tapestri

BMMC Preparation for Tapestri® Single-Cell MRD

User Guide

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Introduction

Acute Myeloid Leukemia (AML) is a complex and dynamic disease with a diverse genetic landscape[1]. The heterogeneity of clonal architecture and the genotypic and phenotypic drifts that can occur during treatment explain why a significant proportion of patients in complete remission subsequently relapse and ultimately succumb to the disease[2].

Measurable residual disease (MRD) testing has become standard in AML and other cancers for detecting residual leukemic clones which persist following therapy and are predictive of relapse. Testing for MRD can shape risk stratification and guide early follow-up treatment decisions to ultimately improve patient outcomes[3].

MRD typically represents an evolved disease state, distinct from the original diagnosis; the high degree of molecular heterogeneity and the rapid evolution of clones in AML present a challenge for MRD monitoring. Current single-analyte approaches used to detect MRD, such as multiparameter flow cytometry (MFC) and bulk next-generation sequencing (NGS), are limited by both false-positive and false-negative results. Moreover, even when these single-analyte assays are concordant on MRD status (e.g., MRD positive versus negative), they are often discordant with patient outcomes (e.g., relapse versus non-relapse), highlighting the fragmented data and incomplete insights provided by these approaches[4,5]. Recently published single-cell data leveraging Tapestri's sensitivity and multiomics capabilities further emphasize the need to assess clinical samples holistically across multiple modalities[6-8].

To more adequately address the existing gaps in MRD detection, Mission Bio has introduced the *Tapestri® Single-Cell MRD AML Multiomics Assay*. The single-cell MRD assay is the industry's first approach to integrate genotypic and immunophenotypic assessment in the same individual cells, enabling scMRD detection with unparalleled resolution, sensitivity, and specificity. This approach enables deconstruction of the complex genetic heterogeneity driving disease transformation and differentiation between leukemic clones and clonal hematopoiesis, ultimately improving the identification of residual disease and potential therapeutic strategies.

About This Guide

Here we introduce a detailed experimental procedure for preparation of cryopreserved bone marrow mononuclear cells (BMMCs), subsequent staining with antibody-oligo conjugates (AOCs), and magnetic-activated cell sorting (MACS) for enrichment of CD34-positive and CD117-positive cells prior to single-cell library preparation on the Mission Bio Tapestri® Platform. This protocol has been validated for BMMCs only, and includes instructions for multiplexing up to three patient samples in a single Tapestri run. Following staining, enrichment, and sample pooling, users should refer to the *Tapestri® Single-Cell DNA + Protein v3 User Guide (PN MB05-0018)* for subsequent protocol steps, starting with *Chapter 2: Encapsulate Cells.*

Materials

Tapestri® Single-Cell MRD AML Kit Components

Component Name	Kit	Storage
○ Blocking Buffer (white cap)	Tapestri® Protein Staining Kit v3	4 °C
TotalSeq™-D scMRD AML Cocktail	TotalSeq™-D scMRD AML Cocktail Box	4 °C
Cell Buffer	Tapestri® Single-Cell DNA Core +4 Kit v3	4 °C

Required Third Party Consumables and Reagents

Component Name	Supplier (Part Number)	Protocol Step
TipOne RPT ultra low retention filter tip	USA Scientific (1180-8810) or Approved Supplier	Reagent handling
1.5 mL Protein LoBind tubes	Eppendorf (22431081) or Approved Supplier	Reagent handling
200 µL Wide bore tip, rack, sterile 1000 µL Wide bore tip, rack, sterile	USA Scientific (1011-8410), USA Scientific (1011-9410) or Approved Supplier	Cell handling
15 mL DNA LoBind conical tubes	Eppendorf (30122208) or Approved Supplier	Cell handling
1.5 mL DNA LoBind Microcentrifuge Tubes	Eppendorf (0030108035) or Approved Supplier	Cell handling
Flowmi™ Cell Strainers for 1000 µL pipette tips, 40 µm	Fisher Scientific (14-100-150)	Cell handling
Sterile single-pack CellTrics™ filters, 30 µm	Sysmex (04-004-2326)	Cell thawing
Fetal Bovine Serum (FBS)	ATCC (30-2021) or Approved Supplier	Cell thawing
Trisodium citrate dihydrate [CAS number 6132-04-3]	Millipore Sigma (1064321000)	Cell thawing
Citric acid monohydrate [CAS number 5949-29-1]	Millipore Sigma (1002431000)	Cell thawing
D-(+)-Glucose monohydrate [CAS number 14431-43-7]	Millipore Sigma (1083421000)	Cell thawing
Propidium Iodide (PI)	Thermo Fisher (P3566) or Approved Supplier	Cell quantification
Hoechst 33342 (OPTIONAL)	Thermo Fisher (H3570) or Approved Supplier	Cell quantification
Trypan Blue (OPTIONAL)	Thermo Fisher (15250061) or Approved Supplier	Cell quantification
Human TruStain FcX (Fc Receptor Blocking Solution)	BioLegend (422301)	Cell staining

