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USER MANUAL

FIXATION

FOR USE WITH

Cell Fixation and Nuclei Fixation

Parse BIOSCIENCES

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Additional Materials and Equipment

The following materials and equipment are required to perform the protocol, but are not provided within the kit. Refer to Appendix A for vendor and catalog numbers for these items. Any questions regarding these items can be directed to support@parsebiosciences.com.

General

Swinging Bucket Centrifuge (capable of holding 15 mL tubes and reaching 4°C)

Tabletop Centrifuge (for spinning down residual liquid in 1.7 mL and 0.2 mL tubes)

Cell Counting Device (e.g. hemocytometer, flow cytometer, etc.)

Water Bath (for thawing frozen cells)

Pipettes

- P1000
- P200
- P20

Pipette tips

Tubes

- Eppendorf DNA/RNA LoBind 1.7 mL tubes
- Falcon 15 mL **polypropylene** centrifuge tubes
  Note: centrifuge tubes must be polypropylene and not polystyrene. Polystyrene centrifuge tubes will lead to substantial cell loss.

If performing fixation on adherent cells:

TrypLE Express (+) phenol Red for detaching cells from culture plates.
Note: use of trypsin is NOT recommended, due to the presence of variable amounts of RNAses in trypsin formulations.

If performing fixation on nuclei, PBMCs, or other cell types prone to clumping:

Bovine Serum Albumin (Bovine Albumin Fraction V, 7.5% solution)

If planning to store samples for later processing:

Access to a -80°C freezer (if storing samples)

“Mr. Frosty” or similar device which enables cooling of a sample at around -1°C/minute after being placed in a -80°C freezer.

SECTION 1

CELL FIXATION

PARTS LIST

NOTES BEFORE STARTING

CELL FIXATION PROTOCOL

Cell Fixation Parts List

Cell Fixation Reagents (-20°C) WF100				
Label	**Component**	**Format**	**Quantity**	**Part Number**
	Cell Buffer	1.5 mL tube	4	WF101
	Fixation Solution	1.5 mL tube	1	WF102
	Permeabilization Solution	1.5 mL tube	1	WF103
	Neutralization Buffer	5 mL tube	4	WF104
	DMSO	1.5 mL tube	1	WF105
Fixation Accessory Box (Room Temp) WF200				
Label	**Component**	**Format**	**Quantity**	**Part Number**
	40 µm strainers	Plastic Bag	8	WF201

Notes Before Starting

User Materials	Before starting an experiment, check the "Additional Materials and Equipment" section and confirm that your lab has all of the supplies that are not provided by the kit. Avoid substituting custom materials for those that are provided in the kit. Each item has been deliberately chosen to attain optimal results.
Centrifuges	Use a swinging bucket centrifuge for all high speed spin steps in this protocol. Use of a fixed-angle centrifuge will lead to substantial cell loss.
Centrifugation Speed	To maximize cell retention, the centrifugation speeds can be adjusted depending on the sample you are working with. Smaller cells may require higher centrifugation speeds to acquire the same retention rates that larger cells accrue with the recommended speeds set here.
Centrifuge Tubes	Ensure that the tubes that will be used are **polypropylene** and not polystyrene. Polystyrene tubes will lead to substantial cell loss.
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. RNases are not inactivated by ethanol or isopropanol, but can be inactivated by specific products such as RNaseZap that can be sprayed on benchtops and used to clean pipettes. Using filtered pipette tips is also recommended to reduce RNase contamination on pipettes.
Cell Detachment	If cell lines are being used, use TrypLE Express (+) phenol Red instead of the standard trypsin when detaching cells from culture plates. Standard trypsin can contain high amounts of RNases, which may reduce the number of genes and transcripts detected per cell.
Addition of BSA (for use with PBMCs only)	Bovine Serum Albumin (BSA) can be helpful to prevent aggregation when working with PBMCs or other cell types prone to clumping. To minimize potential RNase contamination, we recommend using Bovine Albumin Fraction V (7.5% solution). Refer to Appendix A for ordering information.
Cell Strainers	A 40 µm cell strainer will be used in multiple steps. To maximize cell retention, press the pipette tip directly against the strainer. Ensure that ample pressure is applied to hold contact between the tip and the strainer to force liquid through in ~1 second.
Adding Fixation Solution	When adding the fixation solution to your cells be sure to only mix up and down with a pipette exactly **3x**. Additional mixing will lead to substantially elevated doublet rates.

