User Guide | CG001672 | Rev A

Atera
Gene Expression User Guide

For use with:

Atera WTA Human FFPE Assay Bundle (4 rxns) PN-1001124

Atera WTA Human FFPE Assay Bundle (2 rxns) PN-1001155

Atera Select Human Multi-Tissue FFPE Assay Bundle (4 rxns) PN-1001126

Atera Select Human Multi-Tissue FFPE Assay Bundle (2 rxns) PN-1001157

Atera WTA Human FF Assay Bundle (4 rxns) PN-1001125

Atera WTA Human FF Assay Bundle (2 rxns) PN-1001156

Atera Select Human Multi-Tissue FF Assay Bundle (4 rxns) PN-1001127

Atera Select Human Multi-Tissue FF Assay Bundle (2 rxns) PN-1001158

Atera Assay Prep Reagents, 2 rxns PN-1000975

Atera Cell Segmentation Staining Reagents, 2 rxns PN-1001146

Atera WTA Panels*

Atera Select Panels*

*Consult the 10x Genomics Support website for the most current list of available panels and part numbers.

*View **videos for key techniques**. Scan this code or click here.*

Take 1 minute to evaluate this protocol. Scan this code or click here.

Notices

Document Number

CG001672 | Rev A

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Atera Bundles

Atera Bundles	Part Number	Components	#	Component Part Number	Storage
Atera WTA Human FFPE Assay Bundle (4 rxns)	1001124	Atera FFPE Sample Prep Reagents (2 reactions)	2	1001112	–20°C
		Atera Assay Prep Reagents (2 reactions)	2	1000975	–20°C
		Atera Human WTA Panel (2 reactions)	2	1000976	–20°C
		Atera Cell Segmentation Staining Reagents (2 reactions)	2	1001146	–20°C
		Atera Cassette Kit (2 cassettes)	2	1001153	Ambient
		Atera Plate 1 (1 run, 4 slides)	1	1000973	–20°C
		Atera Plate 2 (1 run, 4 slides)	1	1000974	–20°C
		Atera Pouch 123 Bundle (Universal)	1	1001152	Ambient
		Atera Pouch 4 (Large)	1	1001151	–20°C
Atera WTA Human FF Assay Bundle (4 rxns)	1001125	Atera Fresh Frozen Sample Prep Reagents (2 reactions)	2	1001113	–20°C
		Atera Assay Prep Reagents (2 reactions)	2	1000975	–20°C
		Atera Human WTA Panel (2 reactions)	2	1000976	–20°C
		Atera Cell Segmentation Staining Reagents (2 reactions)	2	1001146	–20°C
		Atera Cassette Kit (2 cassettes)	2	1001153	Ambient
		Atera Plate 1 (1 run, 4 slides)	1	1000973	–20°C
		Atera Plate 2 (1 run, 4 slides)	1	1000974	–20°C
		Atera Pouch 123 Bundle (Universal)	1	1001152	Ambient
		Atera Pouch 4 (Large)	1	1001151	–20°C
Atera WTA Human FFPE Assay Bundle (2 rxns)	1001155	Atera FFPE Sample Prep Reagents (2 reactions)	1	1001112	–20°C
		Atera Assay Prep Reagents (2 reactions)	1	1000975	–20°C
		Atera Human WTA Panel (2 reactions)	1	1000976	–20°C
		Atera Cell Segmentation Staining Reagents (2 reactions)	1	1001146	–20°C
		Atera Cassette Kit (2 cassettes)	1	1001153	Ambient
		Atera Plate 1 (1 run, 2 slides)	1	1000971	–20°C
		Atera Plate 2 (1 run, 2 slides)	1	1000972	–20°C
		Atera Pouch 123 Bundle (Universal)	1	1001152	Ambient
		Atera Pouch 4 (Small)	1	1001150	–20°C
Atera WTA Human FF Assay Bundle (2 rxns)	1001156	Atera Fresh Frozen Sample Prep Reagents (2 reactions)	1	1001113	–20°C
		Atera Assay Prep Reagents (2 reactions)	1	1000975	–20°C
		Atera Human WTA Panel (2 reactions)	1	1000976	–20°C
		Atera Cell Segmentation Staining Reagents (2 reactions)	1	1001146	–20°C
		Atera Cassette Kit (2 cassettes)	1	1001153	Ambient
		Atera Plate 1 (1 run, 2 slides)	1	1000971	–20°C
		Atera Plate 2 (1 run, 2 slides)	1	1000972	–20°C
		Atera Pouch 123 Bundle (Universal)	1	1001152	Ambient
		Atera Pouch 4 (Small)	1	1001150	–20°C
Atera Select Human Multi-Tissue FFPE Assay Bundle (4 rxns)	1001126	Atera FFPE Sample Prep Reagents (2 reactions)	2	1001112	–20°C
		Atera Assay Prep Reagents (2 reactions)	2	1000975	–20°C
		Atera Select Human Multi-Tissue Panel (A) (2 reactions)	2	1000977	–20°C
		Atera Cell Segmentation Staining Reagents (2 reactions)	2	1001146	–20°C
		Atera Cassette Kit (2 cassettes)	2	1001153	Ambient
		Atera Plate 1 (1 run, 4 slides)	1	1000973	–20°C
		Atera Pouch 123 Bundle (Universal)	1	1001152	Ambient
		Atera Pouch 4 (Small)	1	1001150	–20°C

Atera Bundles	Part Number	Components	#	Component Part Number	Storage
Atera Select Human Multi-Tissue FF Assay Bundle (4 rxns)	1001127	Atera Fresh Frozen Sample Prep Reagents (2 reactions)	2	1001113	–20°C
		Atera Assay Prep Reagents (2 reactions)	2	1000975	–20°C
		Atera Select Human Multi-Tissue Panel (A) (2 reactions)	2	1000977	–20°C
		Atera Cell Segmentation Staining Reagents (2 reactions)	2	1001146	–20°C
		Atera Cassette Kit (2 cassettes)	2	1001153	Ambient
		Atera Plate 1 (1 run, 4 slides)	1	1000973	–20°C
		Atera Pouch 123 Bundle (Universal)	1	1001152	Ambient
		Atera Pouch 4 (Small)	1	1001150	–20°C
Atera Select Human Multi-Tissue FFPE Assay Bundle (2 rxns)	1001158	Atera FFPE Sample Prep Reagents (2 reactions)	1	1001112	–20°C
		Atera Assay Prep Reagents (2 reactions)	1	1000975	–20°C
		Atera Select Human Multi-Tissue Panel (A) (2 reactions)	1	1000977	–20°C
		Atera Cell Segmentation Staining Reagents (2 reactions)	1	1001146	–20°C
		Atera Cassette Kit (2 cassettes)	1	1001153	Ambient
		Atera Plate 1 (1 run, 2 slides)	1	1000971	–20°C
		Atera Pouch 123 Bundle (Universal)	1	1001152	Ambient
		Atera Pouch 4 (Small)	1	1001150	–20°C
Atera Select Human Multi-Tissue FF Assay Bundle (2 rxns)	1001157	Atera Fresh Frozen Sample Prep Reagents (2 reactions)	1	1001113	–20°C
		Atera Assay Prep Reagents (2 reactions)	1	1000975	–20°C
		Atera Select Human Multi-Tissue Panel (A) (2 reactions)	1	1000977	–20°C
		Atera Cell Segmentation Staining Reagents (2 reactions)	1	1001146	–20°C
		Atera Cassette Kit (2 cassettes)	1	1001153	Ambient
		Atera Plate 1 (1 run, 2 slides)	1	1000971	–20°C
		Atera Pouch 123 Bundle (Universal)	1	1001152	Ambient
		Atera Pouch 4 (Small)	1	1001150	–20°C

Atera Gene Expression Reagent Kits

The Atera Gene Expression workflow also requires Atera Hyb Buffer and Reducing Agent B (FFPE Kit)or Additive A (FF Kit), which can be found in the sample preparation kits. Consult the Atera FF or FFPE Tissue Preparation Handbooks (CG001670 or CG001671) for more information.

When processing four slides, two 2 rxn kits are required. See 4 rxn bundles on Atera Bundles page for other quantities.

Atera Assay Prep Reagents (2 reactions) PN-1000975

Consult SDS for handling and disposal information.

Atera Assay Prep Reagents (2 reactions)
PN-1000975
Shipped on dry ice; Store at −20°C

Cap Color	Reagent	PN	Cap Color	Reagent	PN
○	Post-Hybridization Wash Buffer	2000395	●	Atera Ligation Buffer	2001880
●	Atera Enzyme A	2001915	●	Atera Enzyme B	2001916
●	Amplification Enhancer Buffer	2001234	●	Atera Amplification Enhancer	2001917
●	Amplification Enhancer Wash Buffer	2001236	●	Atera Amp Mix 1	2001976
●	Atera Amp Mix 2	2001881	●	Atera Signal Booster	2001947

Atera Hyb Buffer, which is needed for this protocol, is thawed and prepared in either the Atera Gene Expression - FFPE Tissue Preparation Handbook (CG001671) or the Atera Gene Expression - FF Tissue Preparation Handbook (CG001670).

Optional Atera Cell Segmentation Staining Reagents (2 reactions) PN-1001146

Atera Cell Segmentation Staining Reagents (2 reactions)
PN-1001146
Shipped on dry ice; Store at −20°C

Cap Color	Reagent	PN	Cap Color	Reagent	PN
	Atera Block and Stain Buffer	2001945		Atera Multi-Tissue Stain	2001946
	Atera Multi-Tissue Stain	2001946		Atera Signal Booster	2001947

Atera Cassette Kit (2 cassettes) PN-1001153

Atera Cassette Kit
PN-1001153
Shipped at ambient temp
Store at ambient temp

	#
Atera Cassette Top	2
Atera Cassette Bottom	2
Atera Cassette Insert	4
Atera Cassette Lid	2

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Atera Plates and Pouches

Atera Plates	#	Kit PN	Component PN	Shipping and Storage
Atera Plate 1 (1 run, 2 slides)	1	1000971	2001614	Shipped in dry ice, store at −20°C
Atera Plate 2 (1 run, 2 slides)	1	1000972	2001615	Shipped in dry ice, store at −20°C
Atera Plate 1 (1 run, 4 slides)	1	1000973	2001616	Shipped in dry ice, store at −20°C
Atera Plate 2 (1 run, 4 slides)	1	1000974	2001617	Shipped in dry ice, store at −20°C

Atera Pouches	#	Kit PN	Component PN	Shipping and Storage
Atera Pouch 1 (Universal)	1	1001147	2001931	Shipped and stored at ambient temperature
Atera Pouch 2 (Universal)	1	1001148	2001932	Shipped and stored at ambient temperature
Atera Pouch 3 (Universal)	1	1001149	2001933	Shipped and stored at ambient temperature
Atera Pouch 4 (Small)	1	1001150	2001935	Shipped in dry ice, store at −20°C
Atera Pouch 4 (Large)	1	1001151	2001930	Shipped in dry ice, store at −20°C

Third-Party Items

Successful execution of this workflow requires third-party reagents, kits, and equipment in addition to those provided by 10x Genomics. All necessary third-party reagents and consumables should be obtained before starting this workflow.

Consult the Atera Gene Expression Protocol Planner (CG001669) for a list of the following third-party items:

- Additional reagents, kits, and equipment
- Tested thermal cyclers
- Tested blank slides

10x Genomics has tested all items listed in the Protocol Planner. Unless otherwise noted, 10x Genomics recommends using these items to ensure optimal assay performance.



Substituting materials may adversely affect assay performance.