Component Name	Supplier (Part Number)	Protocol Step
Cell Staining Buffer (CSB)	BioLegend (420201)	Cell staining
MS Columns plus tubes	Miltenyi Biotec (130-122-727)	Cell enrichment
CD34 MicroBead Kit UltraPure, human	Miltenyi Biotec (130-100-453)	Cell enrichment
CD117 MicroBead Kit, human	Miltenyi Biotec (130-091-332)	Cell enrichment
AutoMACS® Rinsing Solution	Miltenyi Biotec (130-091-222)	Cell enrichment
MACS® BSA Stock Solution	Miltenyi Biotec (130-091-376)	Cell enrichment
EasySep™ RBC Depletion Reagent (OPTIONAL)	STEMCELL Technologies (18170)	RBC depletion
EDTA (OPTIONAL)	Thermo Fisher (AM9260G) or Approved Supplier	RBC depletion
DPBS w/o Ca^{2+}/Mg^{2+} (1X) (OPTIONAL)	Thermo Fisher (14190144) or Approved Supplier	RBC depletion; Dead cell removal
EasySep™ Dead Cell Removal (Annexin V) Kit (OPTIONAL)	STEMCELL Technologies (17899)	Dead cell removal
Calcium Chloride (OPTIONAL)	Sigma-Aldrich (21115) or Approved Supplier	Dead cell removal

NOTE ***Unless otherwise noted, do not substitute. Only listed consumables have been validated by Mission Bio.***

Required Benchtop Equipment

Equipment	Supplier (Part Number)
Pipettes, 1 µL – 1000 µL	Mettler-Toledo, Rainin Pipettes, or Approved Supplier
Microcentrifuge (1.5 mL PCR tubes) with temperature control	Thermo Fisher (5406000240) or Approved Supplier
Tube Vortexer	Thermo Fisher (88880017TS) or Approved Supplier
Centrifuge with temperature control and swinging bucket (needs to support 15 mL and 1.5 mL tubes)	Eppendorf (5810 R) or Approved Supplier
Countess™ II Automated Cell Counter or equivalent	Thermo Fisher (AMQAX1000)
OctoMACS™ Separator	Miltenyi Biotec (130-042-109)
MACS® MultiStand	Miltenyi Biotec (130-042-303)
OctoMACS™ Acrylic Tube Rack	Miltenyi Biotec (130-090-448)
1.5 mL tube Magnetic Separation Rack (for use with Dead Cell Removal Kit, OPTIONAL)	New England Biolabs (S1506S) or Approved Supplier

NOTE ***Unless otherwise noted, do not substitute. Only listed equipment have been validated by Mission Bio.***

BMMC Handling Guidelines

The steps provided in this protocol are applicable to mononuclear cells derived from bone marrow aspirates. The cell enrichment strategy described here is designed to increase the relative fraction of cells expressing CD34 and/or CD117 surface antigens. Different sample types may require revised procedures including cell thawing, washing, enrichment, or quantification.

