Cell Thawing Protocols for Single Cell Assays

Introduction

This document outlines four different protocols to thaw cryopreserved cell suspensions for use with 10x Genomics Single Cell assays. One or more protocols may be applicable for a specific sample type and the most appropriate protocol should be used for thawing single cell suspensions for use with 10x Genomics Single Cell assays. This document also provides guidelines in identifying the appropriate protocol(s) by providing a comparison of the protocol features, and indicating the key differences among the four thawing protocols. The protocols described in this document are being used by 10x Genomics. Other thawing methods may also be optimized to be compatible with 10x Genomics Single Cell assays.

Contents

Additional Guidance

Consult the Demonstrated Protocol Cell Preparation Guide (Document CG00053) for Tips & Best Practices on handling cells and for determining accurate target cell counts. Cells carry potentially hazardous pathogens. Follow tissue and material supplier recommendations and local laboratory procedures and regulations for the safe handling, storage, and disposal of biological materials. General guidance on cryopreservation is included in the Appendix.

Cell Sourcing

This protocol was demonstrated using the following cryopreserved samples:

- Human PBMCs, B, and T cells from AllCells, Astarte, and STEMCELL Technologies
- Tumor cells from BioIVT & Discovery Life Sciences
- Mouse primary cells (splenocytes, thymocytes, PBMCs) from BioIVT and C&M LabPro
- Mouse bone marrow cells from StemExpress
- Cell lines from ATCC
- Human bone marrow - Freshly isolated and cryo-preserved in-house

Protocol Comparison

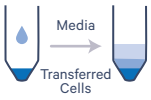
	Thawing Methods			
	PBMCs/Cell lines - Direct Media Thawing	**PBMCs/Cell lines - Dropwise Thawing**	**Tissue Derived Tumor Cells**	**293T/3T3 Cell Line Mixture**
Developed & demonstrated on:				
	• Human/mouse PBMCs bone marrow, B and T cells • Mouse splenocytes, and thymocytes • Human/mouse cell lines (Jurkat, Raji, EL4, A549, H1975, A20, and 293T/3T3s)	Human PBMCs	Human/mouse dissociated tumor cells	Human/mouse - 293T/3T3 cell line mixture
Other recommended sample types				
	Other cell lines and primary cell suspensions lacking fragile cells	Other cell lines and primary cell suspensions lacking fragile cells	Primary cell suspensions with fragile/delicate cells	Robust cell lines
Sample types not recommended				
	Primary cell suspensions with fragile/delicate cells	Primary cell suspensions with fragile/delicate cells	-	Less robust cell lines, any primary cell suspension
Protocol steps: overview and comparison				
The vial is thawed in water bath	✓	✓	✓	✓
Cells are mixed with media to dilute DMSO	✓  Media is added to the cryovials	✓  Cells are transferred to a 50-ml tube; media is added dropwise & in a sequential dilution manner (see page#8)	✓  Cells are transferred to a 50-ml tube; media is added dropwise	 Cells are transferred to a 2-ml tube; media is not added
Cells are centrifuged	✓	✓	✓ Centrifugation conditions for delicate/fragile cells	✓
Cells are washed	✓	✓	✓ A debris removal step is described	✓
Protocol considerations				
	• Versatile and quick	• Slower than Direct Media Thawing protocol • Lengthy protocol	• Includes general washing and better preserves fragile cells • Lengthy protocol	• Few steps • May result in lower viability if used with less robust cell lines

Table 1. Comparison of key features of the four thawing protocols. Every protocol may require additional optimization depending upon the sample type. The four protocols are similar. Two methods are provided for thawing PBMCs/cell lines.

Tips & Best Practices

Tips & Best Practices section includes additional guidance

Signifies critical step requiring accurate execution

Troubleshooting section includes additional guidance

Best Practices

- Best practices for handling any cells or tissues include using sterile techniques, nuclease-free reagents, and consumables.

Thawing Protocols

- The protocols described here are compatible with cryopreserved cell suspensions only.
- More than one thawing protocol may be applicable to a particular sample type. It is recommended to start with the protocol(s) aligned with both the sample type as well and the specific workflow requirements.
- Additional optimization may be required when working with new cell types.
- DMSO is toxic to cells. Perform thawing as quickly as possible.

Pipetting

- Pipette gently and slowly during cell resuspensions to minimize physical damage to cells from shearing forces. Using roughly treated cells as input will compromise system performance.
- Wide-bore pipette tips are recommended for most pipetting steps.

Straining

- Remove large clumps and debris by filtering cell suspensions with an appropriate cell strainer.
- The strainer pore size should be larger than the maximum cell diameter in the sample, but small enough to catch larger clumps.
- Depending on the degree of clumping and the strainer type, the number of cells and the amount of wash solution retained in the strainer can vary.
- Use the MACS SmartStrainer as it generally causes minimal changes to the cell concentration. However, a volume loss of 100 µl or more can occur.
- Use the Flowmi Cell Strainer for low cell suspension volumes to minimize volume losses. However, the cell concentration can decrease by 30% or more. Measure the cell concentration after straining for an accurate cell count.