Cell Fixation Protocol

This protocol begins with a single cell suspension. If you are fixing cells that were frozen, be sure to thaw the cells before proceeding.

*Note: If you are fixing nuclei, proceed to **Nuclei Fixation**.*

1. Prepare for the fixation with the following checklist:
   - ☐ Fill an ice bucket, large enough to hold all of your samples plus additional tubes (each sample will require at least one additional tube during the workflow).
   - ☐ Set your swinging-bucket centrifuge to **4°C.**
   - ☐ Prepare a flow cytometer, hemocytometer, or other device for cell counting.
   - ☐ Prepare all of the samples you plan on fixing in a single cell suspension, and place on ice.
   - ☐ If you are fixing PBMCs, make sure to have BSA available for cell resuspension.
   - ☐ If you plan on storing samples after fixation, take out a "Mr. Frosty" or similar device which enables cooling of a sample at around -1°C/minute. Keep out at room temperature.
2. Take the following items out before proceeding to the next step. It is important that all solutions (except DMSO) are kept on ice after thawing.

Item		Location	Quantity	Format	After taking out:
	40 µm Strainer	Fixation Accessory Box (Room Temp)	2 per number of samples	In plastic bag	Keep at room temperature
	Cell Buffer	Cell Fixation Reagents (-20°C)	1 per number of samples	1.5 mL tube	Thaw, then place on ice.
	Fixation Solution	Cell Fixation Reagents (-20°C)	1	1.5 mL tube	Thaw, then place on ice.
	Permeabilization Solution	Cell Fixation Reagents (-20°C)	1	1.5 mL tube	Thaw, then place on ice.
	Neutralization Buffer	Cell Fixation Reagents (-20°C)	1 per number of samples	5 mL tube	Thaw, then place on ice.
	DMSO	Cell Fixation Reagents (-20°C)	1	1.5 mL tube	Thaw and keep at room temp (Warning: do NOT put on ice!)

CRITICAL! Only proceed if you have taken out items listed in step 2. All items that require thawing should be fully thawed before starting this section.

3. Count the number of cells in your sample. Transfer up to 4 million cells into a 15 mL falcon tube.

   CRITICAL! No more than 4 million cells should be used for any single sample. Exceeding this number may result in substantially elevated doublet rates.

4. Centrifuge tubes in a swinging bucket centrifuge at **4°C f**or **200 x g for 10 minutes**.

Cell Buffer

5. If you are fixing PBMCs, prepare 1 mL of **Cell Buffer** + 0.5% BSA per sample while the cells are spinning. If you are not fixing PBMCs, proceed to step 6.

   CRITICAL! Do NOT add BSA directly to the Cell Buffer tube as you will need Cell Buffer without BSA for later use. Prepare Cell Buffer + BSA in a separate tube and keep the original tube on ice.

6. Remove the supernatant and fully resuspend pellet in **750 µL** of cold **Cell Buffer.**

   Note: If you are fixing PBMCs, the Cell Buffer should contain 0.5% BSA.

   CRITICAL! Failure to fully resuspend cells may result in substantially elevated doublet rates.

7. Pipette cells through a 40 µm strainer into a new 15 mL falcon tube. Place cells back on ice.

   CRITICAL! To ensure that all of the liquid passes through the strainer, press the tip of the pipette against the filter and press the pipette plunger down steadily. All of the liquid should pass through the strainer in ~1 second.

Fix

8. Add **250 µL** of cold **Fixation Solution** and mix immediately by pipetting up and down exactly 3x (with the pipette still set to 250 µL).

   CRITICAL! Do NOT perform additional mixing at this step.