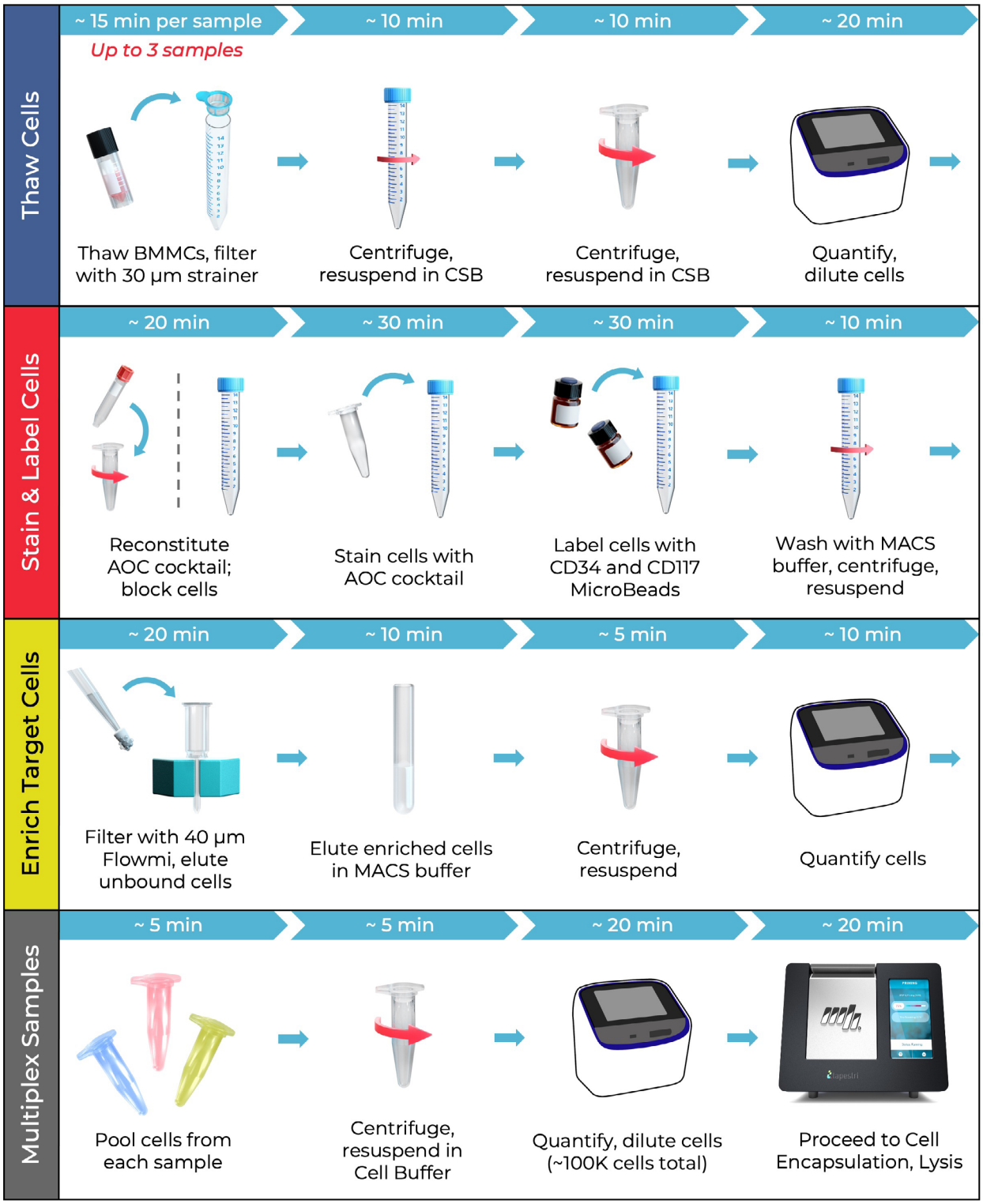
Cell handling

- Avoid the use of samples containing significant debris, dead cells, or fragments of lysed cells.
- For optimal performance, it is important to obtain a single-cell suspension before cell staining and magnetic labeling. Pass cells through a 30 µm filter to remove cell clumps during thawing and manually remove cell clumps throughout the process to prevent non-specific antibody binding and clogging of the enrichment column.
- The workflow is optimized for a starting cell number of 2–5 million. Using a cell input number outside of this range may result in insufficient cell recovery, cell staining, and/or enrichment.
- Using a fixed rotor centrifuge may result in cell loss due to insufficient cell pelleting. It is strongly recommended to use a centrifuge with swinging buckets that supports 15 mL and 1.5 mL tubes to maximize cell recovery.
- Use of wide bore tips to mix and resuspend cells is recommended to maintain cell viability.
- When not actively handling cells, store on ice.

Cell quantification and viability

- Mission Bio strongly recommends the use of an automated cell counter, such as the Countess™ Automated Cell Counter (Thermo Fisher, see *Appendix A: Cell Quantification with Countess™*).
- Mission Bio strongly recommends the use of fluorescent exclusion reagents such as Propidium Iodide (PI) to determine cell death/viability. PI-based assays compared to Trypan Blue-based assays are more robust in accurately determining the percentage of dead/viable cells. Please follow the manufacturer's instructions when using PI-based viability assays.
- Red blood cell contamination of BMMC samples can be determined using a nuclear dye such as Hoechst. If the fraction of red blood cells is higher than 50%, red blood cell depletion is recommended (see *Appendix B: Red Blood Cell Depletion*).
- Cell suspensions should have a viability > 80% prior to cell staining. If viability is lower, dead cell removal is recommended (see *Appendix C: Dead Cell Removal*).
- Final cell concentration values are based on the **total (live + dead)** cell counts, *excluding* red blood cells (non-nucleated, Hoechst-negative).
- Following cell enrichment, up to three samples from unrelated individuals may be multiplexed for one Tapestri run. It is recommended to pool a minimum of 30,000–50,000 cells from each sample for a total input of ~100,000 cells in Mission Bio Cell Buffer (2,800–3,200 cells/µL) for Cell Encapsulation on the Tapestri® instrument.