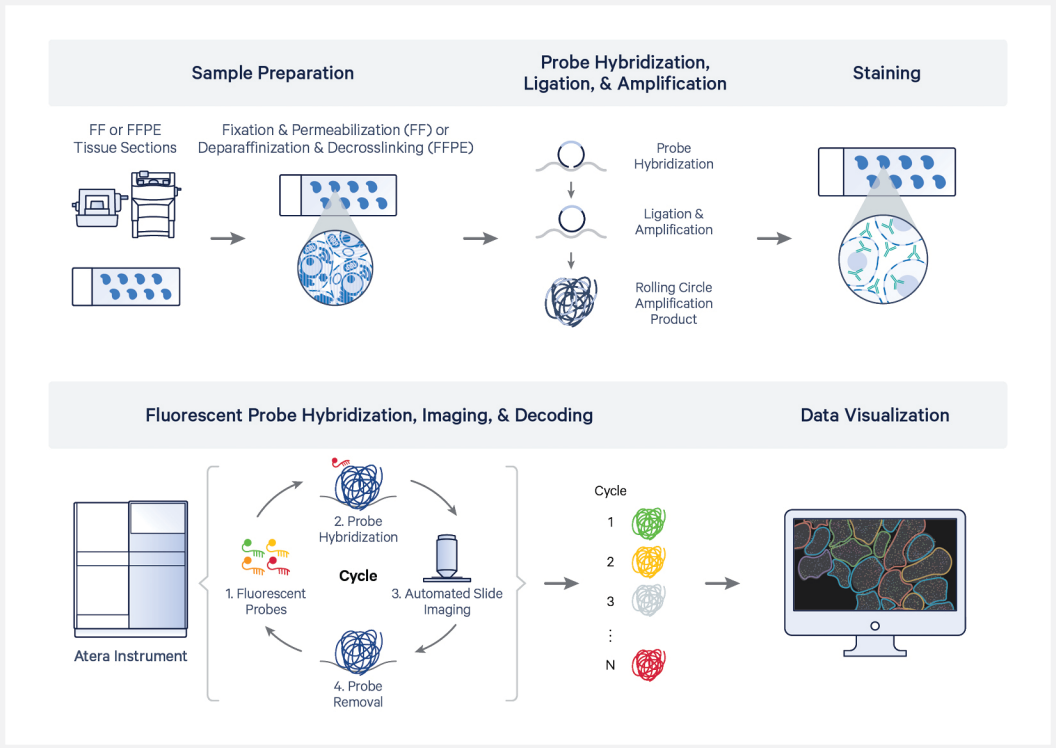
Protocol Steps & Timing

Steps	Timing	Stop & Store
Day 1		
Step 1: Probe Hybridization		
1.1 Custom Probe Preparation (optional) 1.2 Probe Hybridization	15 min 16–24 h (overnight)	
Day 2		
Step 2: Post Hybridization Wash		
2.1 Post Hybridization Wash	30 min	
Step 3: Ligation		
3.1 Ligation	~45 min	
Step 4: Amplification		
4.1 Amplification Enhancement	~135 min	
4.2 Amplification	~130 min	
4.3 Post Amplification Wash	10 min	
4.4 Signal Boost	30 min	
Step 5: Optional - Cell Segmentation Staining		
5.1 Buffer Preparation	10 min	
5.2 Block and Stain	~1 h +16–24 h (overnight)	
5.3 Signal Boost	35 min	
Step 6: Ethanol Drying		
6.1 Ethanol Drying	10 min	STOP 4°C ≤1 week
Step 7: Instrument Loading Preparation		
7.1 Atera Plate Preparation	45 min	
7.2 Atera Pouch Preparation	5 min*	

*3 h is needed if Pouch 4 was not thawed the night before.

Stepwise Objectives

Workflow Overview



Atera Gene Expression assays RNA at the subcellular level by using targeted probes in formalin fixed & paraffin embedded (FFPE) or fresh frozen (FF) tissue sections. FFPE tissue sections placed on slides are deparaffinized and decrosslinked as described in the Atera Gene Expression - FFPE Tissue Preparation Handbook (CG001671). FF tissue sections placed on slides are fixed and pretreated as described in the Atera Gene Expression - Fresh Frozen Tissue Preparation Handbook (CG001670).

Atera probes are added to the tissue to hybridize with the target RNA. Next, probes are ligated and enzymatically amplified, generating multiple copies of the gene-specific barcode for each RNA target.

Slides containing FFPE or FF tissue sections are then loaded for imaging and analysis on the Atera Instrument for high-throughput, automated in situ analysis. Fluorescently labeled oligos bind to the amplified DNA probes.

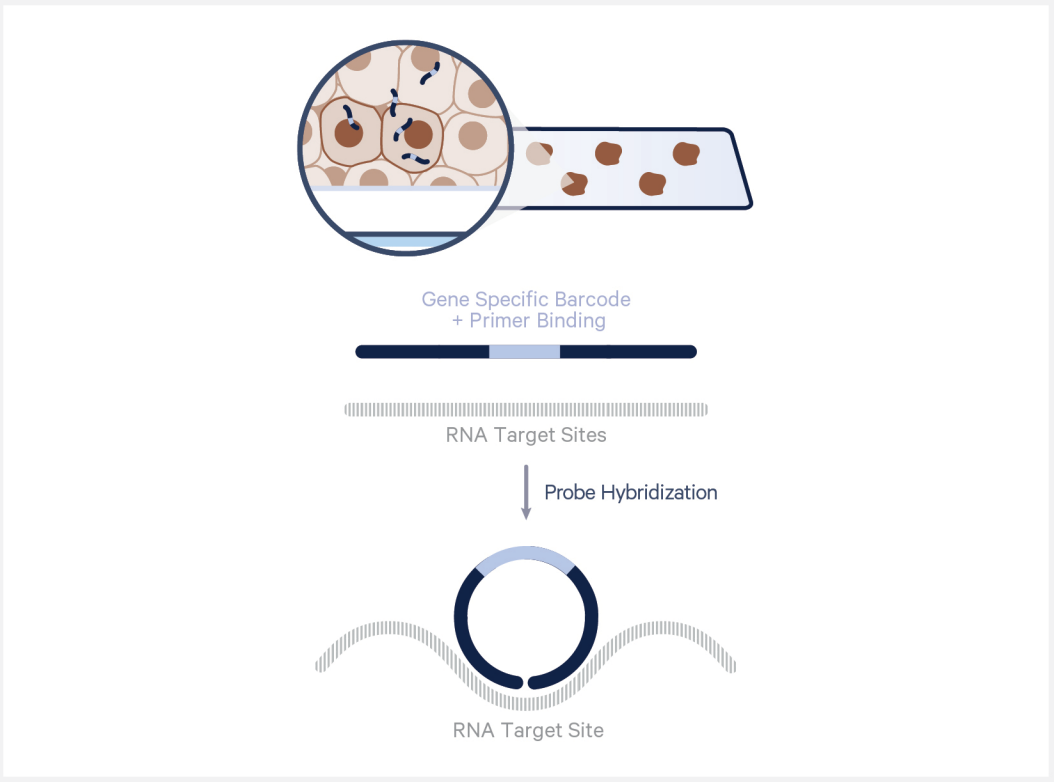
Cyclical rounds of fluorescent probe hybridization, imaging, and removal generate optical signatures specific for each barcode, which are converted into a gene identity. Identified transcripts can be visualized using 10x in situ software. 10x in situ software allows for the simultaneous visualization and analysis of gene expression and cell segmentation data on a per-cell basis.

This document outlines the protocol for generating Atera Gene Expression data from FFPE and FF tissue sections.

The following sections provide a high-level overview of each step in this User Guide.

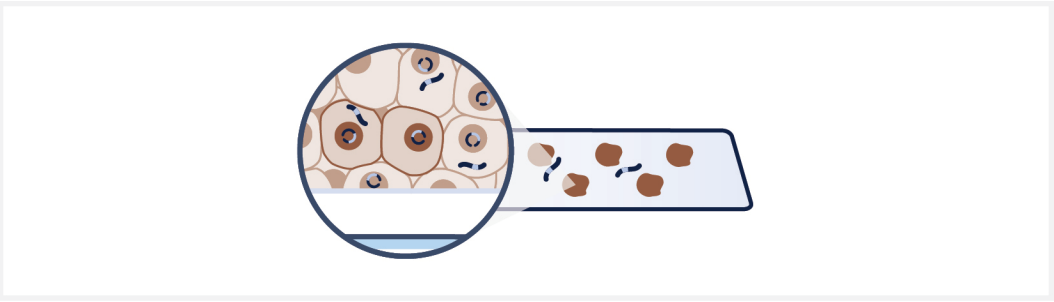
Step 1: Probe Hybridization

Probe panels are added to the FFPE or FF tissue sections, where they bind to target RNA.



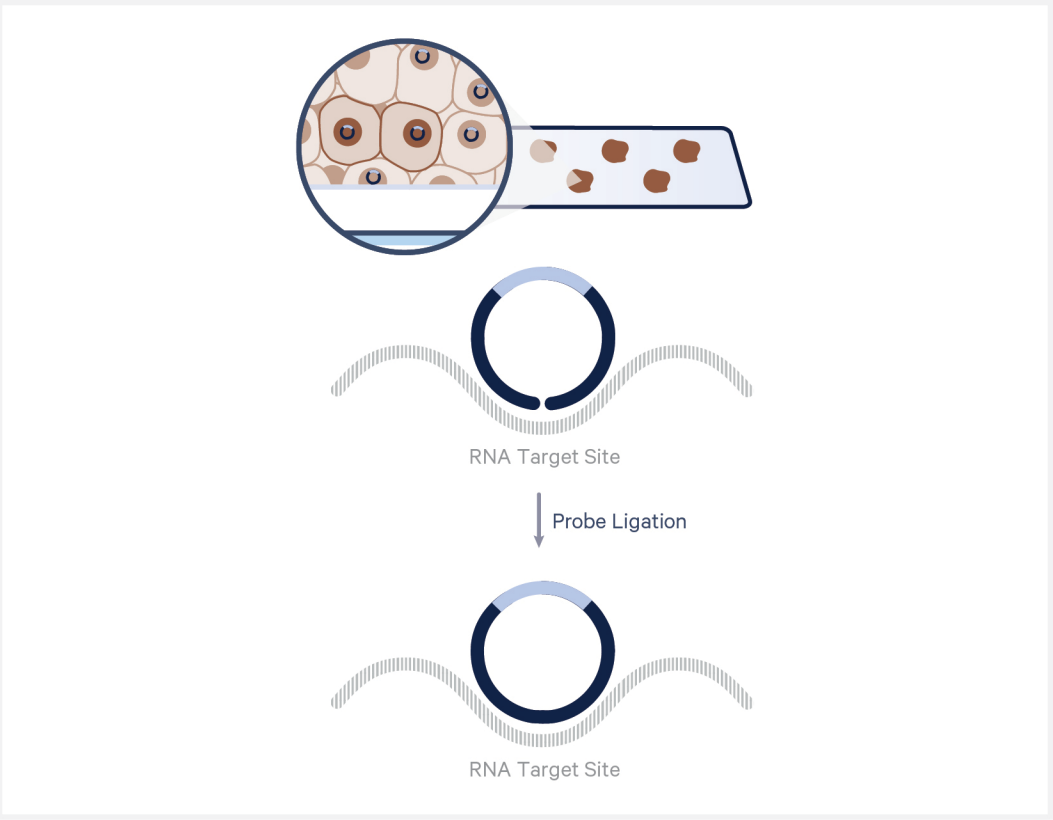
Step 2: Post Hybridization Wash

Excess, unbound probes are washed away in the post hybridization wash step.