Centrifugation Conditions

- Centrifugation conditions depend upon the specific sample type.
- Use of a swinging-bucket rotor is recommended for higher cell recovery.

Wash & Resuspension Buffers

- Wash & resuspension buffer depends upon the cell types.
- These protocols were tested with DMEM, RPMI and/or IMDM cell culture media. Other cell culture media may be compatible. Select an appropriate media for the sample type
- See the applicable thawing protocol for an appropriate wash & ressupension buffer.

1. PBMCs/Cell Lines – Direct Media Thawing

1.0 Overview

This chapter provides guidance on general thawing for robust cells. This protocol is recommended for peripheral blood mononuclear cells (PBMCs)/cell lines for use with 10x Genomics Single Cell assays. While this protocol is specific for PBMCs/cell lines, it was also demonstrated on other primary cells (mouse and human bone marrow, mouse thymocytes, mouse splenocytes, and mouse PBMCs, Table 1) in preparation for use in 10x Genomics Single Cell assays.

1.1 Preparation - Buffers

Thawing & Resuspension	
Media/Buffers	**Composition**
Complete Growth Medium *maintain at 37°C*	10% FBS in cell culture media (e.g., RPMI/DMEM)
PBS + 0.04% BSA *maintain at 4°C*	
*PBS + 1% BSA *maintain at 4°C*	
*PBS + 2-10% FBS *maintain at 4°C*	

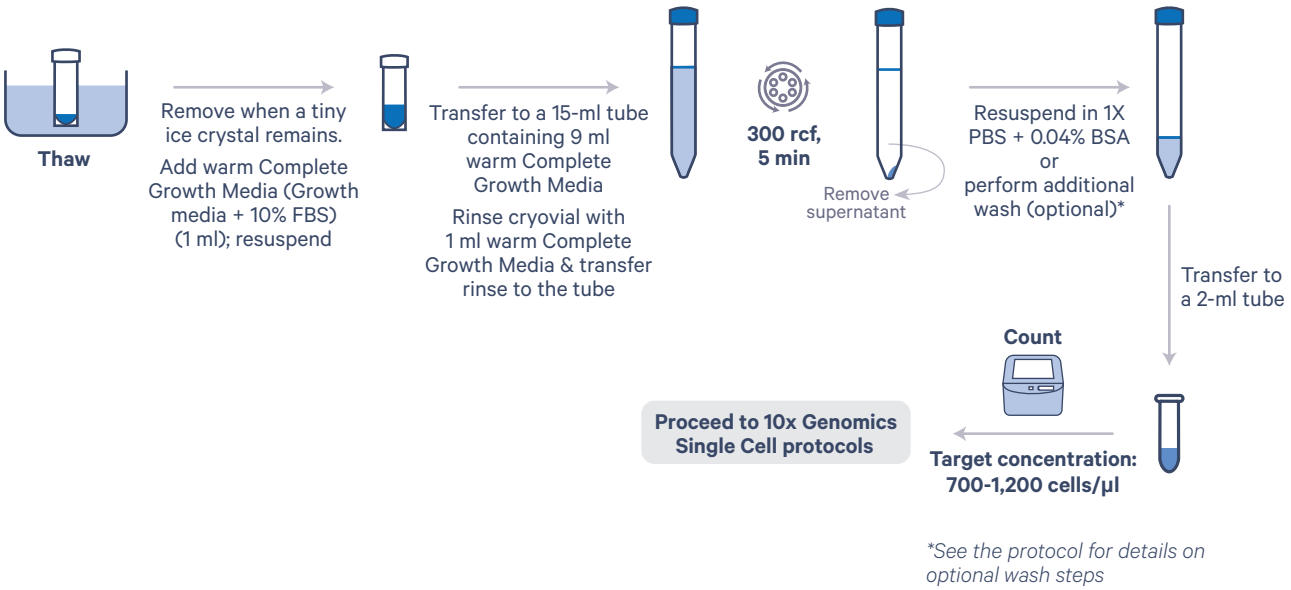
** Alternative buffers*

1.2 Specific Reagents & Consumables

Vendor	Item	Part Number
Thermo Fisher Scientific	Gibco DMEM	11965-092
	UltraPure Bovine Serum Albumin BSA, 50 mg/ml	AM2616
	Trypan Blue Stain (0.4%)	T10282
	Countess II FL Automated Cell Counter	AMAQAF1000
	Countess Cell Counting Chamber Slides	C10228
	Countess 3 FL Automated Cell Counter	AMQAF2000
Corning	Corning RPMI 1640	10-040-CM
	Phosphate-Buffered Saline without Calcium & Magnesium	21-040-CV
VWR	Seradigm Premium Grade Fetal Bovine Serum (FBS)	97068-085
	Sterile Polypropylene Centrifuge Tubes with Flat Caps, 50 ml	82018-050
Eppendorf	DNA LoBind Tubes, 2.0 ml	022431048

1.3 Illustrative Overview

Thawing



See the protocol for details on optional wash steps

1.4 Protocol Steps

Preparation

- Set up a water bath to **37°C** before starting cell thawing
- Prepare ~25 ml warm Complete Growth Media (10% FBS in cell culture media)
- Add 9 ml of the warm Complete Growth Media (10% FBS in cell culture media) to a 15-ml centrifuge tube.