Protocol Overview



Total time until stopping point: ~ 5 hours

Cell Preparation Protocol

1. Prepare Stock and Working Solutions

Cell Preparation Protocol

1. Prepare Stock and Working Solutions

1.1 Prepare 100 mL of thawing media (50% FBS + 50% citrate solution):

» a. Prepare citrate solution:

Reagent	Amount
Trisodium citrate dihydrate	22.0 g
Citric acid monohydrate	8.0 g
D-(+)-Glucose monohydrate	24.5 g
Molecular grade water	Fill to 1 liter

Table 1. Citrate solution components.

NOTE ***Use the solution as-is, no pH adjustment is required. Solution can be made in advance and stored at room temperature for up to 6 months.***

» b. Prepare thawing media:

Reagent	Volume (mL)
Citrate solution from *Step 1.1a*	50
Fetal Bovine Serum (FBS)	50

Table 2. Thawing media components.

NOTE ***Thawing media can be made in advance and stored at 4 °C for up to 6 months.***

1.2 Prepare MACS buffer: add 75 mL of MACS® BSA Stock Solution to 1,450 mL of AutoMACS® Rinsing Solution and invert 5 times to mix. Keep buffer on ice.

NOTE ***This protocol requires 18 mL of MACS buffer per sample. Remaining MACS buffer can be stored at 4 °C for up to 6 months.***

1.3 ***OPTIONAL*** (If using the Countess™ Automated Cell counter along with *Appendix A: Cell Quantification with Countess™*) prepare 100 µL of cell quantification dye solution:

Reagent	Volume (µL)
DPBS	96
Stock Propidium Iodide (PI) Solution	2
Stock Hoechst Solution	2

Table 3. Cell quantification dye solution components.

Cell Preparation Protocol

2. Thaw Cells

2. Thaw Cells

This section describes the steps required to prepare a single-cell suspension, count cells, and assess cell quality and viability. This workflow is optimized for a starting cell number of 2–5 x 10^6 BMMCs at greater than 80% viability.

2.1 Retrieve all reagents required for cell thawing:

- Thawing media (from *Step 1.1b*) → warm to 37 °C in a water bath
- Cell Staining Buffer (CSB, BioLegend) → place on ice

2.2 **Warm thawing media** to 37 °C.

IMPORTANT ***Perform Steps 2.3–2.9 for each sample one at a time. Samples can be processed side-by-side for the remaining steps.***

2.3 Remove cryovial of cells from liquid nitrogen or the -80 °C freezer, **immediately transfer** to a biosafety hood, twist the cap a quarter to relieve pressure, and immediately retighten.

2.4 **Immediately transfer to a 37 °C water bath** and quickly thaw the vial by gently swirling the tube until a small amount of ice remains (< 1 minute). Be sure to avoid submerging the tube completely.

2.5 Remove tube and clean with 70% ethanol.

2.6 Using a wide bore P-1000 tip, add **1 mL of thawing media drop-wise** to the cryovial. **Transfer** the entire contents of the vial to a **15 mL conical tube**.

2.7 Using a wide bore P-1000 tip, **rinse** the vial with **1 mL of thawing media.** Transfer to the 15 mL conical tube containing the cells, drop by drop, making sure to pipette against the wall. Gently shake tube while adding.

2.8 **Add 0.5 mL of thawing media** to the 15 mL tube **every 20-30 seconds until 12 mL total volume** is reached. Gently mix the tube by hand after each addition.

2.9 If there are visible cell clumps, **filter the cells through a 30 µm CellTrics™ filter** into a new 15 mL conical tube:

- a. Place a 30 µm CellTrics™ filter over a 15 mL tube and **prime the filter by adding 1 mL of thawing media.** Allow all the liquid to flow through the filter.
- b. **Discard** the flow-through.
- c. **Transfer the cell suspension** from *Step 2.8* to the 15 mL tube through the primed 30 µm CellTrics™ filter. Allow all the liquid to flow through the filter by gravity.
- d. Discard the filter.