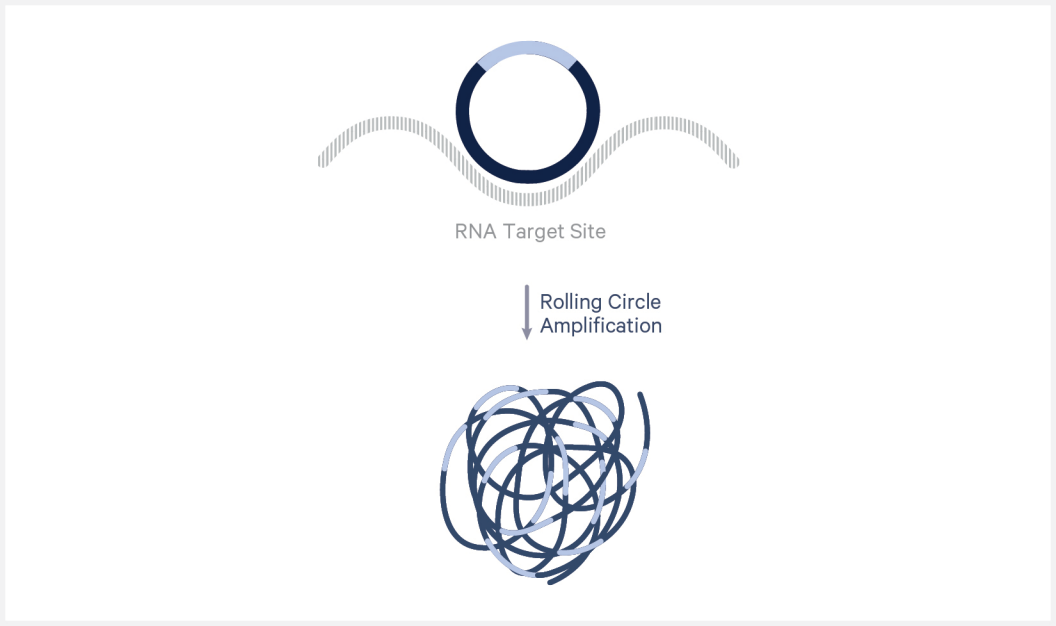
Step 3: Ligation

After removal of unbound probes, a ligase seals the junction between the probes that have hybridized to RNA. Ligation of the probe ends on the targeted RNA region generates a circular DNA probe. This ligation ensures a unique level of probe specificity to the target region.



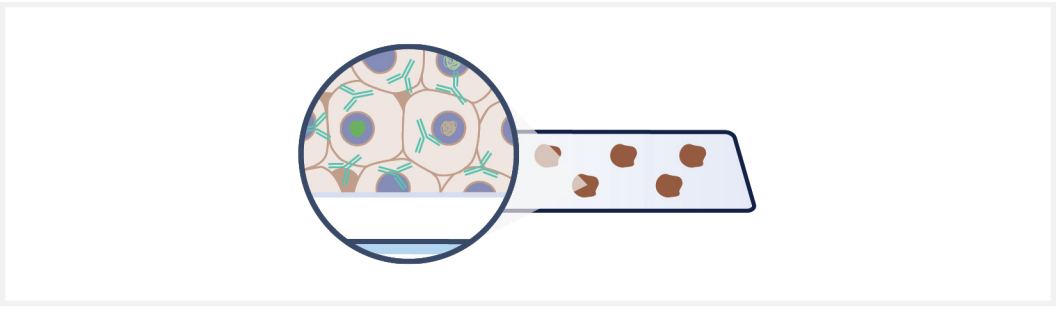
Step 4: Amplification

Ligation products are enzymatically amplified. Hundreds of copies of the gene-specific barcode are generated during the amplification process.



Step 5: Optional - Cell Segmentation Staining

Following a series of ethanol washes, blocking reagent is added to the tissue sections to reduce non-specific binding during staining. Cell segmentation antibodies bind their antigens in an overnight incubation, while excess antibodies are washed away in a post-incubation wash. Stain is then enhanced by the addition of Atera Signal Booster reagent. If cell segmentation staining is skipped, the Atera Instrument performs nuclei and interior 18S rRNA staining.



Step 6: Ethanol Drying

Before instrument loading, tissues are dried with ethanol to promote adhesion onto the slide.

Step 7: Instrument Loading Preparation

Atera Plates and Atera Pouches are prepared before loading onto the Atera Instrument. Consult the Atera Instrument User Guide (CG001673) for more information on instrument operation and loading.

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Tips & Best Practices

Icons



Tips & Best Practices section includes additional guidance



Signifies critical step requiring accurate execution



Troubleshooting section includes additional guidance



Indicates a video is available for the specified technique

Videos

- Videos describing key steps in the protocol can be found here.
- When a relevant video is available for a protocol step, it will be marked with a video icon.

General Reagent Handling

- Fully thaw reagents at indicated temperatures. Thoroughly mix reagents before use.
- When pipette mixing reagents, unless otherwise specified, set pipette to 75% of total volume.
- Keep all enzymes and Master Mixes on ice during setup and use, unless otherwise stated.
- Promptly move reagents back to the recommended storage.

Pipette Calibration

Follow manufacturer's calibration and maintenance schedules.

RNase Decontamination

Before starting, wipe down all surfaces and work areas with RNase Zap RNase decontaminating solution. Keep all surfaces and materials RNase-free.

Probe Panel Handling

- 10x Genomics provides both WTA and Select panels for the Atera assay. Consult the 10x Genomics website for the most current list of available panels.
- Only Atera probe panels are compatible with the Atera workflow.

- Pre-designed Atera probe kits are good for two slides.
- Custom probes are delivered lyophilized at room temperature and should be stored at −20°C upon receipt.
- Custom probes come in 4 rxn tubes, as either a kit of 1 (4 rxn total) or 10 (40 rxn total).
- Custom probes expire two years from the manufacture date in lyophilized form when stored at −20°C. Alternatively, custom probes expire one year from time of resuspension if stored in TE Buffer, pH 8.0 at −20°C or at kit expiration date (whichever comes first).
- Custom probes must be resuspended before use in TE Buffer, pH 8.0. After resuspension, they are stable for short-term at room temperature (<8 h). Resuspended custom probes should be discarded if left at room temperature for more than 8 hours.
- Custom probes (in lyophilized form) accidentally left at room temperature for an extended period of time are likely stable. Contact support@10xgenomics.com if further assistance is needed.

General Slide Handling

- Always wear gloves when handling slides.
- Place tissue sections within the Sample Area on the front of the slide.
- Hold the slide on the label. DO NOT touch the tissue sections.
- Minimize exposure of the slides to sources of particles and fibers.
- Ensure that no absorbent surface is in contact with the reagents on the slide during incubation.
- When pipetting reagent onto a slide, avoid generating bubbles. Avoid pipetting directly onto the tissue unless otherwise specified.
- If slides crack at any point in the workflow, do not proceed.

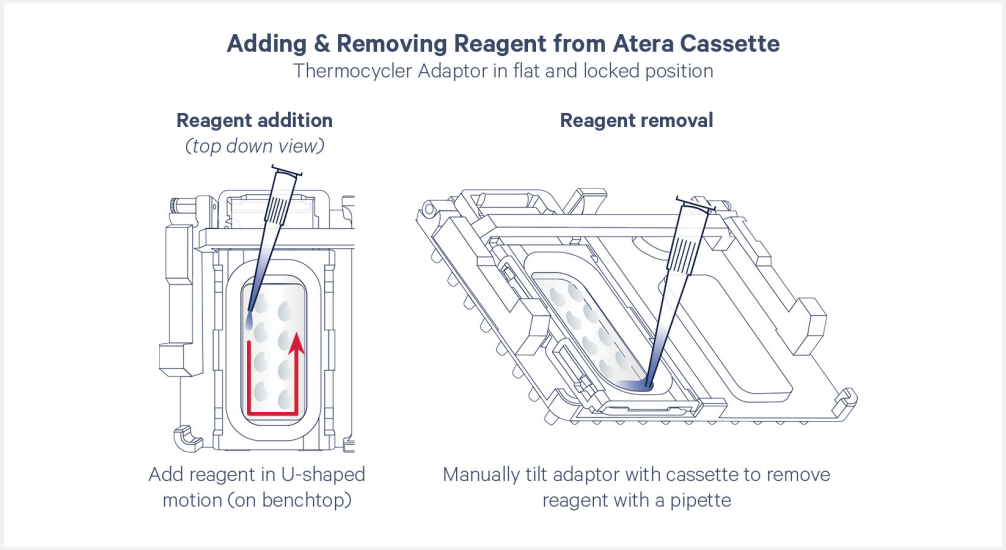
Atera Cassette & Thermocycler Adaptor Handling

- Consult the Atera Cassette Assembly, Disassembly, and Use Quick Reference Card (CG001674) for information on:
  - Cassette assembly and disassembly
  - Atera Cassette Lid application and removal
  - Atera Cassette Insert application and removal
  - Thermocycler Adaptor handling

Adding & Removing Reagents

Adding Reagent to Well

- Ensure Atera Cassette is in Thermocycler Adaptor, the Thermocycler Adaptor is in the flat and locked position.
- Dispense reagents in a U-shaped motion along the side of the well unless otherwise specified.
- Gently tap cassette to disperse reagent evenly.
- Pipette slowly to avoid introducing bubbles.
- DO NOT let sample dry out during any wash steps. Add buffer back to the cassette as quickly as possible once buffer is removed.
- DO NOT touch tissue sections.



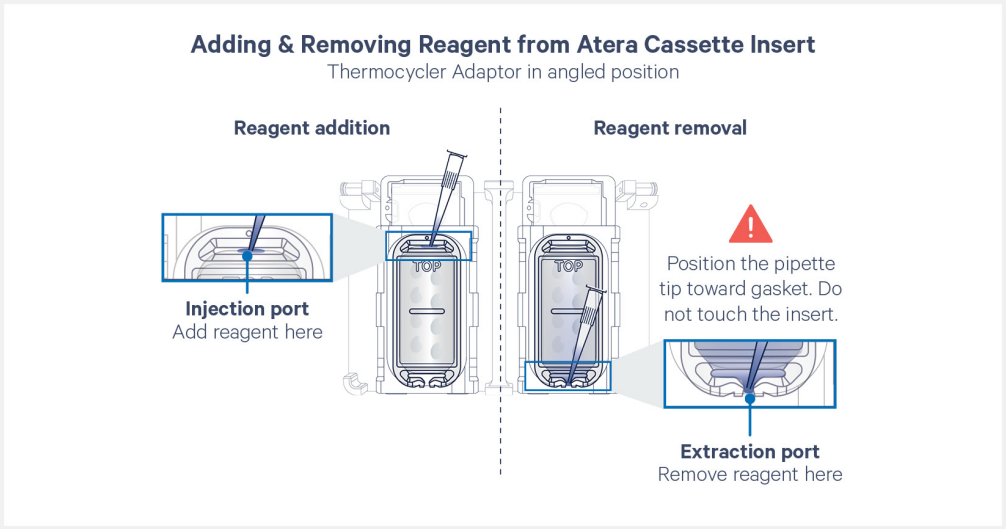
- If moving the adaptor with cassettes to the thermal cycler, ensure it stays level to avoid unnecessary reagent movement.

Removing Reagent from Well

- Slightly tilt the Thermocycler Adaptor with cassettes while removing reagent.
- Place pipette tip on bottom corner of the well while removing reagent.

