User Manual

Version 1.1 - UMFB3300

Evercode™ Whole Blood Fixation

For use with

ECFB3300

ECFB3500

Support Suite

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U.S. Pat. No. 10,900,065

U.S. Pat. No. 11,168,355

U.S. Pat. No. 11,427,856

U.S. Pat. No. 11,634,751

U.S. Pat. No. 11,639,519

U.S. Pat. No. 11,680,283

Patents pending in the U.S. and other countries

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Overview

Workflow

The Evercode Whole Blood Fixation kit stabilizes whole blood shortly after collection for downstream use with Evercode Whole Transcriptome Kits. The quick 3 step workflow is compatible with the 12 reactions and the 48 reactions option, and includes a standard/high input options for increased sample input. Standard input yields ~95,000 PBMCs and high input yields ~500,000 PBMCs. This kit adds increased ease of use and shorter workflow, while conveying all the standard benefits of Parse Evercode Fixation kits — cell integrity, RNA protection, barcoding compatibility, storage flexibility (Figure 1).

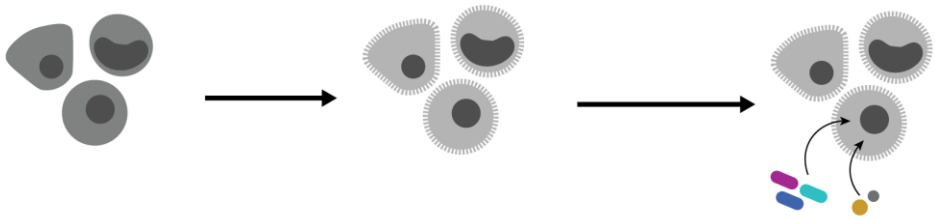

Figure 1: Evercode Whole Blood Fixation. Cells in whole blood are fixed, permeabilized, and isolated before undergoing the split-pool combinatorial barcoding steps.

The figure below provides an overview of the fixation workflow: 188 µL of whole blood can be fixed in a single reaction. Note that cells from more than 188 µL of whole blood may need to be fixed to fully utilize the capacity of the downstream Evercode kits. See Important Guidelines for additional details. If desired, 750 µL of whole blood can be fixed in a single reaction, see Appendix A for details.

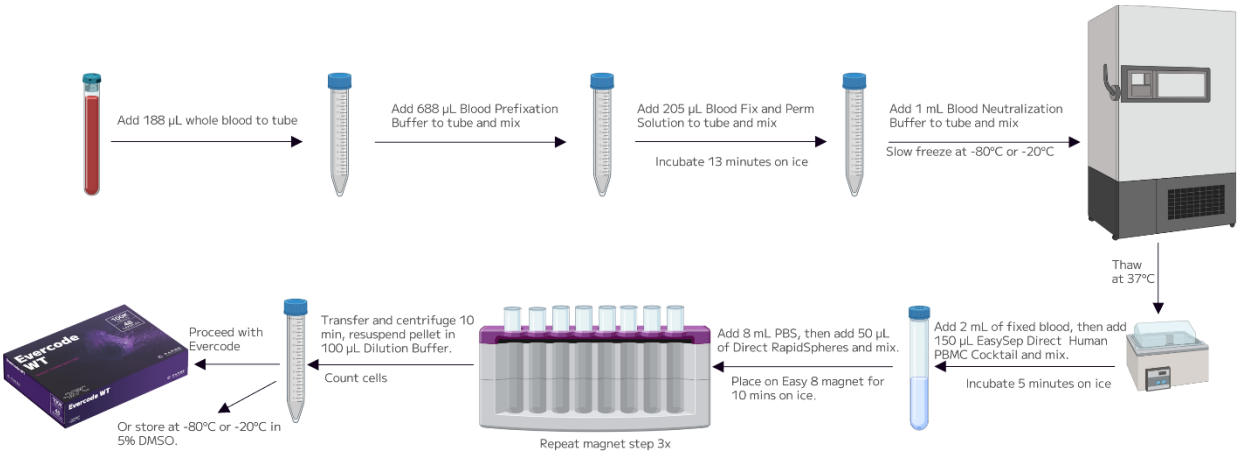

Figure 2: Evercode Whole Blood Fixation Workflow.

Protocol Timing

The table below provides details of the total and hands-on time required for the Whole Blood cell fixation workflow.

SECTION	TOTAL TIME	HANDS-ON TIME	STOPPING POINTS
Section 2.1: Fixation			
Fixation	30 minutes	30 minutes	-20°C or -80°C ≤ 3 months
Section 2.2: Cell Isolation			
Cell Isolation	60-90 minutes	60-90 minutes	-20°C or -80°C ≤ 1 month

Important Guidelines

These guidelines provide additional information to obtain optimal performance beyond the detailed instructions in the protocol. For additional questions not discussed below, please contact us at support@parsebiosciences.com. We also have a library of additional resources and videos on our support site at https://support.parsebiosciences.com/.