IMPORTANT ***If processing multiple samples, place the 15 mL tube on ice and repeat Steps 2.3–2.9 for the next sample until all samples have been thawed.***

2.10 Centrifuge cell suspension at **200 x g for 5 minutes** at room temperature.

2.11 Aspirate all supernatant. **Do not disturb the cell pellet.**

2.12 Using a wide bore P-1000 tip, gently resuspend the cell pellet in **1 mL of CSB**.

NOTE ***If there are visible cell clumps, remove by incubating cell suspension for 1 minute to allow cell clumps to settle before transferring to a new tube in Step 2.13.***

2.13 Transfer cell suspension to a new **1.5 mL DNA LoBind tube**.

2.14 Centrifuge cell suspension at **200 x g for 5 minutes** at room temperature.

2.15 Aspirate all supernatant. **Do not disturb the cell pellet.**

2.16 Gently resuspend the cell pellet in **135 µL of CSB.**

2.17 **Quantify the cells and assess viability** using an automated cell counter or hemocytometer following best practices and the manufacturer's instructions (see *Appendix A: Cell Quantification with Countess™*).

IMPORTANT ***If Hoechst-positive (nucleated) cells are <50%, red blood cell depletion is recommended (see Appendix B: Red Blood Cell Depletion).***

IMPORTANT ***If PI-negative (live) cells are <80%, dead blood cell removal is recommended (see Appendix C: Dead Cell Removal).***

2.18 Dilute cell suspension to **15,000 to 38,500 cells/µL** using CSB in a **minimum volume of 130 µL.**

2.19 **Store the cells on ice** and proceed immediately to *Step 3.1*.

Cell Preparation Protocol

3. Reconstitute AOC Panel and Stain Cells

3. Reconstitute AOC Panel and Stain Cells

In this step, cells are stained with oligo-tagged antibodies. The TotalSeq™-D scMRD AML Cocktail is supplied as lyophilized powder in single reaction vials. The cocktail must be reconstituted prior to staining the cells. One vial of lyophilized cocktail is required for each sample (up to three samples total).

3.1 Retrieve all reagents required for staining the cells:

- Human TruStain FcX (BioLegend) → place on ice
- Blocking Buffer (○) (Protein Staining Kit, Mission Bio) → place on ice
- Cell Staining Buffer (CSB, BioLegend) → place on ice
- TotalSeq™-D scMRD AML Cocktail (4 °C, Mission Bio) → keep at room temperature

Reconstitute Antibody-Oligo Conjugate (AOC) Panel

IMPORTANT ***Perform Steps 3.2–3.8 in parallel for up to three vials of lyophilized AOC cocktail, depending on how many samples are being processed.***

3.2 Retrieve a vial of lyophilized TotalSeq™-D scMRD AML Cocktail and equilibrate to room temperature for 5 minutes.

3.3 Centrifuge the vial at **10,000 x g for 30 seconds** at room temperature.

3.4 Resuspend the lyophilized panel in **60 µL of CSB**. Close the tube with the original cap and vortex for 10 seconds.

3.5 Incubate at **room temperature for 5 minutes**.

3.6 **Vortex** the tube for **10 seconds** and centrifuge at **10,000 x g for 30 seconds** at room temperature.

3.7 **Transfer the entire volume (60 µL)** of reconstituted panel to a **1.5 mL Protein LoBind tube**.

3.8 Centrifuge the tube at **14,000 x g for 15 minutes at 4 °C**. Once completed, the reconstituted cocktail must be used immediately in *Step 3.11*. In the meantime, proceed to the next step.

Stain Cells

3.9 **For each sample**, add the following reagents to a **15 mL DNA LoBind** conical tube:

Reagent	Volume (µL)
Cell Suspension in CSB (15,000–38,500 cells/µL)	130
Blocking Buffer (○)	10
Human TruStain FcX	10
Total Volume	**150**

Table 4. Reagents to block cells.

3.10 **Gently mix** by pipetting up and down with a wide bore P-200 tip and **incubate the solution for 15 minutes on ice**.

3.11 **Transfer 50 µL** of the reconstituted TotalSeq™-D scMRD AML Cocktail (from *Step 3.8*) **to the blocked cell suspension**. Cell staining solution total volume is 200 µL.