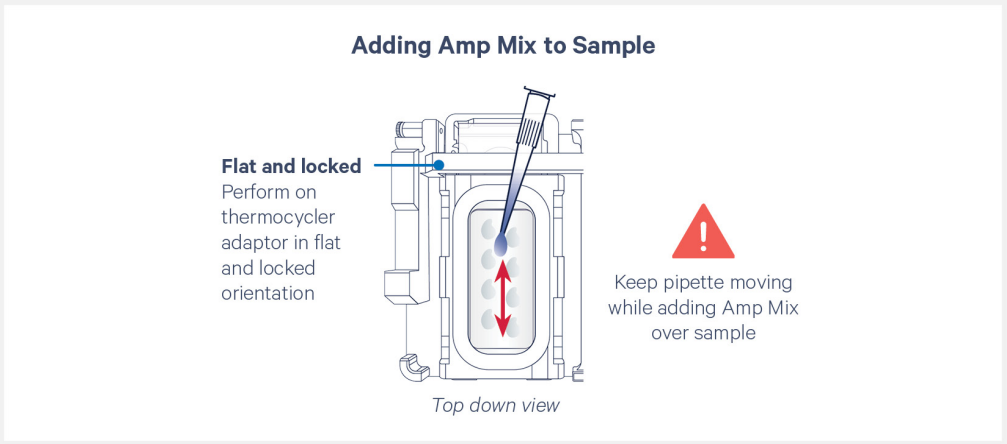
Adding & Removing Reagent to & from Insert

- Add reagent to the injection port when indicated in the protocol.
- Remove reagent from the extraction port when indicated in the protocol.
- Pipette slowly and avoid dispensing past the first stop of the pipette to avoid bubble formation.
- When the insert needs to be removed, additional reagent will be added into the injection port.
- During some steps, adding a small amount of liquid on top of the insert is necessary to avoid drying out the sample. During these steps, DO NOT add the reagent into the injection port. Each protocol step will specify the correct area of the insert to add reagent.



Adding Amp Mix

- Add reagent quickly and evenly over the sample.
- Keep pipette moving while adding reagent to evenly spread the Amp Mix



Processing an Odd Number of Slides

- Atera reagent kits are sufficient for two reactions. If running four slides, two reagent kits are required.
- It is possible to perform the Atera workflow with an odd number (1 or 3) of slides. If working with an odd number of slides, a mock slide is not necessary for thermal cycler incubations.

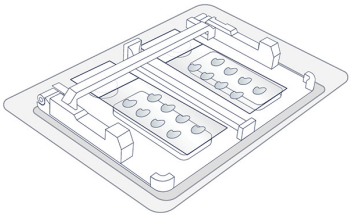
Slide Incubation Guidance

Incubation at a Specified Temperature

- Slide Incubation:
  - Ensure thermal cycler has reached appropriate temperature before starting incubation.
  - Ensure thermal cycler has been calibrated according to manufacturer specifications.
  - After thermal cycler has reached appropriate temperature, place the Atera Thermocycler Adaptor onto the thermal cycler. Ensure that the Atera Thermocycler Adaptor is fully inserted into the thermal cycler and is in contact with the thermal cycler block surface uniformly.

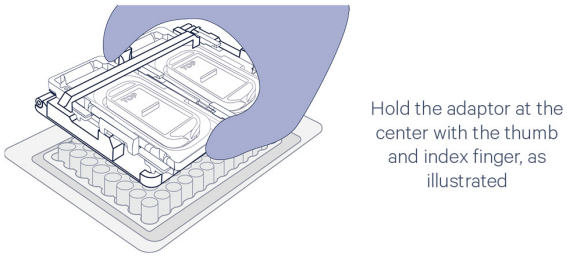
- Position slides on the Atera Thermocycler Adaptor with the tissue side facing up.
- Ensure the Sample Area is aligned with the corresponding area on the Atera Thermocycler Adaptor. DO NOT close the lid.

Slides on Thermocycler Adaptor



- Cassette Incubation:
  - Ensure thermal cyclers have reached appropriate temperature before starting incubation.
  - Ensure thermal cycler has been calibrated according to manufacturer specifications.
  - Place Thermocycler Adaptors flat on the bench.
  - Place the assembled cassettes on the Atera Thermocycler Adaptors with the well facing up. Ensure cassettes are in complete contact with the Atera Thermocycler Adaptors. Cassettes should always be sealed with an Atera Cassette Lid when on the Thermocycler Adaptor unless indicated otherwise.
  - Lock the Thermocycler Adaptor arm over the cassettes.
  - Place Thermocycler Adaptors with cassettes onto the thermal cyclers.

Atera Cassettes on Thermocycler Adaptor



- Before each incubation step, always inspect the adaptors and wipe using a lint-free laboratory wipe. If debris or liquid is noticed, wipe the adaptors using 70% isopropanol, followed by wiping with a lint-free laboratory wipe. Failing to do so could result in slide cracking.

Tightening the Thermal Cycler Lid

For thermal cyclers with adjustable lids, tighten the lid until resistance is felt and before the click is heard.

Incubation at Room Temperature

- Place the assembled cassettes on the Thermocycler Adaptors on a flat, clean, non-absorbent work surface.
- Ensure that no absorbent surface is in contact with the reagents on the slide/cassette during incubation.

Step 1:

Probe Hybridization

1.0 Get Started 28

1.1 Custom Probe Preparation (optional) 30

1.2 Probe Hybridization 31

1.0 Get Started

Workflow Information				
Assay Workflow Date:				
Instrument Run Date:				
Run Name:				
	First Cassette	**Second Cassette**	**Third Cassette**	**Fourth Cassette**
Sample Name:				
Slide ID:				
Fixation Method:				
Tissue Type:				
Panel Name or Custom Panel ID:				

Each 10x Genomics reagent tube is enough for two slides.

Items			10x PN	Preparation & Handling	Storage
Equilibrate to room temperature					
☐		Atera WTA, Select, and Custom Panel Probes	-	This reagent should have been previously thawed in either the Atera Gene Expression - FFPE Tissue Preparation Handbook (CG001671) or FF Tissue Preparation Handbook (CG001670). These instructions are for reference: Thaw probe panels at room temperature. DO NOT vortex. Centrifuge briefly. Consult the 10x Genomics Support Website for the most updated list of available panels and part numbers, as well as information on panel compatibility. See Probe Hybridization on page 31 for additional handling instructions.	–20°C
☐	◯	Reducing Agent B	2000087	Only used if processing a FFPE sample. This reagent should have been previously thawed in the Atera Gene Expression - FFPE Tissue Preparation Handbook (CG001671) and is found in the Atera FFPE Sample Prep Reagents (PN-1001112). These instructions are for reference: Thaw at room temperature. See Probe Hybridization on page 31 for additional handling instructions.	–20°C
☐	◯	Additive A	220093	Only used if processing a FF sample. This reagent should have been previously thawed in the Atera Gene Expression - FF Tissue Preparation Handbook (CG001670) and is found in the Atera FF Sample Prep Reagents (PN-1001113). These instructions are for reference: Thaw at room temperature. Vortex and centrifuge briefly.	–20°C

Items		10x PN	Preparation & Handling	Storage
☐ ◯	Atera Hyb Buffer	2001879	This reagent should have been previously thawed in either the Atera Gene Expression - FFPE Tissue Preparation Handbook (CG001671) or FF Tissue Preparation Handbook (CG001670). These instructions are for reference: Thaw at room temperature. Vortex and centrifuge briefly.	−20°C
Obtain				
☐	Nuclease-free Water	-	-	Ambient
☐	PBS-T	-	PBS-T is prepared in either the Atera Gene Expression - FFPE Tissue Preparation Handbook (CG001671) or FF Tissue Preparation Handbook (CG001670). Consult the relevant document for preparation instructions.	Ambient
☐	Urea	-	-	Ambient
☐	Forceps	-	Optional - use forceps if desired for Atera Cassette Insert application and removal.	Ambient
☐	TE Buffer, TRIS-EDTA, 1X Solution, pH 8.0 (nuclease-free)	-	The pH of the stock solution should be 8.0. Readjusting the pH is NOT recommended.	Ambient
☐	Atera Cassette Lids	-	If working with FFPE samples, a lid has already been used during the sample preparation workflow. Re-use this lid.	Ambient
☐	Atera Thermocycler Adaptor	-	See Tips & Best Practices.	Ambient
☐	Atera Cassette Insert	2001985	**DO NOT** open package until indicated in the protocol.	Ambient
☐	Fume Hood *(optional; only if performing PBS-T washes in the hood)*	-	-	-
☐	Ice Bucket with Ice	-	-	-

1.1 Custom Probe Preparation (optional)

Proceed to Probe Hybridization directly if using pre-designed probes only. Custom probes are delivered lyophilized and must be resuspended before use. Resuspend custom probes according to the instructions below before proceeding with Probe Hybridization. See Probe Panel Handling on page 20 for more information on handling custom probes.

a. Centrifuge custom probe panel tube briefly.

b. Add **70 µl** TE Buffer, pH 8.0 to each tube.

c. Replace cap firmly. Vortex twice for **15 sec** each. Maintain at **room temperature** for **5 min**.

d. Centrifuge custom probe panel tube briefly and maintain at **room temperature**.

e. If custom probes are already resuspended, thaw at room temperature before starting Probe Hybridization.



Custom probes resuspended in TE Buffer, pH 8.0 can be stored at −20°C until the expiration date specified on the kit.

1.2 Probe Hybridization

Before starting this protocol:

- Ensure that tissue sections have been appropriately deparaffinized and decrosslinked if working with FFPE tissues. Consult Atera Gene Expression - FFPE Tissue Preparation Handbook (CG001671) for more information.
- Ensure that tissue sections have been appropriately fixed and permeabilized if working with fresh frozen tissues. Consult Atera Gene Expression - Fresh Frozen Tissue Preparation Handbook (CG001670) for more information.
- Verify that chosen panels are compatible with one another.

DO NOT let sample dry out during any wash steps. Add buffer back to the cassette as quickly as possible once buffer is removed.

a. Prepare one thermal cycler for every two samples with the following incubation protocol and start the program.

Lid Temperature	Reaction Volume	Run Time
50°C	100 µl	-
Step	**Temperature**	**Time** *hh:mm:ss*
Pre-equilibrate	50°C	Hold
Probe Hybridization	50°C	Overnight (16–24 h)
Hold	50°C	Hold

b. Prepare Probe Hybridization Mix according to the options below. Preparation instructions for each option can be found on the following page.

- WTA Panel
- Select Panel
- Custom Panel

Probe Hybridization Mix will be added in two pipetting steps with different volumes.

Prepare either Probe Hybridization Mix option shortly before use and maintain at **room temperature**. Add reagents in the order listed (some overage is included). Pipette mix 5x and centrifuge briefly.

Option 1: Atera WTA Panel + Optional Custom Probe Hybridization Mix



Only use custom probes from Atera Custom RNA Panel C

	Atera WTA + Optional Custom Probe Hybridization Mix	10x PN	1X (µl)	2X (µl)	4X (µl)
◯	Atera Hyb Buffer	2001879	100.0	200.0	400.0
	Urea	-	23.5	47.0	94.0
◯	Reducing Agent B (FFPE) or Additive A (FF)	2000087 or 220093	12.5	25.0	50.0
◯	Atera WTA Probes	-	100.0	200.0	400.0
	Atera Custom RNA Panel C Probes* *If not using custom probes, replace volume with TE Buffer, pH 8.0	-	14.0	28.0	56.0
	Total	**-**	**250.0**	**500.0**	**1,000.0**

Option 2: Atera Select Panel + Optional Custom Probe Hybridization Mix



Panels with the same letter or the same cap color are incompatible. For example, DO NOT combine a Select Probe Panel A with an Atera Custom RNA Panel A.