Sample Input

- We recommend collecting whole blood in 10 mL EDTA vacutainer tubes.
- Invert the whole blood in the collection tube 10x to mix well before aliquoting into the fixation reaction.
- We do not recommend storing the whole blood used in this kit longer than 48 hours prior to fixation. Blood should be stored at 4°C immediately after draw and kept on ice throughout the fixation workflow.
- This protocol accommodates up to 188 µL of whole blood, which typically yields between 75,000-100,000 PBMCs after isolation. This amount can be used to calculate the volumes needed to reach the target cell concentrations for the various Evercode kit sizes shown below. For higher cell input, see Appendix A.

Note: With the standard Whole Blood Fixation kit size the samples will not be concentrated enough to fully load a WT Mega or Penta. We recommend using the high input kit to ensure adequate sample concentration.

- Total blood counts vary substantially between donors, therefore total PBMCs retained from 188 µL of blood will vary. To avoid counting the isolated PBMC twice and/or losing cells, we suggest setting aside a "counting aliquot" after PBMC isolation. This aliquot should be frozen and then thawed specifically to count the cells prior to starting Section 1 of any Evercode assay kit. These counts are used to determine how many cells are loaded in the Round 1 Plate.

CELL CONCENTRATIONS	
Kit size	Minimum Post-Isolation Concentration to Fully Load Kit per µL
Evercode WT Mini	298 cells
Evercode WT	520 cells
Evercode WT Mega	2,126 cells

CELL CONCENTRATIONS	
Evercode WT Mega 384	651 cells
Evercode WT Penta	4,114 cells
Evercode WT Penta 384	3,225 cells

Avoiding RNase Contamination

- Standard precautions should be taken to avoid introducing RNases into samples or reagents throughout the workflow. Always wear proper laboratory gloves and use aseptic technique.
- Although RNases are not inactivated by ethanol or isopropanol, they are inactivated by products such as RNaseZap™ RNase Decontamination Solution (Thermo Fisher Scientific®). These can be sprayed on benchtops and pipettes.
- Nuclease-free, filtered pipette tips should be used to reduce RNase contamination from pipettes.

Cell Counting and Quality Assessment

- As this protocol begins with whole blood fixation, cell counting will not take place until after the cells have been isolated from the blood on the day of barcoding. After centrifugation and resuspension PBMCs should be counted to properly dilute the samples, and imaged to ensure adequate RBC removal.
- Some trace amounts of RBCs at this step are normal and will not negatively impact the quality of PBMC samples in the Evercode kit. Examples of adequate and inadequate amounts of RBC contamination are shown below.
- In case of large RBC contamination, a second round of PBMC cleanup is recommended.

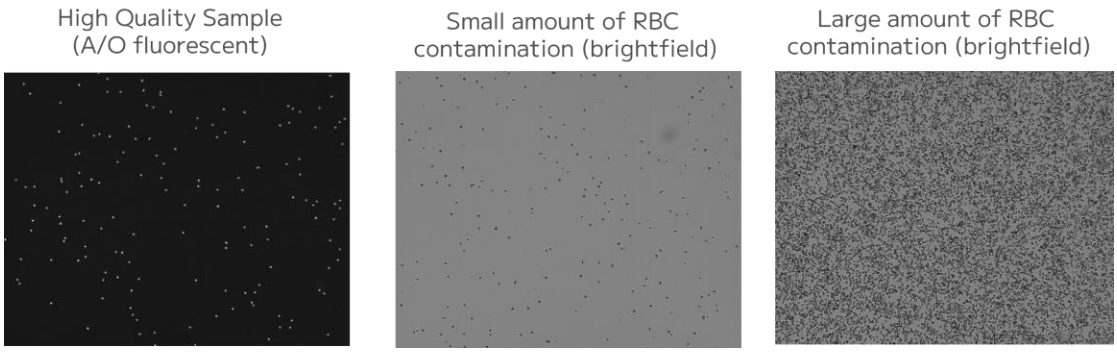

Figure 3: Example of AO/DAPI stained fixed, isolated PBMCs with fluorescent imaging and brightfield.

Centrifugation

- Isolated PBMCs from this whole blood fixation kit should be spun at 400xg for 10 minutes for optimal retention. We recommend using the same speed and duration throughout this and downstream Evercode User Guides.
- A swinging bucket rotor should be used for all high-speed centrifugation steps in this protocol. The use of a fixed-angle rotor will lead to substantial cell loss.