IMPORTANT ***Avoid touching the bottom or sides of the tube containing the reconstituted TotalSeq™-D scMRD AML Cocktail with the pipette tip to avoid pelleted protein aggregates. Aggregates are not visible.***

3.12 **Gently mix** by pipetting up and down with a wide bore P-200 tip.

3.13 **Incubate cell staining solution for 30 minutes on ice**, then proceed to *Step 4.1*.

Cell Preparation Protocol

4. Enrich Cells: Magnetic Labeling and Separation

4. Enrich Cells: Magnetic Labeling and Separation

In this step, cells are labeled with CD34 and CD117 MicroBeads (Miltenyi Biotec), which will preferentially bind to cells expressing these cell surface markers. Cell suspensions are then passed through a magnetic column, wherein unlabeled cells will be washed out as flow-through. Finally, the enriched target cells are eluted into a new tube (off the magnet).

4.1 Retrieve all reagents required for cell enrichment:

- MACS buffer (from *Step 1.2*) → place on ice
- CD34 MicroBeads (Miltenyi Biotec) → place on ice
- CD117 MicroBeads (Miltenyi Biotec) → place on ice

Magnetic Labeling

4.2 With a wide bore P-200 tip, add **200 µL of MACS buffer** to the stained cells and resuspend gently.

NOTE ***If there are visible cell clumps, remove by incubating cell suspension for 1 minute to allow cell clumps to settle, and transfer supernatant to a new tube.***

4.3 Add **50 µL each of CD34 MicroBeads and CD117 MicroBeads** to stained cells. Total volume is 500 µL.

4.4 Gently mix with a wide bore P-1000 tip and incubate the solution for **30 minutes on ice.**

4.5 Add **14 mL of MACS buffer** and centrifuge at **200 x g for 10 minutes at 4 °C.**

4.6 Aspirate and discard supernatant, leaving **~100 µL.**

NOTE ***While the MicroBeads are brown, the cell pellet will not appear brown.***

4.7 Using a wide bore P-1000 tip, add **400 µL of MACS Buffer** and gently resuspend cells. Total volume is ~500 µL.

4.8 **Store the cells on ice** and proceed immediately to *Step 4.9*.

Magnetic Separation

4.9 Set up enrichment columns:

- a. Mount the OctoMACS™ Separator onto the MACS® Multistand.
- b. Insert a **MS column** into the OctoMACS™ Separator **with the column wings oriented to the front**.
- c. Place a **5 mL collection tube** in the OctoMACS™ Acrylic Tube Rack directly below the MS column.

4.10 Prepare the MS column by **rinsing with 500 µL of MACS buffer.** Allow column reservoir to empty before proceeding to the next step.

4.11 Filter and apply 500 µL cell suspension to column:

- a. Prime a **40 µm Flowmi™ cell strainer**: aspirate **500 µL of MACS buffer** with a P-1000 tip, place filter on pipette tip, and **dispense the liquid through the filter.**
- b. **Remove the filter from pipette tip** (do not discard).
- c. Aspirate **500 µL cell suspension** from *Step 4.8* using a P-1000 tip.
- d. Place primed filter on tip and **dispense cell suspension through the filter into the column reservoir.**
- e. Allow column reservoir to empty before proceeding to the next step.

4.12 Wash the column with **500 µL of MACS buffer.**

4.13 Repeat *Step 4.12* twice for a **total of 3 washes**, allowing the column reservoir to empty between washes (2–3 minutes per wash).

IMPORTANT ***Once washes are complete, place the flow-through cells on ice for use in Step 5.5.***

4.14 **Remove MS column** from the magnet and place on a new **5 mL collection tube**.

4.15 Add **1 mL of MACS buffer** to column and allow magnetically labeled cells to **elute off the column by gravity (5–10 minutes).**

4.16 **Place enriched cell suspension on ice** and proceed immediately to *Step 5.1*.

Cell Preparation Protocol

5. Quantify Cells and Multiplex Samples

5. Quantify Cells and Multiplex Samples

In this step, cells from up to three samples are pooled together and cell numbers are adjusted to the correct concentration for Cell Encapsulation on the Tapestri® instrument.

5.1 Retrieve the following reagent required for multiplexing:

- » Cell Buffer (4 °C, Mission Bio) → place on ice

5.2 Transfer enriched cell suspension to a new **1.5 mL DNA LoBind tube** and centrifuge at **200 x g for 5 minutes** in a swinging bucket.

5.3 Carefully **remove the supernatant, leaving 100–150 µL,** and **resuspend cells** in the remaining supernatant with a wide bore P-200 tip.