	Atera Select + Optional Custom Probe Hybridization Mix	10x PN	1X (µl)	2X (µl)	4X (µl)
◯	Atera Hyb Buffer	2001879	100.0	200.0	400.0
	Urea	-	23.5	47.0	94.0
◯	Reducing Agent B (FFPE) or Additive A (FF)	2000087 or 220093	12.5	25.0	50.0
	TE Buffer, pH 8.0	-	86.0	172.0	344.0
Varies	Atera Select OR Custom RNA Panel Probes	-	14.0	28.0	56.0
Varies	Second Custom RNA Panel Probes* *If not using a second panel, replace the volume with TE Buffer, pH 8.0	-	14.0	28.0	56.0
	Total	**-**	**250.0**	**500.0**	**1,000.0**

c. Obtain Atera Cassette Insert package to prepare for application of the Atera Cassette Inserts.

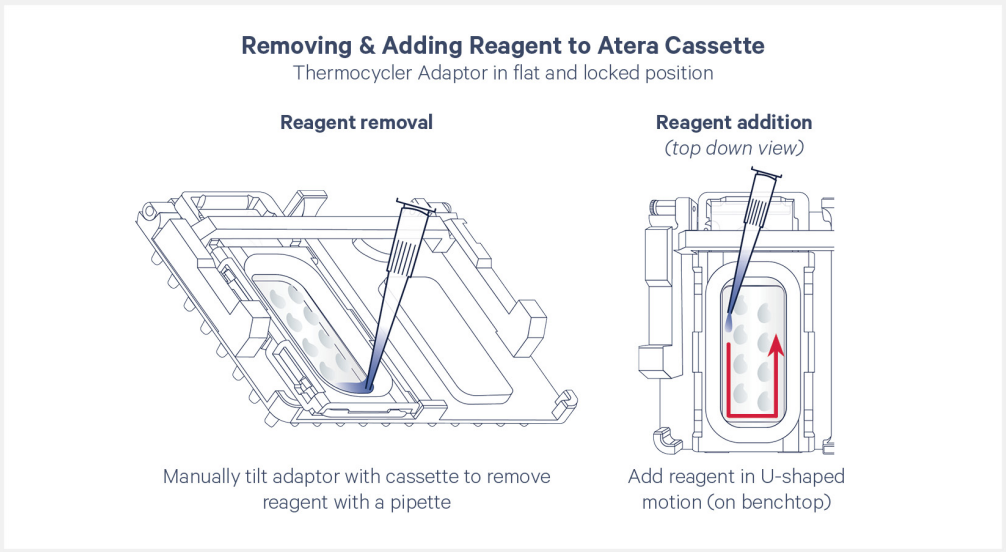
DO NOT open package until instructed.

d. Once Atera Hyb Mix is prepared, remove all buffer from the Atera Cassettes.

e. Add **750 µl** PBS-T to each well.

Optional: photograph the slide against a black background. This image can be used for comparison purposes to identify tissue detachment downstream in the workflow. Work quickly as this is not a safe stopping point. See Troubleshooting for more details.

f. Remove all PBS-T from each cassette well.

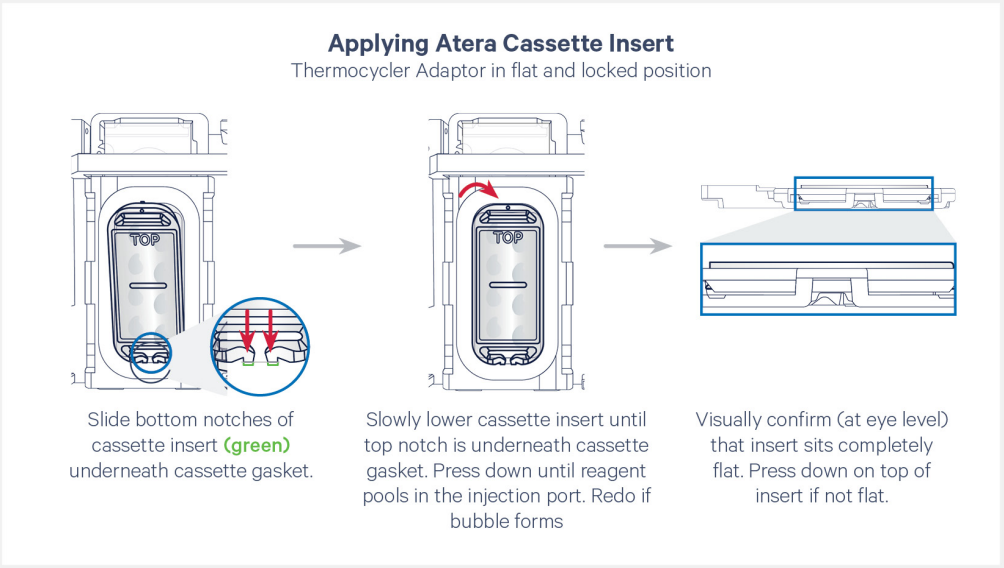


g. Add **300 µl** PBS-T to each well.

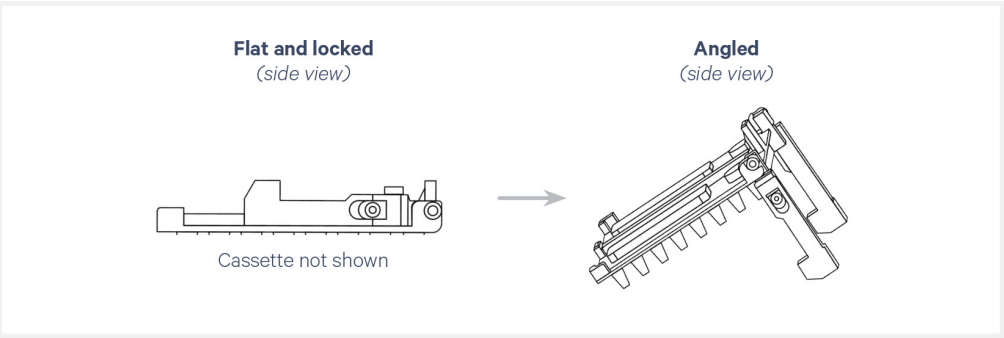
h. Remove Atera Cassette Inserts from their packaging.

Use within 30 min of opening.

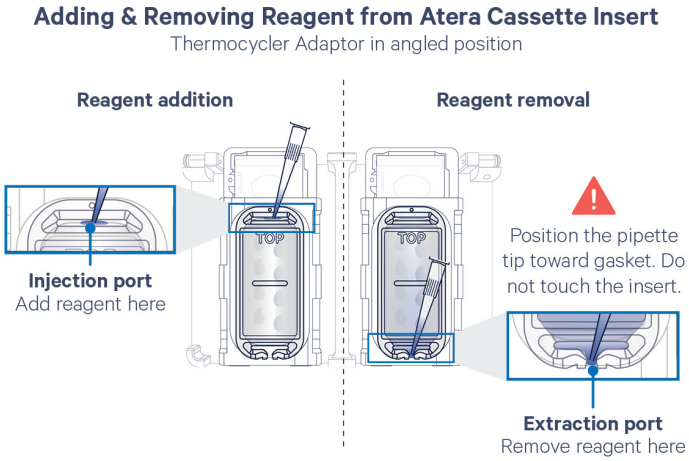
i. Apply Atera Cassette Insert to each Atera Cassette. Consult Atera Cassette Assembly, Disassembly, and Use Quick Reference Card (CG001674) for more information. If bubbles are present, remove insert and reassemble.



j. Adjust Thermocycler Adaptors to angled position.



k. Remove all PBS-T from each Atera Cassette Insert **extraction** port.



Only remove what is easily accessible via the extraction port. Attempting to extract too much liquid from the well may generate a bubble.

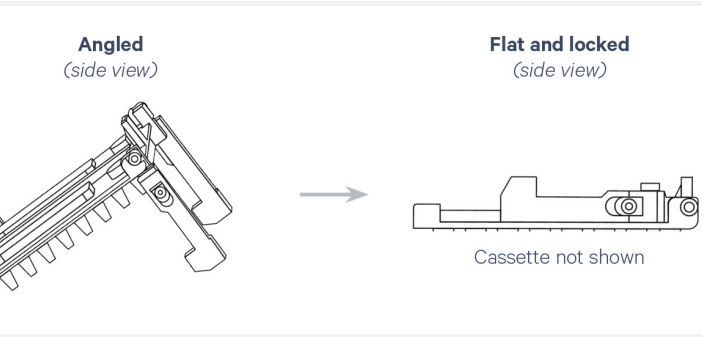
Probe Hybridization Mix Addition 1:

l. Add **170 µl** Probe Hybridization Mix into each Atera Cassette Insert **injection** port and immediately proceed to next step.

m. Remove all displaced reagent from each Atera Cassette Insert **extraction** port. If necessary, repeat removal to ensure all buffer is removed.

Failure to remove remaining displaced buffer may affect assay performance.

n. Adjust Thermocycler Adaptors to flat position and lock arms over cassettes.



Probe Hybridization Mix Addition 2:

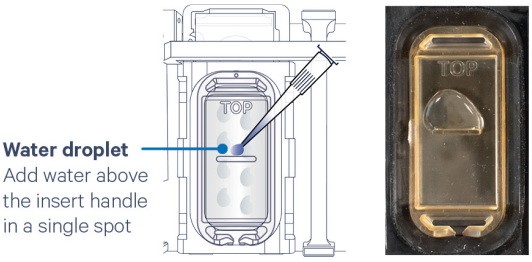
o. Add **60 µl** Probe Hybridization Mix into each Atera Cassette Insert **injection** port.

On top of insert:

p. Add **200 µl** nuclease-free water to a single spot above the insert handle of each Atera Cassette Insert to prevent reagent evaporation during overnight incubation.

Adding Water on top of the Atera Cassette Insert

Thermocycler Adaptor in flat and locked position



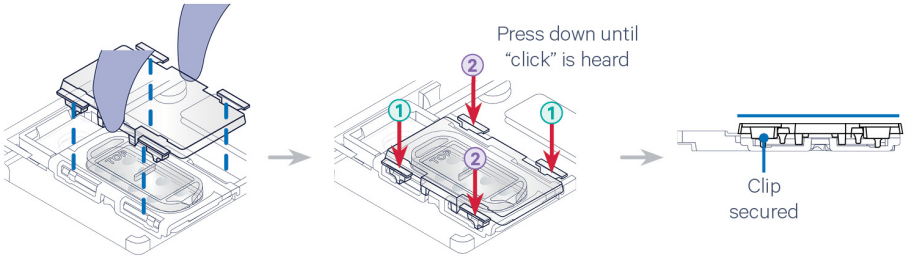
DO NOT add nuclease-free water into the Atera Cassette Insert injection port.

Take a photo of the entire cassette with the nuclease-free water droplet on the insert.

q. Open adaptor arms and apply Atera Cassette Lid on each cassette. If processing FFPE samples, the cassette lid was previously used during the Atera Gene Expression - FFPE Tissue Preparation Handbook (CG001671).

Applying Atera Cassette Lid

Thermocycler Adaptor in flat and unlocked position



Align lid clips to the clips of the cassette top.

Press on two clips **diagonal** from each other until fully clipped in **(1)**. Repeat with remaining two clips **(2)**.

Visually inspect **at eye level on adaptor**. Confirm that all clips are fully secured.

Ensure no bubbles are visible. If bubbles are visible, consult Bubbles During Workflow on page 74. Water may touch the Atera Cassette Lid after lid application. This is normal and does not require lid reapplication.

r. Inspect cassettes at eye level and ensure all clips are secured. After inspection, lock adaptor arms over cassettes.

s. Place Thermocycler Adaptors with cassettes on pre-heated thermal cyclers. Close thermal cycler lids until resistance is felt.

t. Skip Pre-equilibrate step to initiate Probe Hybridization.



u. If loading the Atera Instrument the next day (and optional antibody-based cell segmentation will not be performed), begin thawing Atera Plates and Pouch 4. The required plates vary depending on the type of assay.