10 min

9. Wait **10 minutes** with cells on ice.

Perm

10. Add **80 µL** of cold **Permeabilization Solution**. Mix by pipetting up and down 3x with a P1000 set to 250 µL. Wait **3 minutes** on ice.

11. *Note: Do NOT vortex the Neutralization Buffer. To mix, invert the tube five times.*

Neut Buffer

    Add **4 mL** of cold **Neutralization Buffer** and gently invert the tube once to mix. Place cells back on ice.

12. Centrifuge tube in swinging bucket centrifuge at **4°C** set to **200 x g for 10 minutes.**

13. Aspirate and discard the supernatant using a P1000 pipette.

Cell Buffer

14. Resuspend cells using a **P1000** pipette and place back on ice. If you started with more than 500,000 cells, resuspend in **300 µL** of **Cell Buffer**. If you started with fewer than 500,000 cells, resuspend in **150 µL** of **Cell Buffer.**

    CRITICAL! The Cell Buffer used at this step should NOT contain BSA regardless of the cell type being fixed.

15. Pass cells through a 40 µm strainer into a 1.7 mL tube with a P1000. Keep samples on ice.

Proceed to the steps below only if you are storing your sample(s). Otherwise, keep your cells on ice and proceed to the start of the Parse Single Cell Whole Transcriptome Kit User Manual.

16. Add **DMSO** stepwise to samples as follows:

a. If you started with more than 500,000 cells, add **5 µL** of **DMSO**. If you started with fewer than 500,000 cells, add **2.5 µL** of **DMSO**. Gently flick the tube **3x** to mix, and wait **1 minute** with samples on ice.

b. Repeat the previous step two more times to add a total of **15 µL** of **DMSO** if you started with more than 500,000 cells, or **7.5 µL** of **DMSO** if you started with fewer than 500,000 cells.

c. Mix the final suspension by gently pipetting up and down 5x with a P200 to ensure that the DMSO is well mixed. To avoid creating bubbles, set the P200 to 150 µL if you started with more than 500,000 cells, or 75 µL if you started with fewer than 500,000 cells.

CRITICAL! Do NOT vortex cells.

17. At this step, cells can be split into aliquots before freezing if the cells will be run in multiple barcoding experiments.

18. Place cells into a Mr. Frosty (or similar device which enables cooling at around -1°C/minute) and place into a **-80°C** freezer.

SECTION 2

NUCLEI FIXATION

PARTS LIST

NOTES BEFORE STARTING

NUCLEI FIXATION PROTOCOL

Nuclei Fixation Parts List

Nuclei Fixation Reagents (-20°C) WN100				
Label	**Component**	**Format**	**Quantity**	**Part Number**
	Nuclei Buffer	1.5 mL tube	4	WN101
	Nuclei Fixation Solution	1.5 mL tube	1	WN102
	Nuclei Permeabilization Solution	1.5 mL tube	1	WN103
	Nuclei Neutralization Buffer	5 mL tube	4	WN104
	Nuclei DMSO	1.5 mL tube	1	WN105
Fixation Accessory Box (Room Temp) WF200				
Label	**Component**	**Format**	**Quantity**	**Part Number**
	40 µm strainers	Plastic Bag	8	WF201