5.4 **Quantify the cells and assess viability** using an automated cell counter or hemocytometer following best practices and the manufacturer's instructions (see *Appendix A: Cell Quantification with Countess™*). **No cell dilution is necessary.**

NOTE ***The recommended input for Cell Encapsulation on the Tapestri® instrument is 100,000 cells (30,000 minimum). It is recommended to use 150,000 cells in Step 5.5 below (50,000 minimum), to account for cell loss. Pool cells from up to three samples in equal proportions, if possible. If a minimum of 50,000 total cells is not attainable from the enriched cell fractions (after pooling), it is recommended to add cells from the flow-through (saved at Step 4.13) to reach 50,000 cells. Leftover flow-through cells can then be stored for optional DNA extraction (centrifuge at 500 x g for 5 minutes, remove supernatant, and store at -20 °C; contact support@missionbio.com for additional information).***

5.5 **Pool enriched cells** from up to three samples for a **total of 150,000 cells** in a new **1.5 mL DNA LoBind** tube. *If needed, add flow-through cells from one or more samples (from Step 4.13) to reach a minimum of 50,000 total cells.*

5.6 Centrifuge the multiplexed cell suspension at **200 x g for 5 minutes** at 4 °C in a swinging bucket.

5.7 Carefully remove all supernatant. **Do not disturb the cell pellet.**

5.8 Gently resuspend the pellet in **40 µL of Mission Bio Cell Buffer** by pipetting up and down several times with a wide bore P-200 tip.

5.9 **Quantify the cells and assess viability** using an automated cell counter or hemocytometer following best practices and the manufacturer's instructions (see *Appendix A: Cell Quantification with Countess™*).

5.10 If necessary, dilute cell suspension to **2,800–3,200 cells/µL** using Cell Buffer. **Confirm final concentration using a cell counter or hemocytometer.**

5.11 **Place cells on ice** and proceed **immediately** to Cell Encapsulation on the Tapestri® instrument (*Tapestri® Single-Cell DNA + Protein v3 User Guide, PN MB05-0018, Chapter 2: Encapsulate Cells*).

DNA + Protein Protocol

Appendices

Appendices

Appendix A: Cell Quantification with Countess™

This section describes the steps required to quantify cells, assess viability, and determine the relative proportion of red blood cells in a single cell suspension using the Countess™ Automated Cell Counter.

6.1 Retrieve all reagents required for cell quantification:

- Cell Staining Buffer (CSB, BioLegend) → place on ice
- Cell quantification dye solution (from *Step 1.3*) → place on ice

6.2 **Dilute cells 10x** by adding 5 µL of cells in 45 µL of CSB. Gently mix using a wide bore P-200 tip.

6.3 Mix 5 µL of diluted sample + 5 µL of freshly prepared cell quantification dye solution.

6.4 Load the cell/dye mix (10 µL) onto a Countess™ slide.

6.5 Select the **DAPI and Texas red channels** for quantification.

6.6 **Calculate the sample cell concentration** by multiplying the Countess™ concentration by a factor of 20 (for dilution and dye correction).

6.7 **Calculate the fraction of red blood cells** as the percentage of **cells that are *negative* for DAPI (Hoechst).**

6.8 **Calculate the cell viability** as the percentage of **cells that are *negative* for Texas red (PI).**

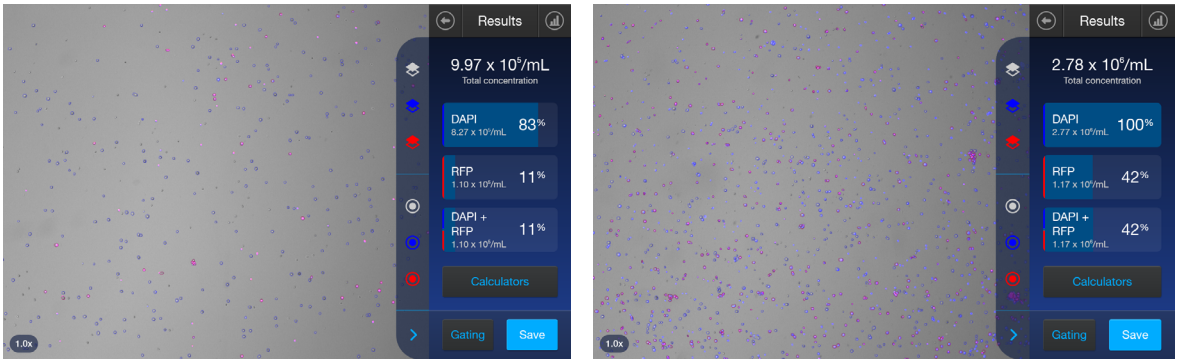


Figure 1. Representative images of high-quality cell suspensions (left image, 89% viable, few multiplets) and low-quality cell suspensions (right image, 58% viable, many multiplets/clumps).