Maximizing Cell Recovery

- It is critical to thoroughly resuspend the cells after centrifugation throughout the protocol. Resuspend by slowly and repeatedly pipetting until no clumps are visible. Ideally, this should be verified with microscopy.
- To minimize cell loss from cell adherence to tubes, carefully pipette along the bottom and sides of tubes.
- We do not recommend wide bore pipette tips as they make it difficult to resuspend cell pellets adequately.
- Use 15 mL and 50 mL polypropylene centrifuge tubes, as polystyrene tubes will lead to substantial sample loss.
- The first time performing this assay, post-centrifugation supernatants should be kept and counted to ensure centrifugation speeds/times are optimized.

Reagent Stability

- Reagents in this kit may be freeze/thawed up to 2 times. If the kit will be used more than 2 times, we recommend aliquoting reagents.

Storage of Fixed Samples

- Fixed whole blood samples may be stored for up to 3 months either at -80°C or a -20°C with no changes in quality. Stability testing is ongoing and samples may be stable for longer.
- Fixed whole blood samples may be stored at 4°C for up to three days with negligible impact on sample quality if shipment to another location is required. Do not thaw frozen samples for shipment and then refreeze them.

Part List

The Evercode Whole Blood Fixation v3, 12 reactions tube workflow requires the Evercode Whole Blood Fixation Reagents box.

Whole Blood Fixation Reagents, 12 reactions. Store at -20°C, PN ECFB3300

LABEL	ITEM	PN	FORMAT	QTY
	Blood Prefixation Buffer	BF101	15 mL bottle	1
	Blood Fix and Perm Buffer	BF102	8 mL bottle	1
	Blood Neutralization Buffer	BF103	15 mL bottle	1
	Sample Dilution Buffer	MG105	2 mL tube	1

The Evercode Whole Blood Fixation v3, 48 reactions tube workflow requires the Evercode Whole Blood Fixation Reagents box.

Whole Blood Fixation Reagents, 48 reactions. Store at -20°C, PN ECFB3500

LABEL	ITEM	PN	FORMAT	QTY
	Blood Prefixation Buffer	BF101	15 mL bottle	4
	Blood Fix and Perm Buffer	BF102	8 mL bottle	4
	Blood Neutralization Buffer	BF103	15 mL bottle	4
	Sample Dilution Buffer	MG105	2 mL tube	3

*The -20°C Sample Dilution Accessory Kit is optional and can be purchased separately if needed to dilute concentrated samples.

User Supplied Equipment and Materials

The following materials and equipment are required to perform the protocol but are not provided within the kit. This list does not include standard laboratory equipment, such as freezers.

Reagents

ITEM	SUPPLIER	PN	NOTES
EasySep™ Direct Human PBMC Isolation Kit	STEMCELL Technologies	19654	Centrifuge Rapid Spheres for 30 seconds immediately prior to use. Store at 4°C. Each kit can process 30 reactions. Two kits are needed when processing 48 samples.
RNaseZap™ RNase Decontamination Solution	Thermo Fisher Scientific	AM9780	Or equivalent RNase decontamination solution.
Trypan Blue	Various Suppliers	Varies	Or alternative viability dyes, such as AO/PI.
Isopropyl alcohol	Various Suppliers	Varies	(Optional) If using a Mr. Frosty Freezing Container.
1X PBS	Various Suppliers	Varies	
DMSO	Various Suppliers	Varies	(Optional) If using the optional stopping point between PBMC Isolation and Barcoding.

Equipment

ITEM	SUPPLIER	PN	NOTES
EasyEights™ EasySep™ Magnet	STEMCELL Technologies	18103	Supports more than two reactions simultaneously for the standard 12-reaction and 48-reaction kits.
"The Big Easy" EasySep™ Magnet		18001	Supports processing up to two reactions at a time with the standard 12-reaction input kit.
"Easy50" EasySep™ Magnet		18002	Supports high-input workflows for both the 12-reaction and 48-reaction kits.
Centrifuge with Swinging Bucket Rotor			Compatible with 15 mL and 50 mL tubes and capable of reaching 4°C.

ITEM	SUPPLIER	PN	NOTES
Styrofoam Cooler	Various Suppliers	Varies	
1-channel: P20, P200, P1000	Various Suppliers	Varies	
Hemocytometer	Sigma-Aldrich®	Z359629	Or other cell counting device.
Mr. Frosty™ Freezing Container	Thermo Fisher Scientific®	5100-0001	Needed if storing fixed samples before processing with an Evercode kit. Or an equivalent device that cools samples at about -1°C/minute to minimize cell damage.
Water Bath	Various Suppliers	Varies	Or equivalent thermomixer, heat block, or bead bath capable of holding temperature at 37°C.