Thawing

a. Remove cryovials from storage and immediately thaw in the water bath at **37°C** until a tiny ice crystal remains.

DO NOT submerge the entire vial in the water bath. Remove from the water bath when a tiny ice crystal remains.

b. Using a wide-bore pipette tip, add **1 ml** warm Complete Growth Media to the cells in cryovial. Slowly resuspend with warm media to melt the remaining ice.

c. Transfer the entire volume to the 15-ml centrifuge tube containing **9 ml** warm Complete Growth Media.

d. Rinse the cryovial with **1 ml** warm Complete Growth Media (used above) and transfer the rinse to the 15-ml tube.

e. Centrifuge at **300 rcf** for **5 min**.

f. Remove supernatant without disrupting the pellet.

Cell pellet may be present on the side or on the bottom of the tube.

g. Perform optional additional wash steps or directly proceed to step i.

h. Optional additional wash steps recommended to remove leftover DMSO:

- Using a regular-bore pipette tip, add **1 ml** Complete Growth Media to each tube and gently pipette mix 5x to resuspend cell pellet. Alternatively, PBS + 2-10% FBS or PBS + 0.04%-1% BSA can also be used for washing.
- Transfer to a 2-ml tube.

  To achieve higher cell recovery in low cell input samples, rinse the 15-ml tube with 1 ml Complete Growth Media (or PBS + 2-10% FBS or PBS + 0.04% -1% BSA) and transfer the rinse to the 2-ml tube.
- Centrifuge at **300 rcf** for **5 min**.
- Remove supernatant without disrupting the pellet and proceed to step i.

i. Using a regular-bore pipette tip, add appropriate volume 1X PBS + 0.04% BSA to each tube to achieve a final cell concentration 700-1,200 cells/µl.
Alternatively, PBS + 1% BSA or PBS + 2-10% FBS can also be used for primary cells that need higher concentration of FBS/BSA to maintain viability. Gently pipette mix 5x to resuspend cell pellet and maintain on ice.

j. Determine the cell concentration using an automated cell counter or a hemocytometer.

k. Proceed immediately to the 10x Genomics Single Cell protocols.

2. PBMCs/Cell Lines – Dropwise Thawing

2.0 Overview

This protocol outlines an alternate thawing protocol for peripheral blood mononuclear cells (PBMCs) for use with 10x Genomics Single Cell protocols. While this protocol is specific to PBMCs, it may be used as a basis for handling other primary cells (see Table 1) in preparation for use in the 10x Genomics Single Cell assays.

2.1 Preparation - Buffers

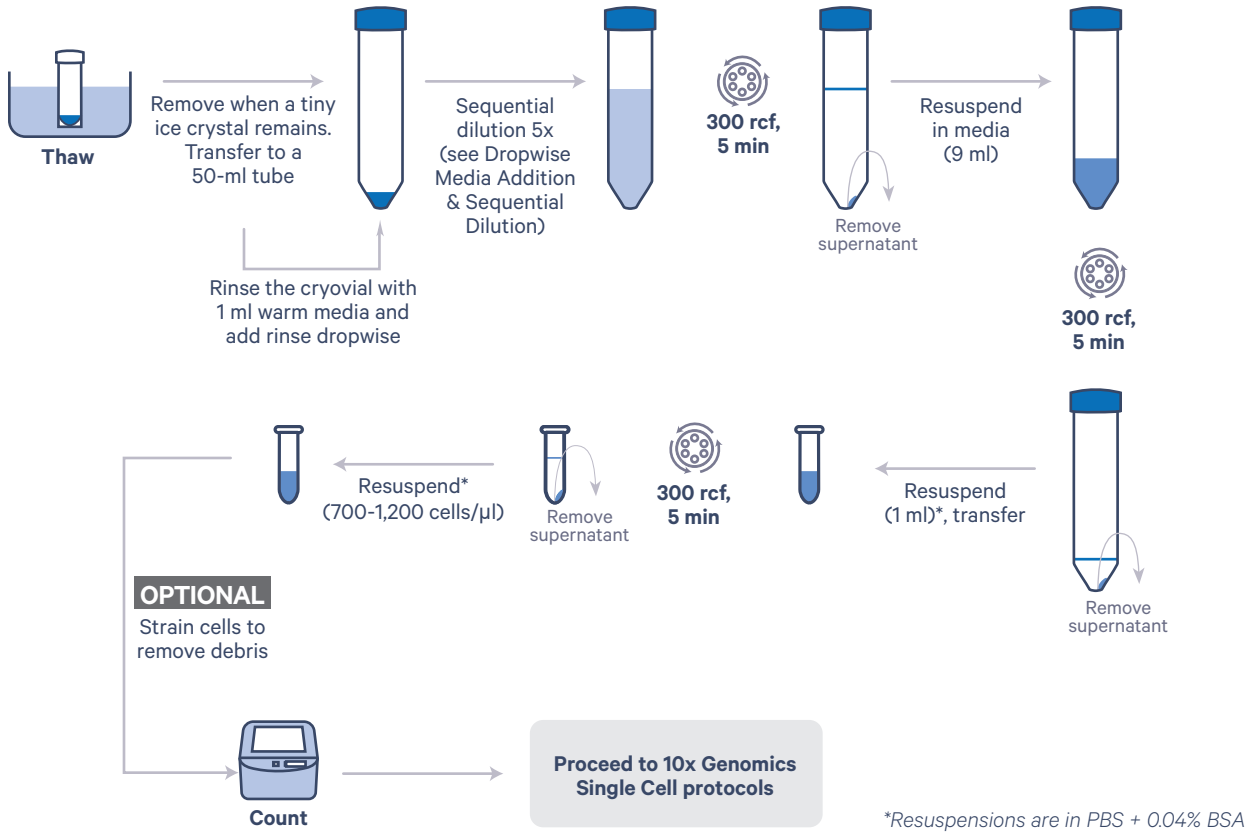
Thawing & Resuspension	
Media/Buffers	**Composition**
Complete Growth Medium *maintain at 37°C*	10% FBS in cell culture media (e.g., IMDM/RPMI)
PBS + 0.04% BSA *maintain at 4°C*	