Assay	Required Plates	Preparation
Atera WTA, 2 rxn	Plate 1, 2 rxn AND Plate 2, 2 rxn	Keep plates in original packing and thaw **overnight (16–24 h)** at **4°C**
Atera WTA, 4 rxn	Plate 1, 4 rxn AND Plate 2, 4 rxn	
Atera Select, 2 rxn	Plate 1, 2 rxn	
Atera Select, 4 rxn	Plate 1, 4 rxn	

Pouch	Preparation
Pouch 4	Thaw **overnight (16–24 h)** at **room temperature**

Step 2:

Post Hybridization Wash

2.0 Get Started 39

2.1 Post Hybridization Wash 40

2.0 Get Started

Each 10x Genomics reagent tube is enough for two slides.

Items		10x PN	Preparation & Handling	Storage
Equilibrate to room temperature				
☐ ◯	Post Hybridization Wash Buffer	2000395	Thaw at 37°C for 10 min or until thawed completely. Vortex and centrifuge briefly. Keep the buffer at room temperature after thawing.	–20°C
Obtain				
☐	PBS-T	-	Prepared in either the Atera Gene Expression - FFPE Tissue Preparation Handbook (CG001671) or FF Tissue Preparation Handbook (CG001670).	Ambient
☐	Forceps	-	Optional - use forceps if desired for Atera Cassette Insert application and removal.	Ambient

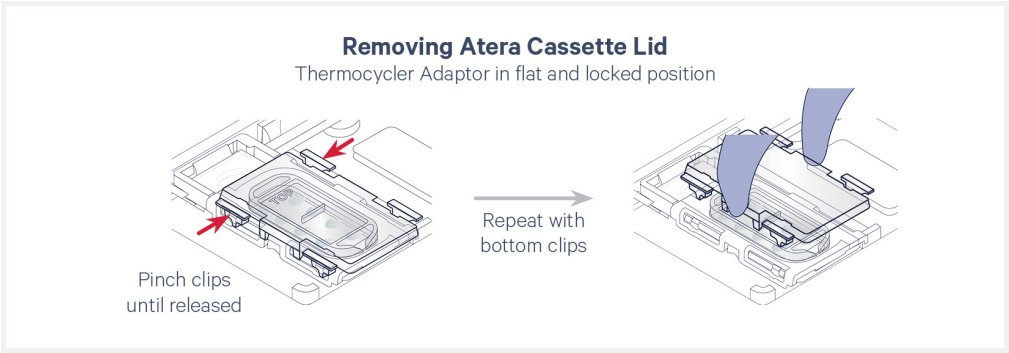
2.1 Post Hybridization Wash

DO NOT let samples dry out during any wash steps. Add buffer back to the cassette as quickly as possible once buffer is removed.

a. Remove Thermocycler Adaptors with cassettes and place on a clean, flat surface.

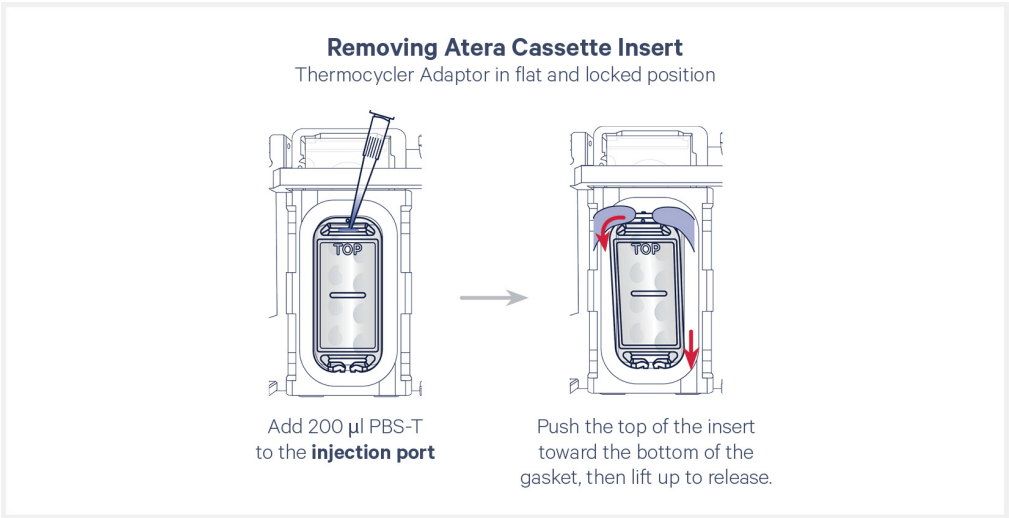
Move quickly to PBS-T washes. Some bubble formation during hybridization is normal. See Bubbles During Workflow on page 74 for more information.

b. Remove Atera Cassette Lids and remove any condensation with a lint-free laboratory wipe. **Save lids** for later steps.



c. Examine the Atera Cassette Inserts for any remaining water. If necessary, remove condensation with a pipette.

d. Add **200 µl** PBS-T into each Atera Cassette Insert **injection** port.



e. Remove each Atera Cassette Insert and discard.

f. Remove all buffer from each well.

Two PBS-T washes (PBS-T prepared in either the Atera Gene Expression - FFPE Tissue Preparation Handbook (CG001671) or FF Tissue Preparation Handbook (CG001670):

g. **Wash 1: Immediately** add **750 µl** PBS-T to each well. Incubate for **1 min** at **room temperature.** Remove all PBS-T.

h. **Wash 2:** Add **750 µl** PBS-T to each well. Incubate for **1 min** at **room temperature**. Buffer removal will occur after preparing thermal cycler programs to complete Wash 2.

i. Prepare one thermal cycler for every two samples with the following incubation protocol and start the program.

Lid Temperature	Reaction Volume	Run Time
35°C (lid may be set to the lowest temperature if the instrument does not enable 35°C)	100 µl	-
Step	**Temperature**	**Time** *hh:mm:ss*
Pre-equilibrate	35°C	Hold
Post Hybridization Wash	35°C	00:15:00
Hold	35°C	Hold

j. Remove all PBS-T to complete Wash 2.

k. Add **750 µl** Post Hybridization Wash Buffer to each well.

l. Apply previously used Atera Cassette Lids onto cassettes.

m. Place Thermocycler Adaptors with cassettes on pre-heated thermal cyclers. Close thermal cycler lids.

n. Skip Pre-equilibrate step to initiate Post Hybridization Wash.

TIPS

Retrieve Ligation reagents and start preparing Ligation Mix as detailed in Get Started on page 43 and Ligation on page 44.

o. After Post Hybridization Wash is complete, **immediately** proceed to next step.

The hold time after Post Hybridization Wash incubation should not exceed 2–3 min.

10x GENOMICS

Step 3:

Ligation

3.0 Get Started 43

3.1 Ligation 44

3.0 Get Started

Each 10x Genomics reagent tube is enough for two slides.

Items		10x PN	Preparation & Handling	Storage
Equilibrate to room temperature				
☐	Atera Ligation Buffer	2001880	Ligation Buffer may not be completely frozen during −20°C storage. Thaw at room temperature. Vortex and centrifuge briefly. Maintain at room temperature after thawing.	−20°C
Place on ice				
☐	Atera Enzyme A	2001915	Pipette mix and centrifuge briefly. Maintain on ice until ready to use.	−20°C
☐	Atera Enzyme B	2001916	Pipette mix and centrifuge briefly. Maintain on ice until ready to use.	−20°C
Obtain				
☐	PBS-T	-	Prepared in either the Atera Gene Expression - FFPE Tissue Preparation Handbook (CG001671) or FF Tissue Preparation Handbook (CG001670).	Ambient
☐	Ice Bucket with Ice	-	-	-

3.1 Ligation

DO NOT let samples dry out during any wash steps. Add buffer back to the cassette as quickly as possible once buffer is removed.

a. Remove Thermocycler Adaptors with cassettes and place on a clean, flat surface.

b. Remove Atera Cassette Lids. **Save lids** for later steps.

c. Remove Post Hybridization Wash Buffer from each well.

Three PBS-T washes (PBS-T prepared in either the Atera Gene Expression - FFPE Tissue Preparation Handbook (CG001671) or FF Tissue Preparation Handbook (CG001670):

d. **Wash 1: Immediately** add **750 µl** PBS-T to each well. Incubate for **1 min** at **room temperature**. Remove all PBS-T.

e. **Wash 2:** Add **750 µl** PBS-T to each well. Incubate for **1 min** at **room temperature**. Remove all PBS-T.

f. **Wash 3:** Add **750 µl** PBS-T to each well (buffer removal will occur after preparing thermal cycler programs to complete Wash 3).

g. Prepare Ligation Mix shortly before using (for maximum efficiency, start preparing this mix as indicated during the Post Hybridization Wash). Add reagents in the order listed (some overage is included). Pipette mix 10x and centrifuge briefly. Maintain on ice.

	Ligation Mix	10x PN	1X (µl)	2X (µl)	4X (µl)
	Atera Ligation Buffer	2001880	722.0	1,444.0	2,888.0
	Atera Enzyme A	2001915	20.5	41.0	82.0
	Atera Enzyme B	2001916	82.5	165.0	330.0
	Total	**-**	**825.0**	**1,650.0**	**3,300.0**

h. Prepare one thermal cycler for every two samples with the following incubation protocol and start the program.

Lid Temperature	Reaction Volume	Run Time
42°C	100 µl*	-
Step	**Temperature**	**Time** *hh:mm:ss*
Pre-equilibrate	42°C	Hold
Ligation	42°C	00:30:00
Hold	42°C	Hold

**Max volume that can be set for the Bio-Rad C1000.*

i. Remove PBS-T to complete Wash 3.

j. Add **750 µl** Ligation Mix to each well.

k. Apply previously used Atera Cassette Lids onto cassettes.

l. Place Thermocycler Adaptors with cassettes on pre-heated thermal cyclers. Close thermal cycler lids.

m. Skip Pre-equilibrate step to initiate Ligation.

TIPS

Start thawing Amplification Enhancement and Amplification reagents (except Atera Signal Booster) at the beginning of Ligation incubation as outlined in *Get Started on page 47**.*

n. After Ligation is complete, **immediately** proceed to next step.

Step 4:

Amplification

4.0 Get Started 47

4.1 Amplification Enhancement 48

4.2 Amplification 50

4.3 Post Amplification Wash 52

4.4 Signal Boost 53

4.0 Get Started

Each 10x Genomics reagent tube is enough for two slides.

Item	10x PN	Preparation & Handling	Storage
Equilibrate to room temperature			
☐ Amplification Enhancer Buffer	2001234	Thaw at **37°C** for **5 min**. Maintain at room temperature. Vortex and centrifuge briefly before use.	–20°C
☐ Amplification Enhancer Wash Buffer	2001236	Thaw at room temperature. Vortex and centrifuge briefly. Maintain at room temperature.	–20°C
☐ Atera Amp Mix 1	2001976	Thaw at room temperature. Vortex and centrifuge briefly. Maintain at room temperature.	–20°C
☐ Atera Amp Mix 2	2001881	Thaw at room temperature. Vortex and centrifuge briefly. Maintain at room temperature.	–20°C
Place on ice			
☐ Atera Amp Enhancer	2001917	Pipette mix and centrifuge briefly. Maintain on ice.	–20°C
☐ Atera Signal Booster	2001947	Retrieve when indicated during the protocol (during Amplification incubation). Maintain on ice.	–20°C
Obtain			
☐ PBS-T	-	Prepared in either the Atera Gene Expression - FFPE Tissue Preparation Handbook (CG001671) or FF Tissue Preparation Handbook (CG001670).	Ambient
☐ TE Buffer, TRIS-EDTA, 1X Solution, pH 8.0 (nuclease-free)	-	-	Ambient
☐ Ice Bucket with Ice	-	-	-

4.1 Amplification Enhancement

DO NOT let samples dry out during any wash steps. Add buffer back to the cassette as quickly as possible once buffer is removed.

a. Remove Thermocycler Adaptors with cassettes and place on a clean, flat surface.

b. Remove Atera Cassette Lids. **Save lids** for later steps.

c. Remove all Ligation Mix from each well.