Appendix B: Red Blood Cell Depletion

This section describes the steps required to deplete red blood cells (RBCs) from a cell suspension.

7.1 Retrieve all reagents required for red blood cell removal:

- DPBS w/o Ca^{2+}/Mg^{2+} (1X) → keep at room temperature
- FBS → keep at room temperature
- 0.5 M EDTA → keep at room temperature
- EasySep™ RBC Depletion Reagent (STEMCELL Technologies) → keep at room temperature

7.2 Prepare 1 mL of DPBS + 2% FBS by **adding 20 µL of FBS to 980 µL of DPBS.**

7.3 Centrifuge cells at **200 x g for 5 minutes** at room temperature.

7.4 Carefully remove all supernatant. **Do not disturb the cell pellet.**

7.5 Add **500 µL of DPBS + 2% FBS solution** and gently resuspend cell pellet by **pipetting up and down 5x** with a wide bore P-1000 tip.

7.6 **Add 6 µL of 0.5 M EDTA** and mix by **pipetting up and down 5x** with a wide bore P-1000 tip.

7.7 Add **500 µL of DPBS + 2% FBS solution**.

7.8 **Vortex Depeletion Reagent** thoroughly for **30 seconds.**

7.9 Add **25 µL of Depletion Reagent** and mix using a wide bore P-1000 tip. **Immediately transfer the tube to the magnetic stand,** leaving the lid open.

7.10 Once the solution is clear (3–5 min), **transfer supernatant** into a new **1.5 mL DNA LoBind** tube. Proceed with *Step 2.14*.

NOTE ***If a significant amount of RBCs remain (>50%), repeat Steps 7.8–7.10.***

Appendix C: Dead Cell Removal

This section describes the steps required to remove dead cells from a single cell suspension.

8.1 Retrieve all reagents required for dead cell removal:

- DPBS w/o Ca^{2+}/Mg^{2+} (1X) → keep at room temperature
- FBS → keep at room temperature
- 1 M $CaCl_2$ → keep at room temperature
- EasySep™ Dead Cell Removal (Annexin V) Kit (STEMCELL Technologies) → keep at room temperature

8.2 Prepare 2 mL of DPBS + 2% FBS by **adding 40 µL of FBS to 1,960 µL of DPBS.**

8.3 Prepare 1 mL of 50 mM $CaCl_2$ resuspension buffer by **adding 50 µL of 1 M $CaCl_2$ to 950 µL of DPBS + 2% FBS.**

8.4 **Centrifuge cells at 200 x g for 5 minutes at room temperature.**

8.5 **Carefully remove** all supernatant. **Do not disturb the cell pellet.**

8.6 For *Steps 8.7–8.14*, refer to the following volumes for each reagent:

Total cells	DPBS + 2% FBS (Volume A)	Resus. Buffer (Volume B)	Annexin V (Volume C)	Biotin (Volume D)	RapidSpheres (Volume E)
< 10^7	100 µL	2 uL	5 uL	5 uL	10 uL
≥ 10^7	1 mL	20 uL	50 uL	50 uL	100 uL

Table 6. Reagent volumes for dead cell removal.

8.7 **Add Volume A of DPBS + 2% FBS solution** and gently resuspend cell pellet by **pipetting up and down 5x** with a wide bore tip.

8.8 **Add Volume B of resuspension buffer (2% FBS + 50 mM $CaCl_2$)** and mix by **pipetting up and down 5x** with a wide bore tip.

8.9 **Transfer** cells into a new **1.5 mL DNA LoBind** tube.

8.10 Add **Volume C of annexin V.**

8.11 Add **Volume D of biotin** and mix by **pipetting up and down 5x** with a wide bore tip.

8.12 Incubate for 3 minutes at room temperature.

8.13 **Vortex RapidSpheres™** thoroughly for **30 seconds.**

8.14 Add **Volume E of RapidSpheres™** and mix using a wide bore tip. **Immediately transfer the tube to the magnetic stand,** leaving the lid open.

8.15 Once the solution is clear (3-5 min), **transfer supernatant** into a new **1.5 mL DNA LoBind** tube. Proceed with *Step 2.14*.

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