Consumables

ITEM	SUPPLIER	PN	NOTES
Pipette Tips TR LTS 20 µL, 200 µL, 1,000 µL	Rainin®	17014961, 17014963, 17014967	Or appropriate DNA low-binding, DNase/RNase-free, and filtered pipette tips. Do not use wide bore tips.
Falcon® High Clarity PP Centrifuge Tubes, 15 mL	Corning	352097	Or equivalent 15 mL centrifuge tubes. If using the high input protocol in Appendix C, do not substitute polystyrene tubes as it will lead to substantial cell loss.
Falcon® Round-Bottom Tubes, 14 mL	STEMCELL Technologies	100-0086 OR 38008 OR 38026	
Serological Pipettes (10 mL, 50 mL)	Thermo Scientific™	170356N 170358N	Or equivalent individually wrapped, RNase/DNase free.
Falcon® High Clarity PP Centrifuge Tubes, 50 mL	Corning	352070	Or equivalent 50 mL centrifuge tubes.
Blood Collection Tubes (BD vacutainer EDTA tubes 10 mL)	BD	366643	We do not recommend using Streck collection tubes.
Protein LoBind® Tubes, 1.5 mL	Eppendorf®	022431081	

Section 1: Fixation

1.1. Whole Blood Fixation

Whole blood is transferred from Blood Collection Vacutainer EDTA Tubes to 15 mL conical polypropylene tubes. Reagents are added to fix and permeabilize cells, and then to neutralize these reactions. Fixed whole blood is stored at -80°C or at -20°C.

To fix cells:

1. Gather the following reagents.

ITEM	SOURCE	FORMAT	HANDLING AND STORAGE
Blood Prefixation Buffer	Whole Blood Fixation Reagents (-20°C)	15 mL bottle	Thaw at room temperature then immediately store on ice. Mix by inverting each tube/bottle. Do not vortex.
Blood Fix and Perm Solution	Whole Blood Fixation Reagents (-20°C)	8 mL bottle	
Blood Neutralization Buffer	Whole Blood Fixation Reagents (-20°C)	15 mL bottle	

2. Fill a bucket with ice.
3. Place a styrofoam cooler at room temperature.
4. Invert whole blood in sample collection tube 10x to mix. Visually check that whole blood is homogenous with no signs of coagulation.
5. Transfer **188 µL** of whole blood from each sample into a 15 mL polypropylene conical centrifuge tube. Keep it on ice.
6. Add **688 µL** of Blood Prefixation Buffer to each tube and mix immediately by pipetting 5x with a P1000 set to 688 µL. Store on ice.
7. Add **205 µL** of Blood Fix and Perm Solution to each tube and mix immediately by pipetting 5x with a P1000 set to 500 µL.
8. Incubate on ice for **13 minutes.**
9. Add **1 mL** of Blood Neutralization Buffer to each tube. Immediately mix thoroughly by pipetting 5x with a P1000 set to 1000 µL.

10. Place the tubes with samples in a tube holder in a room temperature styrofoam cooler, close the lid, and store at -80°C to slowly cool the samples.

CRITICAL! Storing samples directly in the freezer without controlled cooling may lead to cell damage and compromise data quality.

Safe stopping point: Samples are stable for up to 3 months either at -80°C or at -20°C.

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1.2. Post Fixation PBMC Isolation

PBMC isolation from fixed whole blood is performed using magnetic beads. Prior to barcoding, samples should be thawed, processed, and resuspended in Sample Dilution Buffer. The Sample Dilution Buffer used to resuspend the fixed and isolated PBMCs is part of the barcoding kits. The desired Evercode kit should be purchased before starting the post-fixation PBMC isolation.

The protocol described below details the steps for PBMC isolation using the EasyEights magnet and differs from the manufacturer's instructions. If processing 2 or fewer samples at a time, The Big Easy magnet may be used instead, following instructions from Appendix B.

To isolate PBMCs:

1. Cool a centrifuge with swinging bucket rotors to 4°C.
2. Set a water bath to 37°C
3. Fill a bucket with ice.
4. Prepare an automated fluorescent cell counter, hemocytometer, flow cytometer, or other cell counting device.
5. Gather the following items and handle as indicated below.

ITEM	SOURCE	FORMAT	HANDLING AND STORAGE
1X PBS	Various Suppliers		
EasySep™ Direct Human PBMC Isolation Kit	STEMCELL Technologies		For handling the components, follow the kit manufacturer instructions.
STEMCELL Easy8 Magnet	STEMCELL Technologies		
Falcon® Round-Bottom Tubes, 14 mL	STEMCELL Technologies		
Sample Dilution buffer	-20°C Reagents		Thaw at room temperature then store on ice. Mix by inverting 3x.