2.2 Specific Reagents & Consumables

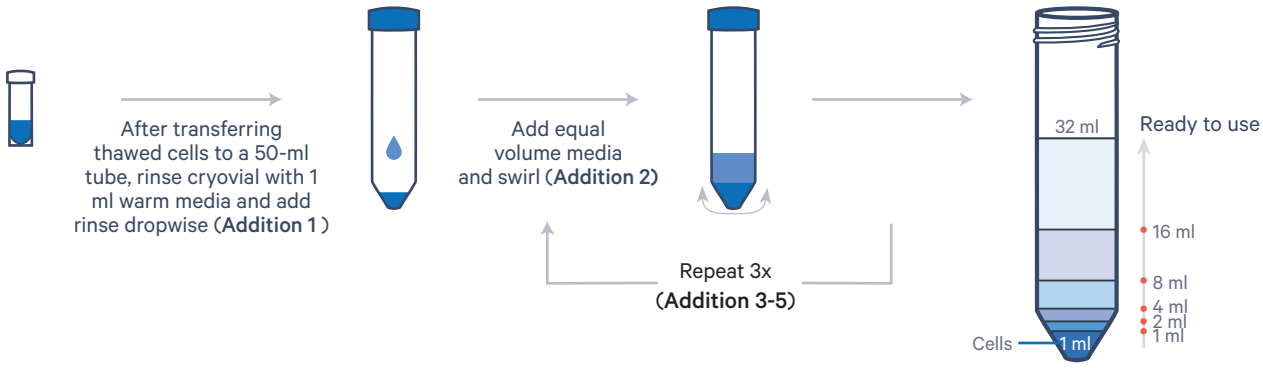
Vendor	Item	Part Number
Thermo Fisher Scientific	Gibco IMDM	12440-053
	UltraPure Bovine Serum Albumin BSA, 50 mg/ml	AM2616
	Trypan Blue Stain (0.4%)	T10282
	Countess II FL Automated Cell Counter	AMAQAF1000
	Countess Cell Counting Chamber Slides	C10228
	Countess 3 FL Automated Cell Counter	AMQAF2000
Miltenyi	MACS SmartStrainers, 30 µm	130-098-458
Bel-Art	Flowmi Cell Strainer, 40 µm alternative to Miltenyi product	H13680-0040
Corning	Corning RPMI 1640	10-040-CM
	Phosphate-Buffered Saline without Calcium & Magnesium	21-040-CV
VWR	Seradigm Premium Grade Fetal Bovine Serum (FBS)	97068-085
	Sterile Polypropylene Centrifuge Tubes with Flat Caps, 50 ml	82018-050
Eppendorf	DNA LoBind Tubes, 2.0 ml	022431048

2.3 Illustrative Overview

Thawing & Resuspension 30–45 min



*Dropwise Media Addition & Sequential Dilution



2.4 Protocol Steps

Preparation

- Set up a water bath to **37°C** before starting cell thawing
- Prepare ~50 ml warm Complete Growth Media (10% FBS in cell culture media)

Thawing

All cell washes are performed at room temperature.

a. Remove cryovials from storage and immediately thaw in the water bath at **37°C** until a tiny ice crystal remains.