Notes Before Starting

User Materials	Before starting an experiment, check the "Additional Materials and Equipment" section and confirm that your lab has all of the supplies that are not provided by the kit. Avoid substituting custom materials for those that are provided in the kit. Each item has been deliberately chosen to attain optimal results.
Centrifuges	Use a swinging bucket centrifuge for all high speed spin steps in this protocol. Use of a fixed-angle centrifuge will lead to substantial nuclei loss.
Centrifugation Speed	To maximize nuclei retention, the centrifugation speeds can be adjusted depending on the sample you are working with. Smaller nuclei may require higher centrifugation speeds to acquire the same retention rates that larger nuclei accrue with the recommended speeds set here.
Centrifuge Tubes	Ensure that the tubes that will be used are **polypropylene** and not polystyrene. Polystyrene tubes will lead to substantial nuclei loss.
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. RNases are not inactivated by ethanol or isopropanol, but can be inactivated by specific products such as RNaseZap that can be sprayed on benchtops and used to clean pipettes. Using filtered pipette tips is also recommended to reduce RNase contamination on pipettes.
Cell Detachment	If cell lines are being used, use TrypLE Express (+) phenol Red instead of the standard trypsin when detaching cells from culture plates. Standard trypsin can contain high amounts of RNases, which may reduce the number of genes and transcripts detected per cell.
Addition of BSA	Bovine Serum Albumin (BSA) can be helpful to prevent aggregation when working with nuclei. To minimize potential RNase contamination, we recommend using Bovine Albumin Fraction V (7.5% solution). Refer to Appendix A for ordering information.
Cell Strainers	A 40 µm cell strainer will be used in multiple steps. To maximize nuclei retention, press the pipette tip directly against the strainer. Ensure that ample pressure is applied to hold contact between the tip and the strainer to force liquid through in ~1 second.
Adding Fixation Solution	When adding the fixation solution to your nuclei be sure to only mix up and down with a pipette exactly **3x**. Additional mixing will lead to substantially elevated doublet rates.

Nuclei Fixation Protocol

This protocol begins with a single nucleus suspension.

1. Prepare for the fixation with the following checklist:
   - ☐ Fill an ice bucket, large enough to hold all of your samples plus additional tubes (each sample will require at least one additional tube during the workflow).
   - ☐ Set your swinging-bucket centrifuge to **4°C.**
   - ☐ Prepare a flow cytometer, hemocytometer, or other device for nuclei counting.
   - ☐ Prepare all of the samples you plan on fixing in a single nucleus suspension, and place on ice.
   - ☐ Make sure to have BSA available for nuclei resuspension.
   - ☐ If you plan on storing samples after fixation, take out a “Mr. Frosty” or similar device which enables cooling of a sample at around -1°C/minute. Keep out at room temperature.
2. Take the following items out before proceeding to the next step. It is important that all solutions (except nuclei DMSO) are kept on ice after thawing.

Item		Location	Quantity	Format	After taking out:
	40 um Strainer	Fixation Accessory Box (Room Temp)	2 per number of samples	In plastic bag	Keep at room temperature
	Nuclei Buffer	Nuclei Fixation Reagents (-20°C)	1 per number of samples	1.5 mL tube	Thaw, then place on ice.
	Nuclei Fixation Solution	Nuclei Fixation Reagents (-20°C)	1	1.5 mL tube	Thaw, then place on ice.
	Nuclei Permeabilization Solution	Nuclei Fixation Reagents (-20°C)	1	1.5 mL tube	Thaw, then place on ice.
	Nuclei Neutralization Buffer	Nuclei Fixation Reagents (-20°C)	1 per number of samples	5 mL tube	Thaw, then place on ice.
	Nuclei DMSO	Nuclei Fixation Reagents (-20°C)	1	1.5 mL tube	Thaw and keep at room temp (Warning: do NOT put on ice!)

CRITICAL! Only proceed if you have taken out items listed in step 2. All items that require thawing should be fully thawed before starting this section.

3. Count the number of nuclei in your sample. Transfer up to 4 million nuclei into a 15 mL falcon tube.

   CRITICAL! No more than 4 million nuclei should be used for any single sample. Exceeding this number may result in substantially elevated doublet rates.

4. Centrifuge tubes in a swinging bucket centrifuge at **4°C f**or **200 x g for 10 minutes.**

Nuclei Buffer

5. While nuclei are spinning, prepare 1 mL of **Nuclei Buffer** + 0.75% BSA per sample being fixed.

   CRITICAL! Do NOT add BSA directly to the Nuclei Buffer tube as you will need Nuclei Buffer without BSA for later use. Prepare Nuclei Buffer + BSA in a separate tube and keep the original tube on ice.

6. Remove the supernatant and fully resuspend pellet in **750 µL** of cold **Nuclei Buffer.**

   Note: The Nuclei Buffer at this step should contain 0.75% BSA.

   CRITICAL! Failure to fully resuspend nuclei may result in substantially elevated doublet rates.