6. Thaw the previously fixed whole blood samples in a water bath set to 37°C until all ice crystals dissolve. Thoroughly mix each sample by pipetting and store on ice.

7. Transfer **2 mL** of each fixed whole blood sample into individual 14 mL Falcon® Round-Bottom tubes.

8. Add **150 µL** of EasySep™ Direct Human PBMC Cocktail to each tube(s) and mix 5x immediately with a P1000 set to 1000 µL while on ice.

9. Incubate on ice for **5 minutes**.

10. With the samples still on ice, add **8 mL** of 1x PBS to each sample using a 10 mL serological pipette.

11. Vortex Direct RapidSpheres™ for **30 seconds** to mix.

12. Add **50 µL** of Direct RapidSpheres™ to each tube and mix immediately by pipetting 5x (or until well mixed) with 10 mL serological pipette.

13. Place the tube(s) (without lid) on the EasyEights™ EasySep™ Magnet for **10 minutes**.

CRITICAL! Keep samples and magnet on ice for these incubation periods.

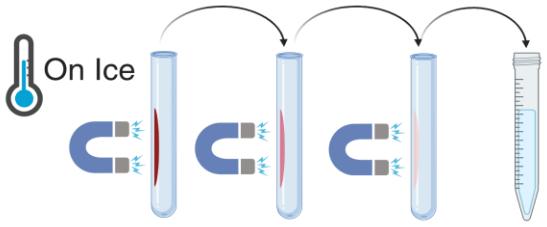
14. Using a 10 mL serological pipette, transfer enriched cell suspension to a new 14 mL Falcon® Round-Bottom Tube.

CRITICAL! Switch serological pipette between each sample.

15. Repeat steps 12-14 twice more for a total of 3 rounds of enrichment.

16. After the 3rd round of enrichment, carefully pipette the enriched cell suspension into a conical 15 mL polypropylene centrifuge tube using a 10 mL serological pipette (see image below).

CRITICAL! Collect the entire supernatant, all at once, into a single pipette (for EasyEights™ 14 mL tube, use a 10 mL serological pipette)



17. Centrifuge the 15 mL tube in a swinging bucket rotor for **10 minutes** at **400 x g** at 4°C. Immediately move to the next step after centrifugation.

18. Remove first **9 mL** supernatant post centrifugation using a 10 mL serological pipette. Use a P200 for the final **1 mL** of volume, leaving **~20 µL** maximum supernatant remaining.

CRITICAL! Switch pipettes and tips between each sample.

19. Resuspend pellets in **100 µL** Sample Dilution Buffer and mix well, switching tip between each sample.

20. Count cells using an automated cell counter and fluorescent dye (or equivalent cell counting device) and fill the Sample Loading Table with obtained cell counts.

CRITICAL! Do NOT count the cells when planning to freeze the PBMC post-isolation. Instead, proceed directly to step 22.

21. Proceed with barcoding with Evercode WT kits. If storing sample, proceed to the next step.

22. After resuspending enriched sample in Sample Dilution Buffer, add **to each sample DMSO** up to a final concentration of **5%** and transfer to 1.5 mL tubes. For each sample, set aside a **10 µL** "counting aliquot". This will be used to count cells and fill the Sample Loading Table with obtained cell counts prior to barcoding. Place the samples and the counting aliquots in a styrofoam cooler and store them at –80°C or -20°C.

Safe stopping point: enriched samples resuspended in Sample Dilution Buffer and **5% DMSO** stored either at -80°C or at -20°C for up to 1 month.

Appendices

Appendix A: High Input Workflow Set Up

If desired, 750 µL of whole blood can be fixed in a single reaction. However, this necessitates scaling up the reagent volume by 4x, which limits a 12 reaction kit to fixing only 3 samples. When using the 48 reaction kit, the total number of reactions possible is 12.

The figure below outlines the protocol for the high input fixation workflow.