DO NOT submerge the entire vial in the water bath. Remove from the water bath when a tiny ice crystal remains.

b. In a biosafety hood, slowly transfer thawed cells to a 50-ml conical tube using a wide-bore pipette tip. Rinse the cryovial with **1 ml** warm Complete Growth Medium and add the rinse dropwise (**1 drop** per **5 sec**) to the 50-ml conical tube while gently swirling the tube.

c. Sequentially dilute cells in the 50-ml conical tube by incremental 1:1 volume additions of media for a total of 5 times (including dilution at step b) with ~1 min wait between additions. Add media at a speed of **1 ml/3-5 sec** to the tube and swirl.

For example, slowly add 2 ml medium into the existing 2 ml cell suspension, gently swirl for 5 sec, and leave the tube at room temperature for ~1 min. This is considered the second serial dilution. Repeat 3 more times adding 4, 8, and then 16 ml to achieve a final volume of ~32 ml.

d. Centrifuge at **300 rcf** for **5 min**.

e. Remove most of the supernatant, leaving **~1 ml** and resuspend cell pellet in this volume using a regular-bore pipette tip.

Cell pellet may be present on the side or on the bottom of the tube.

f. Add an additional **9 ml** Complete Growth Medium (at a speed of **1 ml/ 3–5 sec**) to achieve a total volume of **~10 ml**.

g. Centrifuge at **300 rcf** for **5 min**.

h. Remove the supernatant without disrupting the cell pellet.

i. Using a wide-bore pipette tip, add **1 ml** 1X PBS + 0.04% BSA and gently pipette mix 5x.

j. Transfer the cells into a 2-ml microcentrifuge tube. Rinse the 50-ml conical tube with 0.5 ml 1X PBS + 0.04% BSA and transfer the rinse into the 2-ml tube containing cells.

k. Centrifuge at **300 rcf** for **5 min**.

l. Remove the supernatant without disrupting the cell pellet.

m. Based on starting cell concentration and assuming ~50% cell loss, add an appropriate volume 1X PBS + 0.04% BSA to obtain a concentration of 700-1,200 cells/µl. Gently pipette mix using a regular-bore pipette tip until a single cell suspension is achieved.

OPTIONAL *If cell debris and large clumps are present, pass the sample through a 40 µm Flowmi Cell Strainer.*

n. Determine the cell concentration using an automated cell counter or a hemocytometer. The targeted final concentration is 700-1,200 cells/µl.

o. Once the final cell concentration is achieved, place cells on ice. Proceed immediately to the 10x Genomics Single Cell protocols.

If the cell concentration was adjusted based on the initial count, the final cell suspension should be counted again.

3. Tissue Derived Tumor Cells Thawing

3.0 Overview

This protocol outlines the thawing of extremely fragile cells or primary cells with a high nonviable cell content for use with 10x Genomics Single Cell assays. The protocol was demonstrated using frozen dissociated tumor cells from glioblastoma, renal cell carcinoma, and endometrial cancer patients. While this protocol is specific to dissociated tumor cells, it may be used as a basis for handling other primary/fragile cells (see Table 1) in preparation for use in the 10x Genomics Single Cell assays.