Three PBS-T washes (PBS-T prepared in either the Atera Gene Expression - FFPE Tissue Preparation Handbook (CG001671) or FF Tissue Preparation Handbook (CG001670):

d. **Wash 1: Immediately** add **750 µl** PBS-T to each well. Incubate for **1 min** at **room temperature**. Remove all PBS-T.

e. **Wash 2:** Add **750 µl** PBS-T to each well. Incubate for **1 min** at **room temperature**. Remove all PBS-T.

f. **Wash 3:** Add **750 µl** PBS-T to each well (buffer removal will occur after preparing Amplification Enhancer Mix and thermal cycler programs to complete Wash 3).

g. Prepare one thermal cycler for every two samples with the following incubation protocol and start the program.

Lid Temperature	Reaction Volume	Run Time
Off	100 µl	-
Step	**Temperature**	**Time** *hh:mm:ss*
Pre-equilibrate	4°C	Hold
Amplification Enhancement	4°C	02:00:00

h. Prepare Amplification Enhancer Mix on ice shortly before use. Add reagents in the order listed (some overage is included). Pipette mix 10x and centrifuge briefly. Maintain on ice.

	Amplification Enhancer Mix	10x PN	1X (µl)	2X (µl)	4X (µl)
●	Amplification Enhancer Buffer	2001234	720.0	1,440.0	2,880.0
●	Atera Amp Enhancer	2001917	80.0	160.0	320.0
	Total	**-**	**800.0**	**1,600.0**	**3,200.0**

i. Remove all PBS-T to complete Wash 3.

j. **Immediately** add **750 µl** Amplification Enhancer Mix to each well.

k. Apply previously used Atera Cassette Lids on the Atera Cassettes.

l. Place Thermocycler Adaptors with cassettes onto pre-cooled thermal cyclers. Close thermal cycler lids.

m. Skip Pre-equilibrate step to initiate Amplification Enhancement.

n. After Amplification Enhancement is complete, **immediately** proceed to next step.

4.2 Amplification

DO NOT let samples dry out during any wash steps. Add buffer back to the cassette as quickly as possible once buffer is removed.

a. Remove Thermocycler Adaptors with cassettes and place on a clean, flat surface.

b. Remove Atera Cassette lids. **Save lids** for use in later steps.

c. Remove all Amplification Enhancer Mix from each well.

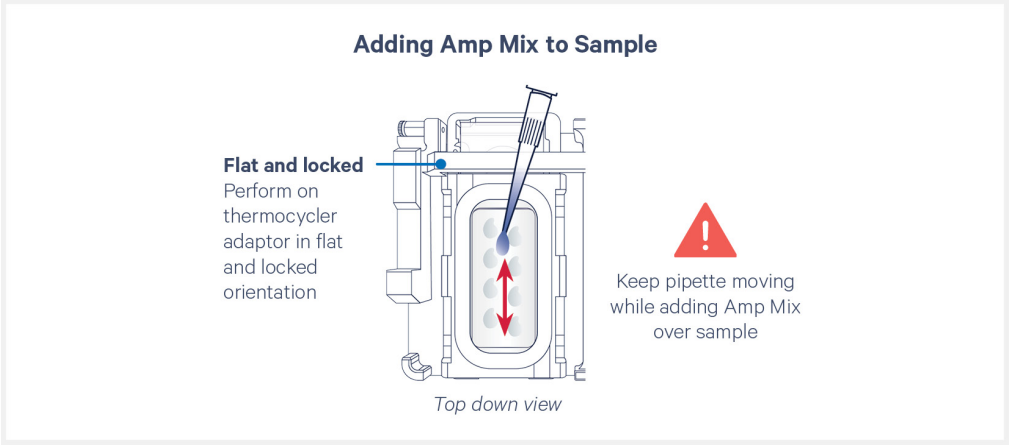
d. **Immediately** add **750 µl** Amplification Enhancer Wash Buffer to each well.

e. Incubate for **1 min** at **room temperature**.

Two-step Amplification:

f. Remove all Amplification Enhancer Wash Buffer from each well.

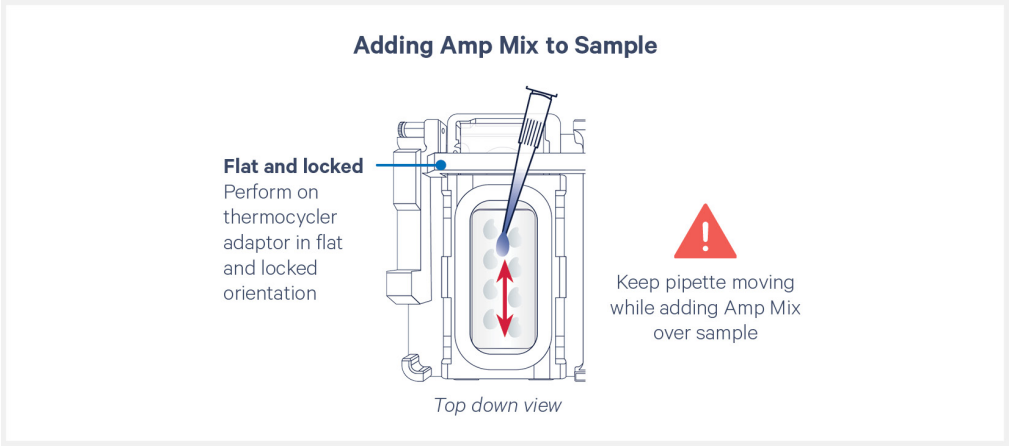
g. **Immediately** add **500 µl** ● Atera Amp Mix 1 quickly and evenly over each sample. Keep pipette moving while adding reagent to evenly spread Amp Mix 1.



DO NOT pipette Atera Amp Mix 1 along the side of the cassette. Amplification is the only step where pipetting over the sample is required.

h. Incubate for **1 min** at **room temperature**.

i. Without removing Atera Amp Mix 1, **immediately** add **500 µl** ● Atera Amp Mix 2 quickly and evenly over each sample. Keep pipette moving while adding reagent to evenly spread Amp Mix 2.



DO NOT pipette Atera Amp Mix 2 along the side of the cassette. Amplification is the only step where pipetting over the sample is required.

j. Prepare one thermal cycler for every two samples with the following incubation protocol and start the program.

Lid Temperature	Reaction Volume	Run Time
30°C (lid may be set to the lowest temperature if the instrument does not enable 30°C)	100 µl*	-
Step	**Temperature**	**Time** *hh:mm:ss*
Pre-equilibrate	30°C	Hold
Amplification	30°C	02:00:00
Hold	30°C	Hold

**Max volume that can be set for the Bio-Rad C1000.*

k. Apply previously used Atera Cassette Lids onto Atera Cassettes.

l. Place Thermocycler Adaptors with cassettes on the thermal cyclers. Close thermal cycler lids.

m. Skip Pre-equilibrate step to initiate Amplification.

Retrieve Atera Signal Booster and place on ice before the end of the Amplification incubation.

TIPS *If performing Cell Segmentation Staining, start thawing Block and Stain Buffer during Amplification as outlined in Get Started on page 55.*

n. After Amplification is complete, **immediately** proceed to the next step.

4.3 Post Amplification Wash

DO NOT let samples dry out during any wash steps. Add buffer back to the cassette as quickly as possible once buffer is removed.

a. Remove Thermocycler Adaptors with cassettes and place on a clean, flat surface.

b. Remove Atera Cassette lids. **Save lids** for later steps.

c. Remove all Atera Amp Mix from each well.

Three TE Buffer, pH 8.0 Washes:

d. **Wash 1:** Add **750 µl** TE Buffer, pH 8.0 to each well. Incubate **1 min** at **room temperature**. Remove all TE buffer.

e. **Wash 2:** Add **750 µl** TE Buffer, pH 8.0 to each well. Incubate **1 min** at **room temperature**. Remove all TE buffer.

f. **Wash 3:** Add **750 µl** TE Buffer, pH 8.0 to each well. Incubate **1 min** at **room temperature**. Remove all TE buffer.

Three PBS-T Washes (PBS-T prepared in either the Atera Gene Expression - FFPE Tissue Preparation Handbook (CG001671) or FF Tissue Preparation Handbook (CG001670)):

g. **Wash 1:** Add **750 µl** PBS-T to each well. Incubate for **1 min** at **room temperature**. Remove all PBS-T.

h. **Wash 2:** Add **750 µl** PBS-T to each well. Incubate for **1 min** at **room temperature**. Remove all PBS-T.

i. **Wash 3:** Add **750 µl** PBS-T to each well (buffer removal will occur after preparing Atera Signal Booster to complete Wash 3).

4.4 Signal Boost

a. Prepare Atera Signal Booster while samples are in PBS-T. Centrifuge briefly. Visually inspect the tube to ensure white powder is at the bottom of the tube before opening. Add **1,100 µl** 1X PBS. Pipette mix 5x and centrifuge for **5 sec**.

One Atera Signal Booster is good for two slides. Prepare two Atera Signal Booster tubes if working with four slides.

	Atera Signal Booster	10x PN	1X PBS (µl)
●	Atera Signal Booster	2001947	1,100

b. Remove all PBS-T from each well to complete Wash 3.

c. Add **500 µl** Atera Signal Booster to each well.

d. Apply Atera Cassette Lids to each cassette.

e. Inspect cassette at eye level and ensure all clips are secured. After inspection, lock adaptor arms over cassettes.

f. Incubate for **20 min** at **room temperature**.

g. Remove Atera Cassette Lids and remove all Atera Signal Booster from each well. **Save lids** for later steps.

Three PBS-T Washes (PBS-T prepared in either the Atera Gene Expression - FFPE Tissue Preparation Handbook (CG001671) or FF Tissue Preparation Handbook (CG001670)):

h. **Wash 1:** Add **750 µl** PBS-T to each well. Incubate for **1 min** at **room temperature**. Remove all PBS-T.

i. **Wash 2:** Add **750 µl** PBS-T to each well. Incubate for **1 min** at **room temperature**. Remove all PBS-T.

j. **Wash 3:** Add **750 µl** PBS-T to each well (buffer removal will occur after the next step to complete Wash 3).

k. Proceed to either Optional - Cell Segmentation Staining on page 54 or directly to Ethanol Drying on page 64 if not performing cell segmentation staining. Maintain the cassette with PBS-T at room temperature (previously used lid applied) while preparing buffers for the next step.

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Step 5:

Optional - Cell Segmentation Staining

5.0 Get Started 55

5.1 Buffer Preparation 56

5.2 Block and Stain 57

5.3 Signal Boost 60

5.0 Get Started

Each 10x Genomics reagent tube, except for the Atera Multi-Tissue Stain, is enough for two slides. Atera Multi-Tissue Stain is enough for one slide.