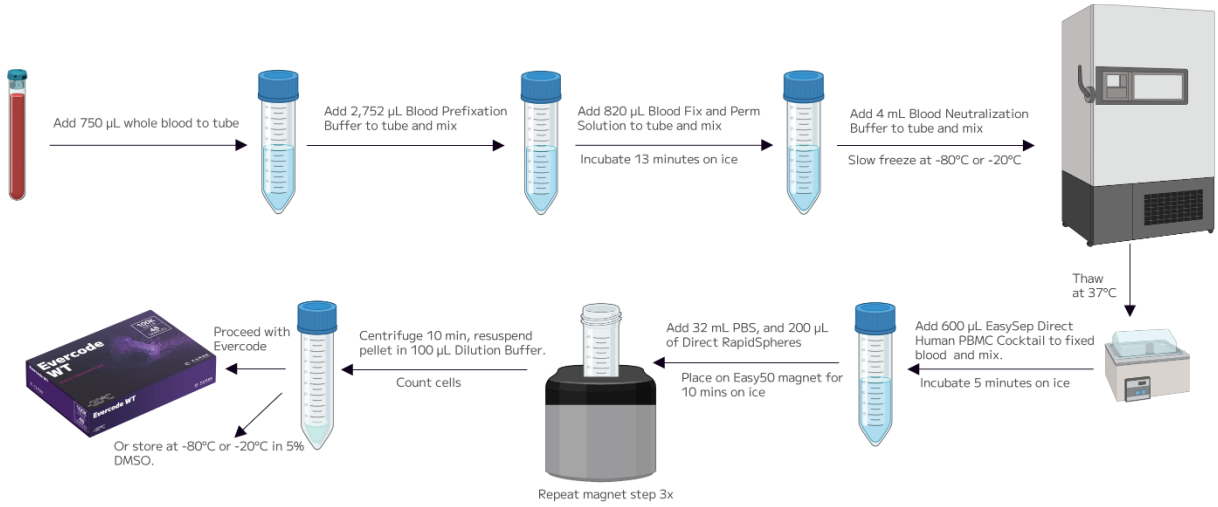


Figure 5: High Input Evercode Whole Blood Fixation Workflow.

Appendix A1: High Input Workflow

Whole blood is transferred from Blood Collection Vacutainer EDTA Tubes to 50 mL conical polypropylene tubes. Reagents are added to fix and permeabilize cells, and then to neutralize these reactions. Fixed whole blood is stored at -80°C.

To fix cells:

1. Fill a bucket with ice.
2. Place a styrofoam cooler at room temperature.
3. Invert blood collection tube 10x to mix. Visually check that whole blood is homogenous with no signs of coagulation.
4. Transfer **750 µL** of Whole Blood from each sample into a 50 mL polypropylene conical centrifuge tube. Keep in on ice.
5. Add **2,752 µL** of Blood Prefixation Buffer to each tube and mix immediately by pipetting 5x with a P1000 set to 1000 µL. Store on ice.
6. Add **820 µL** of Blood Fix and Perm Solution to each tube and mix immediately by pipetting 5x with a P1000 set to 1000 µL.
7. Incubate on ice for **13 minutes**.
8. Add **4 mL** of Blood Neutralization Buffer to each tube. Immediately mix thoroughly by capping and inverting the tube 3x.
9. Place the tubes with samples in a tube holder in a room temperature styrofoam cooler, close the lid, and store at -80°C to slowly cool the samples.

CRITICAL! Storing samples directly in the freezer without controlled cooling may lead to cell damage and compromise data quality.

Safe stopping point: Samples are stable for up to 3 months either at -80°C or at -20°C.

Appendix A2: Post Fixation PBMC Isolation - High Input Workflow

PBMC isolation from fixed whole blood is performed using magnetic beads. Prior to barcoding, samples should be thawed, processed, and resuspended in Sample Dilution Buffer. The Sample Dilution Buffer used to resuspend the fixed and isolated PBMCs is part of the barcoding kits. The desired Evercode kit should be purchased before starting the post-fixation PBMC isolation.

The protocol described below details the steps for PBMC isolation and differs from the manufacturer's instructions.

To isolate PBMCs:

1. Cool a centrifuge with swinging bucket rotors to 4°C.
2. Set a water bath to 37°C
3. Fill a bucket with ice.
4. Prepare an automated fluorescent cell counter, hemocytometer, flow cytometer, or other cell counting device.
5. Gather the following items and handle as indicated below.

ITEM	SOURCE	FORMAT	HANDLING AND STORAGE
1X PBC	Various Suppliers		
EasySep™ Direct Human PBMC Isolation Kit	STEMCELL Technologies		For handling the components, follow the kit manufacturer instructions.
STEMCELL Easy50 Magnet	STEMCELL Technologies		
Falcon® Tubes, 50 mL	Corning		
Sample Dilution buffer	-20°C Reagents		Thaw at room temperature then store on ice. Mix by inverting 3x.

6. Thaw the previously fixed whole blood samples in a water bath set to 37°C until all ice crystals dissolve. Thoroughly mix each sample by pipetting and store on ice.
7. Add **600 µL** of EasySep™ Direct Human PBMC Cocktail to each tube and mix 10x immediately with a P1000 set to 1000 µL while on ice.

8. Incubate on ice for **5 minutes.**

9. While on ice, add **32 mL** of 1x PBS to each sample using a 50 mL serological pipette.

10. Vortex the Direct RapidSpheres™ for **30 seconds** to mix.

11. Add **200 µL** of Direct RapidSpheres™ to each tube and mix immediately by pipetting up and down 5x (or until well mixed) with 50 mL serological pipette.