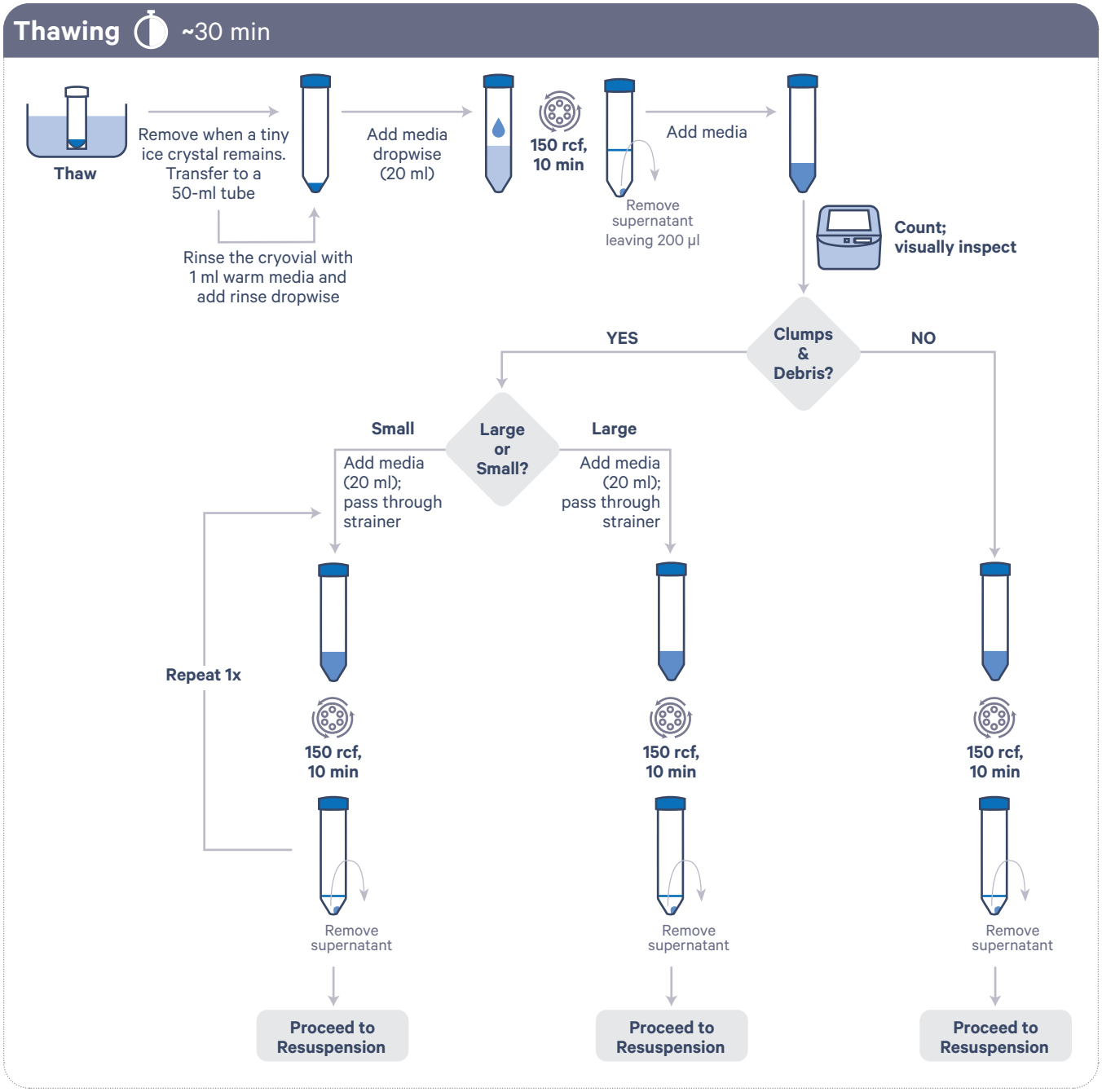
3.1 Preparation - Buffers

Thawing & Resuspension	
Media/Buffers	**Composition**
Complete Growth Medium *maintain at 37°C*	10% FBS in cell culture media (e.g., IMDM/RPMI)

3.2 Specific Reagents & Consumables

Vendor	Item	Part Number
Thermo Fisher Scientific	Gibco IMDM	12440-053
	Trypan Blue Stain (0.4%)	T10282
	Countess II FL Automated Cell Counter	AMAQAF1000
	Countess Cell Counting Chamber Slides	C10228
	Countess 3 FL Automated Cell Counter	AMQAF2000
Miltenyi	MACS SmartStrainers, 30 µm	130-098-458
Bel-Art	Flowmi Cell Strainer, 40 µm alternative to Miltenyi product	H13680-0040
Corning	Corning RPMI 1640	10-040-CM
VWR	Seradigm Premium Grade Fetal Bovine Serum (FBS)	97068-085
	Sterile Polypropylene Centrifuge Tubes with Flat Caps, 50 ml	82018-050
Eppendorf	DNA LoBind Tubes, 2.0 ml	022431048

3.3 Illustrative Overview



3.4 Protocol Steps

Preparation

- Set up a water bath to **37°C** before starting cell thawing
- Prepare ~50 ml warm Complete Growth Media (10% FBS in cell culture media)

Thawing

All cell washes are performed at room temperature.

a. Remove cryovials from storage and immediately thaw in the water bath at **37°C** until a tiny ice crystal remains. Gently swirl the cryovial while in water bath.

DO NOT submerge the entire vial in the water bath. Remove from the water bath when a tiny ice crystal remains.

b. In a biosafety hood, gently transfer thawed cells to a 50-ml centrifuge tube using a wide-bore pipette tip. Rinse the cryovial with **1 ml** warm Complete Growth Medium and add the rinse dropwise (**1 drop** per **5 sec**) to the 50-ml tube while gently swirling the tube.

c. Dilute cells in the 50-ml tube by adding **20 ml** warm Complete Growth Media. Add media dropwise. Slowly increase addition speed as the volume in the tube increases. Gently mix by inverting the tube 2x.

d. Centrifuge at **150 rcf** for **10 min**. A swinging bucket rotor can be used to increase cell recovery.

Pellet
may not be visible

e. Remove most of the supernatant, leaving **~200 µl** and resuspend cell pellet in this volume using a regular-bore pipette tip.

Cell pellet may be present on the side or on the bottom of the tube.

f. Based on starting cell concentration and viability, add an appropriate volume Complete Growth Medium to obtain a concentration of ~2,500 cells/µl.
For example, 5 x 10^6 cells with a 70% viability can be resuspended in ~2.0 ml of medium.

g. Determine the cell concentration using an automated cell counter or a hemocytometer. The viability should be ~70% or more.

h. Visually inspect cells for the presence of cellular clumps or debris.

