place the sample into the "Big Easy" EasySep™ magnet.
8. Incubate at room temperature for 5 minutes.
9. Picking up the magnet and the tube together, pour the cell suspension in to a new 14 mL round-bottom tube.
10. Add 100  $\mu$ L of RapidSpheres to sample, mix by 1 mL pipette, and then immediately place the tube into the magnet.
11. Incubate at room temperature for 5 minutes.
12. Picking up the magnet and the tube together, pour the cell suspension into a new 14 mL round-bottom tube.
13. Immediately place the new tube back into the magnet.
14. Incubate at room temperature for 5 minutes.
15. Picking up the magnet and the tube together, pour the cell suspension into a new 14 mL round-bottom tube.
16. Centrifuge the tube at 400 x g for 10 minutes (4 °C).
17. Remove and discard supernatant.
18. Wash cells by resuspending in 1 mL of PBS.
19. Centrifuge at 400 x g for 5min (4 °C).
20. Remove supernatant and use the PBMCs immediately for surface staining studies (flow cytometry) or freeze at -80 °C for downstream RNA or DNA isolation.

## gDNA Isolation with DNeasy® Blood & Tissue Kit.

Modified from the DNeasy Blood & Tissue Kit "Tissue" protocol, Cat. No. 69504.

1. Thaw WBC pellet on ice.
2. Resuspend WBCs in 200  $\mu$ L Buffer RTL.
3. Add 20  $\mu$ L Proteinase K and 200  $\mu$ L Buffer AL to each sample and vortex well.
4. Place samples in 56 °C water bath and incubate overnight.
5. Following incubation, remove samples from water bath, add 200  $\mu$ L 100% ethanol and vortex well to combine.
6. Transfer entire contents of tube to a DNeasy Mini spin column.
7. Spin at 6,000 x g for 1 minute.
8. Discard collection tube and flow-through and place spin column in a new 2 mL collection tube.
9. Add 500  $\mu$ L Buffer AW1.
10. Spin at 6,000 x g for 1 minute.
11. Add 500  $\mu$ L Buffer AW2.
12. Spin at 20,000 x g for 1 minute.
13. Discard collection tube and flow-through and place spin column in a new 2 mL collection tube.
14. Dry the membrane by spinning an additional 2 minutes at 20,000 x g.
15. Elute DNA by adding 100  $\mu$ L Buffer AE directly to the spin column membrane. Incubate for 1 minute at room temperature.
16. Centrifuge for 1 minute at 6,000 x g.

**cRNA Extraction with Quick-RNA™ FFPE Miniprep Kit, Cat. No. R1008.**

1. Thaw WBC pellet on ice.
2. To each sample, add 95 µL nuclease-free water, 95 µL 2X digestion buffer, and 10 µL Proteinase K. Resuspend pellet using pipette.
3. Place in 55 °C water bath and incubate for 1 hour.
4. Incubate at 65 °C for an additional 15 minutes.
5. Remove samples from water bath and add 300 µL RNA Lysis Buffer. Mix well by vortex.
6. Transfer sample into a Spin-Away Filter (yellow) in a collection tube.
7. Spin at 12,000 x g for 30 seconds. **Save the flow-through.**
8. To the flow-through, add 600 µL 100% ethanol and mix well.
9. Transfer the mixture to a Zymo-spin IIIICG column (green).
10. Spin at 12,000 x g for 30 seconds. Discard flow-through.
11. Wash the column with 400 µL RNA Wash Buffer.
12. Spin at 12,000 x g for 30 seconds. Discard flow-through.
13. Add 75 µL DNA Digestion Buffer and 5 µL DNase 1 to each sample. Incubate at room temperature for 15 minutes.
14. Add 400 µL RNA Prep Buffer.
15. Spin at 12,000 x g for 30 seconds. Discard the flow-through.
16. Add 700 µL RNA Wash Buffer.
17. Spin at 12,000 x g for 30 seconds. Discard the flow-through.
18. Add 400 µL RNA Wash Buffer.
19. Spin at 12,000 x g for 1 minute to dry the membrane. Discard the flow-through and collection tube. Place column in a new 1.5 mL tube.
20. Add 100 µL DNase/RNase-free water directly to the filter. Incubate for 1 minute at room temperature.
21. Centrifuge at 12,000 x g for 1 minute.