7. Pipette nuclei through a 40 µm strainer into a new 15 mL falcon tube. Place nuclei back on ice.

   CRITICAL! To ensure that all of the liquid passes through the strainer, press the tip of the pipette against the filter and press the pipette plunger down steadily. All of the liquid should pass through the strainer in ~1 second.

Nuclei Fix

8. Add **250 µL** of cold **Nuclei Fixation Solution** and mix immediately by pipetting up and down exactly 3x (with the pipette still set to 250 µL).

   CRITICAL! Do NOT perform additional mixing at this step.

10 min

9. Wait **10 minutes** with nuclei on ice.

Nuclei Perm

10. Add **80 µL** of cold **Nuclei Permeabilization Solution**. Mix by pipetting up and down 3x with a P1000 set to 250 µL. Wait 3 minutes on ice.

    Note: Do NOT vortex the Nuclei Neutralization Buffer. To mix, invert the tube five times.

Nuclei Neut

11. Add **4 mL** of cold **Nuclei Neutralization Buffer** and gently invert the tube once to mix. Place nuclei back on ice.

12. Centrifuge tube in swinging bucket centrifuge at **4°C** set to **200 x g for 10 minutes.**

13. Aspirate and discard the supernatant using a P1000 pipette.

Nuclei Buffer

14. Resuspend nuclei using a **P1000** pipette and place back on ice. If you started with more than 500,000 nuclei, resuspend in **300 µL** of **Nuclei Buffer**. If you started with fewer than 500,000 nuclei, resuspend in **150 µL** of **Nuclei Buffer.**

    CRITICAL! The Nuclei Buffer used at this step should NOT contain BSA.

15. Pass nuclei through a 40 µm strainer into a **1.7 mL** tube with a P1000. Keep samples on ice.

Proceed to the steps below only if you are storing your sample(s). Otherwise, keep your nuclei on ice and proceed to the start of the Parse Single Cell Whole Transcriptome Kit User Manual.

16. Add **Nuclei DMSO** stepwise to samples as follows:

a. If you started with more than 500,000 nuclei, add **5 µL** of **Nuclei DMSO**. If you started with fewer than 500,000 nuclei, add **2.5 µL** of **Nuclei DMSO**. Gently flick the tube **3x** to mix, and wait **1 minute** with samples on ice.

b. Repeat the previous step two more times to add a total of **15 µL** of **Nuclei DMSO** if you started with more than 500,000 nuclei, or **7.5 µL** of **Nuclei DMSO** if you started with fewer than 500,000 nuclei.

c. Mix the final suspension by gently pipetting up and down 5x with a P200 to ensure that the Nuclei DMSO is well mixed. To avoid creating bubbles, set the P200 to 150 µL if you started with more than 500,000 nuclei, or 75 µL if you started with fewer than 500,000 nuclei.

CRITICAL! Do NOT vortex nuclei.

17. At this step, nuclei can be split into aliquots before freezing if the nuclei will be run in multiple barcoding experiments.

18. Place nuclei into a Mr. Frosty (or similar device which enables cooling at around -1°C/minute) and place into a **-80°C** freezer.

APPENDIX

APPENDIX A: ORDERING ADDITIONAL REAGENTS

Appendix A: Ordering Additional Reagents

Material	Vendor	Catalog Number
15 mL polypropylene Falcon tubes	Corning	352097
1.7 mL Eppendorf tubes	Eppendorf	22431021
Filter pipette tips 20 uL	Rainin	17014961
Filter pipette tips 200 uL	Rainin	17014963
Filter pipette tips 1000 uL	Rainin	17014967
Hemocytometer	Sigma-Aldrich	Z359629-1EA
Isopropyl alcohol (for Mr. Frosty)	Sigma-Aldrich	635639
Bovine Albumin Fraction V (7.5% solution)	Thermo Fisher Scientific	15260037
Mr. Frosty	Thermo Fisher Scientific	5100-0001
RNaseZap	Thermo Fisher Scientific	AM9780
TrypLE™ (+) phenol Red Express Enzyme (1X)	Thermo Fisher Scientific	12605010

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