If no visible cell clumps or debris are present	If large cell clumps or debris are present:	If small cell debris is present:
i. Centrifuge at 150 rcf for 10 min. ii. Remove the supernatant without disrupting the cell pellet. iii. Proceed to Resuspension.	i. Add 20 ml warm media to the cells. ii. Pass the sample through a 30 µm MACS SmartStrainer. iii. Centrifuge at 150 rcf for 10 min. iv. Remove the supernatant without disrupting the cell pellet. v. Proceed to Resuspension.	i. Add 20 ml warm media to the cells. ii. Pass the sample through a 30 µm MACS SmartStrainer. iii. Centrifuge at 150 rcf for 10 min. iv. Remove the supernatant without disrupting the cell pellet. **v. Repeat i-iv** to perform a second wash. vi. Proceed to Resuspension.

Resuspension

a. Based on the starting cell concentration and assuming ~50% cell loss, add an appropriate volume Complete Growth Medium to obtain a concentration of 700-1,200 cells/µl.

Gently pipette mix using a regular-bore pipette tip until a single cell suspension is achieved.

b. Determine cell concentration and viability using an automated cell counter or a hemocytometer.

The targeted final concentration is 700-1,200 cells/µl.

c. Once the final cell concentration is achieved, place cells on ice.

If the cell concentration was adjusted based on the initial count, the final cell suspension should be counted again.

d. Proceed immediately to the 10x Genomics Single Cell protocols.

4. 293T/3T3 Cell Lines Mixture Thawing

4.0 Overview

10x Genomics routinely uses a 1:1 mixture of human and mouse cells to validate the technical performance of the 10x Genomics Single Cell Solutions. This protocol outlines thawing of 1:1 mixtures of human and mouse cells in preparation for use in 10x Genomics Single Cell protocols. While this protocol is specific to the 293T/3T3 cell lines mixture, it may be used as a basis for handling other cell types (see Table 1) in preparation for use in the 10x Genomics Single Cell assays.