12. Place the tube (without lid) on the Easy50™ EasySep™ Magnet for **10 minutes** on ice.

CRITICAL! Keep samples and magnet on ice for these incubation periods.

13. Using a 50 mL serological pipette, transfer all but **~2 mL** of enriched cell suspension to a new 50 mL conical tube.

CRITICAL! Do not pipette the last ~2 mL of volume at this step, as this will transfer additional RBCs to enriched PBMC solution.

14. Add **200 µL** more Direct RapidSpheres™ to the 50 mL conical tube containing the enriched cell suspension and mix immediately by pipetting up and down with 50 mL serological pipette.

15. Place the tube (without lid) on the Easy50™ EasySep™ Magnet for **10 minutes**.

16. Using a 50 mL serological pipette, transfer all enriched cell suspension to a new 50 mL conical tube.

17. Repeat steps 14-16 for one final bead enrichment.

CRITICAL! Collect the entire supernatant, all at once, into a single pipette (for Easy50™ 50 mL tube, use a 50 mL serological pipette).

18. Centrifuge the 50 mL tube in a swinging bucket rotor for **10 minutes** at **400 x g** at 4°C. Immediately move to the next step after centrifugation.

19. Remove first **45 mL** supernatant post spin using a 50 mL serological pipette. Use a P1000 for the final 5 mL of volume, leaving ~50 µL maximum supernatant remaining.

20. Resuspend pellets in a **100 µL** Sample Dilution Buffer and mix well.

21. Count cells using an automated cell counter and fluorescent dye (or equivalent cell counting device) and fill the Sample Loading Table with obtained cell counts.

CRITICAL! Do NOT count the cells when planning to freeze the PBMC post-isolation. Instead, proceed directly to step 23.

22. Proceed with barcoding with Evercode WT kits. If storing sample, proceed to the next step.

23. After resuspending enriched sample in Sample Dilution Buffer, add **to each sample DMSO** up to a final concentration of **5%** and transfer to 1.5 mL tube**s**. For each sample, set aside a **10 µL** "counting aliquot". This will be used to count cells and fill the Sample Loading Table with obtained cell counts prior to barcoding. Place the samples and the counting aliquots in a styrofoam cooler and store them at –80°C.

Safe stopping point: enriched samples resuspended in Sample Dilution Buffer and **5% DMSO** stored either at -80°C or at -20°C for up to 1 month.

Appendix B: Single Magnet Workflow

If desired, cell isolation from fixed whole blood samples can be performed on one sample at a time using the STEMCELL Big Easy magnet, rather than the Easy8 Magnet. This magnet has slightly different parameters for use as detailed below. If processing more than two whole blood fixed samples at once, we recommend using the Easy8 magnet to minimize sample processing time and the results time samples spend on ice. The figure below outlines the protocol for using the Big Easy magnet.

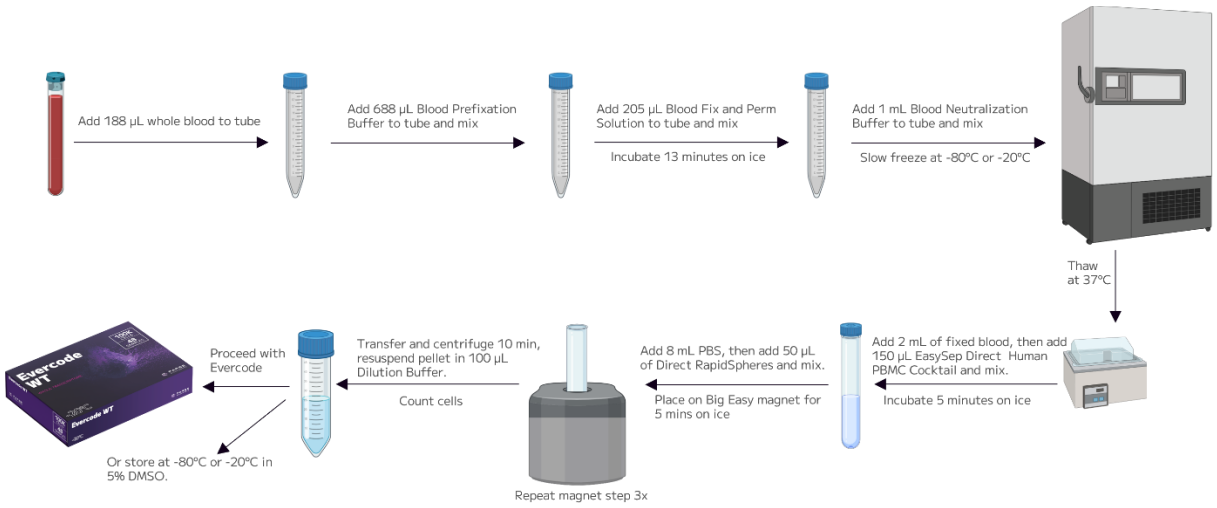


Figure 5: Cell Isolation workflow using the Big Easy magnet.

Appendix B1: Single Tube Cell Isolation

Whole blood is thawed and transferred from 15 mL conical polypropylene tubes to 14 mL round bottom tubes. PBMC isolation from fixed whole blood is performed using magnetic beads. Samples will be then resuspended in Sample Dilution Buffer prior to barcoding. The Sample Dilution Buffer used to resuspend the fixed and isolated PBMCs is part of the barcoding kits. The desired Evercode kit should be purchased before starting the post-fixation PBMC isolation.

The protocol described below details the steps for PBMC isolation and differs from the manufacturer's instructions.

To isolate PBMCs:

1. Cool a centrifuge with swinging bucket rotors to 4°C.
2. Set a water bath to 37°C
3. Fill a bucket with ice.
4. Prepare an automated fluorescent cell counter, hemocytometer, flow cytometer, or other cell counting device.
5. Gather the following items and handle as indicated below.

ITEM	SOURCE	FORMAT	HANDLING AND STORAGE
1X PBS	Various Suppliers		
EasySep™ Direct Human PBMC Isolation Kit	STEMCELL Technologies		For handling the components, follow the kit manufacturer instructions.
STEMCELL Big Easy Magnet	STEMCELL Technologies		
Falcon® Round-Bottom Tubes, 14 mL	STEMCELL Technologies		
Sample Dilution Buffer	-20°C Reagents		Thaw at room temperature then store on ice. Mix by inverting 3x.

6. Thaw the previously fixed whole blood samples in a water bath set to 37°C until all ice crystals dissolve. Thoroughly mix each sample by pipetting and store on ice.
7. Transfer **2 mL** of fixed whole blood samples to 14 mL Falcon® Round-Bottom tubes.

8. Add **150 µL** of EasySep™ Direct Human PBMC Cocktail to each tube and mix 5x immediately with a P1000 set to 1000 µL while on ice.

9. Incubate on ice for **5 minutes**.

10. While on ice, add **8 mL** of 1x PBS to each sample using a 10 mL serological pipette.

11. Vortex rapid spheres for **30 seconds** to mix.

12. Add **50 µL** of Direct RapidSpheres™ to each tube and mix immediately by pipetting with 10 mL serological pipette.

13. Place the tube (without lid) on the Big Easy™ EasySep™ Magnet for **5 minutes**.

14. Pick up the EasySep® Magnet, and in one continuous motion invert the magnet and tube, pouring the enriched cell suspension into a new 14 mL Falcon® Round-Bottom Tube (see diagram below).

15. Repeat steps 12-13 once more for a total of 2 isolations.

16. Carefully pipette the enriched cell suspension into a 15 mL polypropylene centrifuge tube using a 10 mL serological pipette.

CRITICAL! Collect the entire supernatant in a single, continuous pour.

17. Centrifuge the 15 mL tube in a swinging bucket rotor for **10 minutes** at **400 x g** at 4°C. Immediately move to the next step after centrifugation.

18. Remove first **9 mL** supernatant post spin using a 10 mL serological pipette. Use a P200 for the final **1 mL** of volume, leaving **~20 µL** maximum supernatant remaining.

19. Resuspend pellets in **100 µL** Sample Dilution Buffer and mix well.

20. Count cells using an automated cell counter and fluorescent dye (or equivalent cell counting device) and fill the Sample Loading Table with obtained cell counts.

CRITICAL! Do NOT count the cells when planning to freeze the PBMC post-isolation. Instead, proceed directly to step 22.

21. Proceed with Barcoding with Evercode WT kits. If storing sample, proceed to the next step.

22. After resuspending enriched sample in Sample Dilution Buffer, add **to each sample DMSO** up to a final concentration of **5%** and transfer to 1.5 mL tube**s**. For each sample, set aside a **10 µL** "counting aliquot". This will be used to count cells and fill the Sample Loading Table with obtained cell counts prior to barcoding. Place the samples and the counting aliquots in a styrofoam cooler and store them at –80°C.

Safe stopping point: Enriched sample resuspended in Sample Dilution Buffer and 5% DMSO stored either at -80°C or at -20°C for up to 1 month.

Appendix C: Revision History

Version	Description	Date
1.0	Initial Release	December 2025
1.1	Updated temperature and storage time	February 2026

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