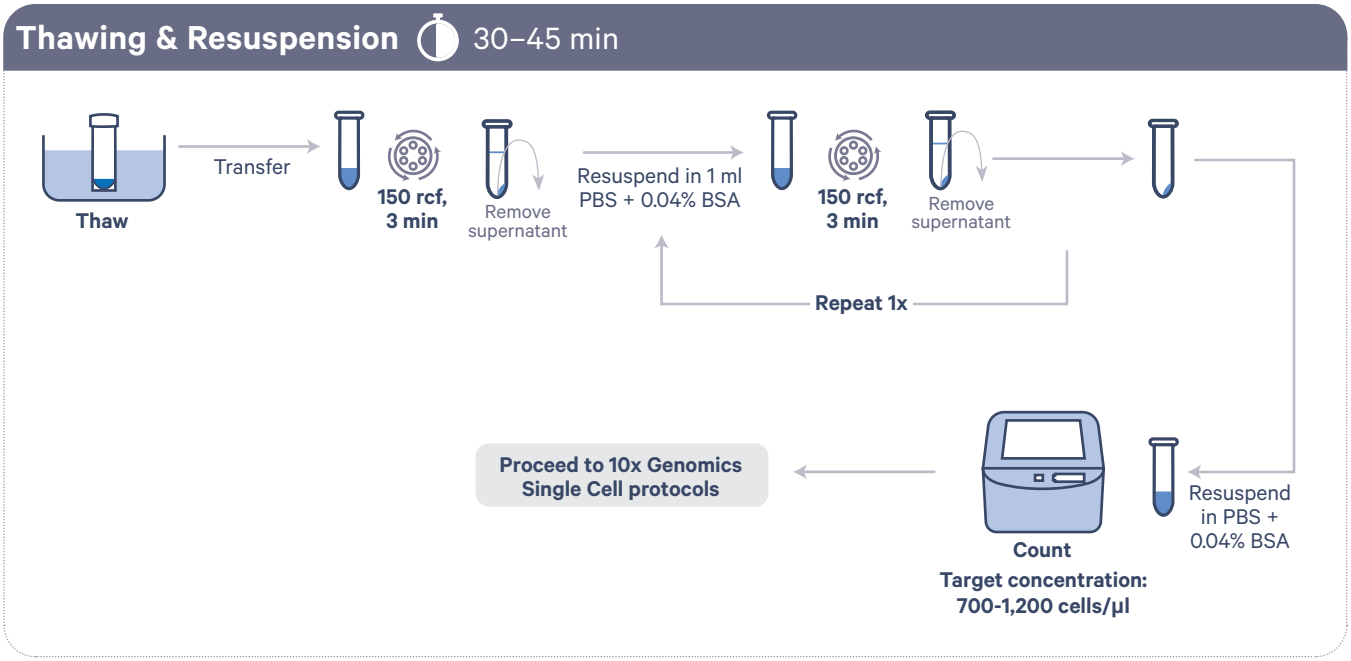
4.1 Preparation - Buffers

Thawing & Resuspension
Media/Buffers
PBS + 0.04% BSA *maintain at 4°C*

4.2 Specific Reagents & Consumables

Vendor	Item	Part Number
Thermo Fisher Scientific	Trypan Blue Stain (0.4%)	T10282
	UltraPure Bovine Serum Albumin BSA, 50 mg/ml	AM2616
	Countess II FL Automated Cell Counter	AMAQAF1000
	Countess Cell Counting Chamber Slides	C10228
	Countess 3 FL Automated Cell Counter	AMQAF2000
Corning	Phosphate-Buffered Saline without Calcium & Magnesium	21-040-CV
Millipore Sigma	Bovine Serum Albumin in DPBS (10%) alternative to Thermo Fisher product	A1595
	Phosphate-Buffered Saline (PBS) with 10% Bovine Albumin alternative to Thermo Fisher product	SRE0036
Eppendorf	DNA LoBind Tubes, 2.0 ml	022431048

4.3 Illustrative Overview



4.4 Protocol Steps

Thawing

Set up a water bath to **37°C** before starting cell thawing. All cell washes are performed at room temperature.

a. Remove cryovials from storage and immediately thaw in the water bath at **37°C** until a tiny ice crystal remains.

DO NOT submerge the entire vial in the water bath. Remove from the water bath when a tiny ice crystal remains.

b. Using a wide-bore pipette tip, pipette mix the cells and transfer the entire volume to a 2-ml tube.

c. Centrifuge at **150 rcf** for **3 min**.

d. Remove supernatant without disrupting the pellet.

Cell pellet may be present on the side or on the bottom of the tube.

Pellet
may not be visible

e. Using a wide-bore pipette tip, add **1 ml** 1X PBS + 0.04% BSA to each tube and gently pipette mix 5x to resuspend cell pellet. Pool the tubes if necessary.

f. Centrifuge at **150 rcf** for **3 min**. Remove the supernatant.

g. **Repeat e-f** for a total of two washes.

h. Based on starting cell concentration and assuming ~50% cell loss, add an appropriate volume 1X PBS + 0.04% BSA to obtain a cell concentration of 700-1,200 cells/µl. Gently pipette mix using a regular-bore pipette tip until a single cell suspension is achieved.

DO NOT invert the tube, as cells can stick to the sides of the tube, thereby changing the cell concentration.

i. Determine the cell concentration using an automated cell counter or a hemocytometer. The targeted final cell concentration is 700-1,200 cells/µl.

j. Once the final cell concentration is achieved, place cells on ice.
If the cell concentration was adjusted based on the initial count, the final cell suspension should be counted again.

k. Proceed **immediately** to the 10x Genomics Single Cell protocols.

Appendix

Cryopreservation Protocol

Prepare:

Pre-cool a cell freezing container by placing it on ice before starting cryopreservation.
It is recommended to start with >90% cell viability. If viability is <80%, consider dead cell removal.

Specific Reagents & Consumables

Vendor	Item	Part Number
Thermo Fisher Scientific	Nunc Biobanking & Cell Culture Cryogenic Tubes, 1.8 ml	368632
	Countess II FL Automated Cell Counter	AMAQAF1000
	Countess Cell Counting Chamber Slides	C10228
	Countess 3 FL Automated Cell Counter	AMQAF2000
	Dimethyl Sulfoxide (DMSO), for molecular biology	ICN19141880
	Mr. Frosty Freezing Container	5100-0001
Corning	Phosphate-Buffered Saline without Calcium & Magnesium	21-040-CV
Millipore Sigma	Biocision CoolCell FTS30 Cell Freezing Container	BSC-170
Eppendorf	Cryostor CS10 (Alternatively, use 90% FBS + 10% DMSO)	7930

a. Determine cell concentration and viability using a hemocytometer or an automated cell counter.

b. Centrifuge the cells at **300 rcf** for **5 min**.

c. Using a pipette, remove the supernatant without disturbing the pellet.

d. Resuspend cells in a cryopreservation medium (or 90% FBS + 10% DMSO) to a minimum concentration of $1x10^6$ cells/ml.

e. Maintain cells on ice

f. Gently pipette mix the cells

g. Aliquot 0.5-1 ml each into cryogenic storage vials.

h. Cells are frozen slowly at 1°C/min. This can be achieved using a programmable cooler (CryoMed Controlled Rate Freezer) or by placing vials in an insulated box (CoolCell or Mr. Frosty) placed in a –80°C freezer.

i. Once vials have reached –80°C throughout (based on the freezer program, or after ~24 hr at -80°C), transfer to liquid nitrogen storage.

Document Revision Summary

Document Number	CG000447
Title	Cell Thawing Protocols for Single Cell Assays
Revision	Rev B
Revision Date	February 2024
General Changes	Updated for general minor consistency of language and terms throughout.
Specific Changes	Added Cryopreservation Protocol under Appendix section Added Countess 3 FL Automated Cell Counter in Reagents Table

Contact:
support@10xgenomics.com
10x Genomics
6230 Stoneridge Mall Road
Pleasanton, CA 94588 USA