Item		10x PN	Preparation & Handling	Storage
Place on Ice				
☐	Atera Block and Stain Buffer	2001945	Thaw at room temperature for 30 min. Vortex and centrifuge briefly. Maintain on ice.	–20°C
☐	Atera Multi-Tissue Stain	2001946	Prepare according to Block and Stain on page 57. Maintain on ice. One tube is needed per slide.	–20°C
☐	Atera Signal Booster	2001947	To be prepared the **next day** during Signal Boost. Obtain after staining is complete. Maintain on ice.	–20°C
Obtain				
☐	Atera Cassette Insert	2001985	**DO NOT** open package.	Ambient
☐	Nuclease-free Water	-	-	Ambient
☐	100% Ethanol	-	-	Ambient
☐	1X PBS	-	Prepared in either the Atera Gene Expression - FFPE Tissue Preparation Handbook (CG001671) or FF Tissue Preparation Handbook (CG001670). To be obtained **next day** for Stain Enhancement.	Ambient
☐	PBS-T	-	Prepared in either the Atera Gene Expression - FFPE Tissue Preparation Handbook (CG001671) or FF Tissue Preparation Handbook (CG001670).	Ambient
☐	Forceps	-	Optional - use forceps if desired for Atera Cassette Insert application and removal.	Ambient
☐	Ice Bucket with Ice	-	-	Ambient

If loading the Atera Instrument the next day, begin thawing Atera Plates and Pouch 4. The required plates vary depending on the type of assay.

Assay	Required Plates	Preparation
Atera WTA, 2 rxn	Plate 1, 2 rxn AND Plate 2, 2 rxn	Keep plates in original packing and thaw **overnight (16–24 h)** at **4°C**
Atera WTA, 4 rxn	Plate 1, 4 rxn AND Plate 2, 4 rxn	
Atera Select, 2 rxn	Plate 1, 2 rxn	
Atera Select, 4 rxn	Plate 1, 4 rxn	

Pouch	Preparation
Pouch 4	Thaw **overnight (16–24 h)** at **room temperature**

5.1 Buffer Preparation

a. Retrieve PBS-Tween (PBS-T) prepared in either the Atera Gene Expression - FFPE Tissue Preparation Handbook (CG001671) or FF Tissue Preparation Handbook (CG001670).

b. Prepare ethanol dilutions using nuclease-free water (some overage is included). Vortex and centrifuge briefly. Maintain at room temperature.

For Ethanol Washes		
Items		**Preparation & Handling**
	100% Ethanol	Prepare 20 ml 100% ethanol for four slides. This 100% ethanol will also be used during the Ethanol Drying step.
	70% Ethanol	Prepare 20 ml 70% ethanol for four slides. This 70% ethanol will also be used during the Ethanol Drying step.

70% Ethanol						
Items		**Stock**	**Final**	**1X (ml)**	**2X (ml)**	**4X (ml)**
	Ethanol	100%	70%	3.5	7.0	14.0
	Nuclease-free water	-	-	1.5	3.0	6.0
	Total	**-**	**-**	**5.0**	**10.0**	**20.0**

c. Prepare 1X Diluted Atera Block and Stain Buffer in a centrifuge tube for both blocking and staining steps. Add reagents in the order listed (some overage is included). Vortex and centrifuge briefly. Maintain on ice.

1X Diluted Atera Block and Stain Buffer							
Items			**10x PN**	**Stock**	**Final**	**1X (µl)**	**2X (µl)**	**4X (µl)**
		Nuclease-free Water	-	-	-	875	1,750	3,500
	●	Atera Block and Stain Buffer	2001945	-	-	275	550	1,100
		Total	**-**	**-**	**-**	**1,150**	**2,300**	**4,600**

5.2 Block and Stain

a. Retrieve Atera Cassettes from the previous step and place on a flat, clean work surface.

b. Remove all PBS-T buffer from each well.

DO NOT let tissue dry out during the ethanol wash steps and between removal of ethanol and addition of PBS-T. Left over ethanol volumes may be used for ethanol wash steps in Ethanol Drying on page 64.

Four ethanol washes:

c. **Wash 1:** Add **750 µl** 70% ethanol to each well. Incubate for **2 min** at **room temperature**. Remove the ethanol.

d. **Wash 2:** Add **750 µl** 100% ethanol to each well. Incubate for **2 min** at **room temperature**. Remove the ethanol.

e. **Wash 3:** Add **750 µl** 100% ethanol to each well. Incubate for **2 min** at **room temperature**. Remove the ethanol.

f. **Wash 4:** Add **750 µl** 70% ethanol to each well. Incubate for **2 min** at **room temperature**. Remove the ethanol.

g. **Immediately** add **750 µl** PBS-T to each well. Incubate for **1 min** at **room temperature**. Remove all PBS-T.

h. Add **750 µl** 1X Diluted Atera Block and Stain Buffer (from Buffer Preparation on the previous page) to each well.

Keep remaining volume for later indicated steps.

i. Apply the previously used Atera Cassette Lids on the Atera Cassettes and incubate for **1 h** at **room temperature**.

j. Centrifuge Atera Multi-Tissue Stain tube for **5 sec**.

Complete Atera Multi-Tissue Stain prep steps (steps j–n) during 1 hour block (step i).

k. Add **250 µl** 1X Diluted Atera Block and Stain Buffer (from Buffer Preparation on the previous page) to Atera Multi-Tissue Stain tube.

One Atera Multi-Tissue Stain Tube is good for one slide. Prepare four Atera Multi-Tissue Stain tubes if working with four slides.

l. Pipette mix 15x (pipette set to 175 µl) and centrifuge briefly. Avoid bubbles if possible.

	Atera Multi-Tissue Stain	10x PN	1X Diluted Atera Block and Stain Buffer (µl)
●	Atera Multi-Tissue Stain	2001946	250.0

DO NOT add any additional antibodies to the Atera Multi-Tissue Stain, as this can compromise assay performance.

m. Incubate resuspended Atera Multi-Tissue Stain for **30 min** at **room temperature**.

n. Centrifuge Atera Multi-Tissue Stain for **10 min** at **14,000 rcf** at **4°C**. Maintain on ice until ready to use.

o. Obtain Atera Cassette Insert packages to prepare for application.

p. Retrieve Atera Cassettes after 1 h incubation.

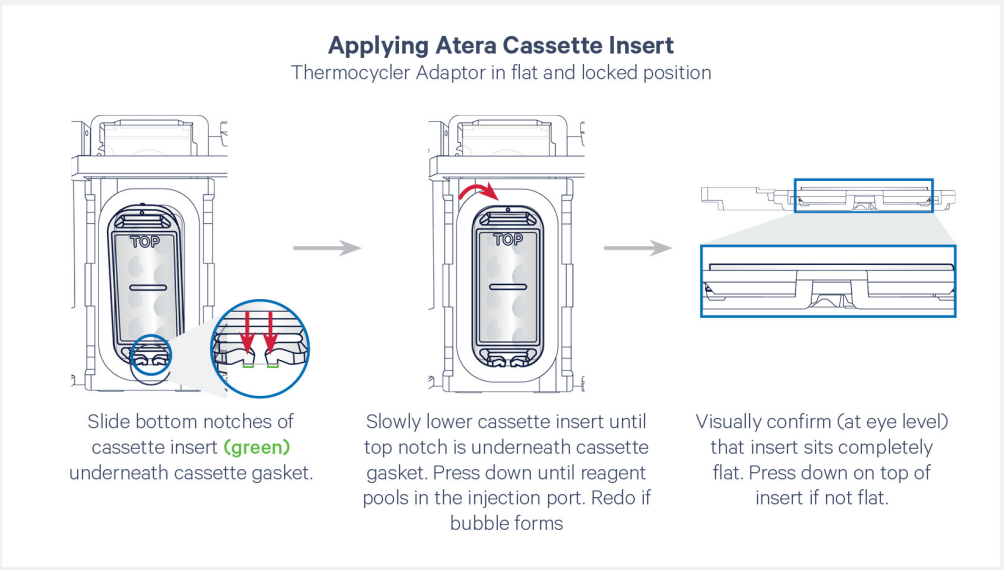
q. Remove Atera Cassette Inserts from their packaging.

Use within 30 min of opening.

r. Remove Atera Cassette Lids. **Save the lid** for later steps.

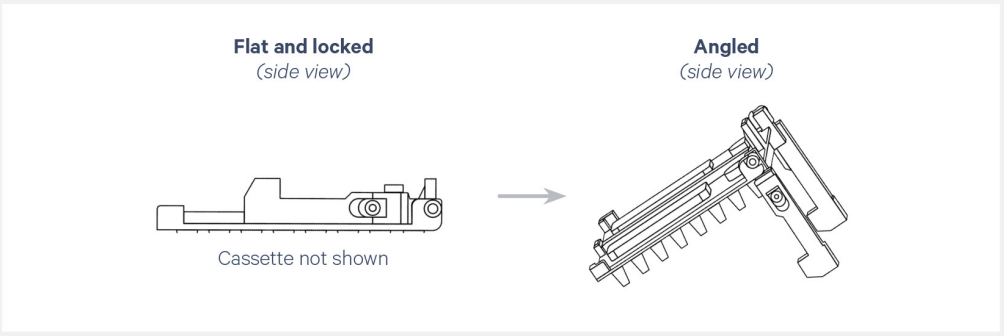
s. Using a pipette, remove **450 µl** 1X Diluted Atera Block and Stain Buffer from each well, leaving behind **300 µl**. DO NOT let the tissue sections dry out.

t. Apply a new Atera Cassette Insert to each cassette. Consult Atera Cassette Assembly, Disassembly, and Use Quick Reference Card (CG001674) for more information. If bubbles are present, remove insert and reassemble.



u. Adjust Thermocycler Adaptors to angled position.

Atera Multi-Tissue Stain will be added in two pipetting steps with different volumes.



v. **Immediately** remove any reagent from each Atera Cassette Insert **extraction** port.

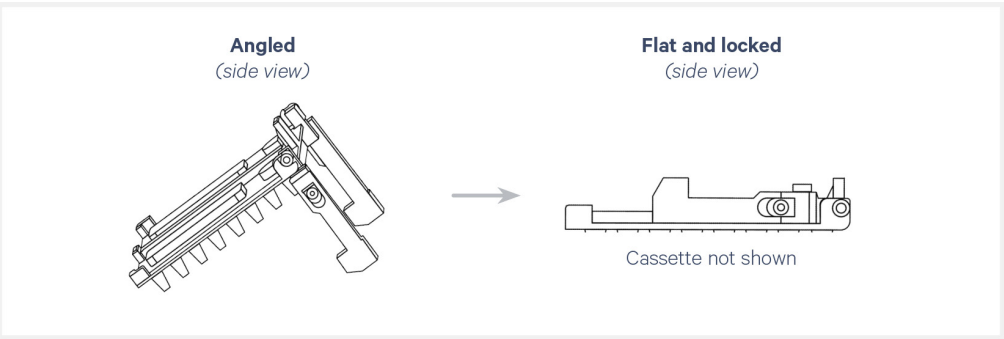
w. Add **170 µl** Atera Multi-Tissue Stain to each Atera Cassette Insert **injection** port.

DO NOT pipette from bottom of Atera Multi-Tissue Stain tube.

x. **Immediately** remove all displaced buffer from each Atera Cassette Insert **extraction** port. If necessary, repeat removal to ensure all buffer is removed.

Failure to remove remaining displaced buffer may affect assay performance.

y. Adjust Thermocycler Adaptors to flat position and lock arms over cassettes.



z. Add **60 µl** Atera Multi-Tissue Stain into each Atera Cassette Insert **injection** port.

aa. Apply the previously used Atera Cassette Lids on the cassettes.

ab. Incubate the Atera Cassettes on Thermocycler Adaptors **overnight (16–24 h)** at **4°C** (in a refrigerator or incubator).

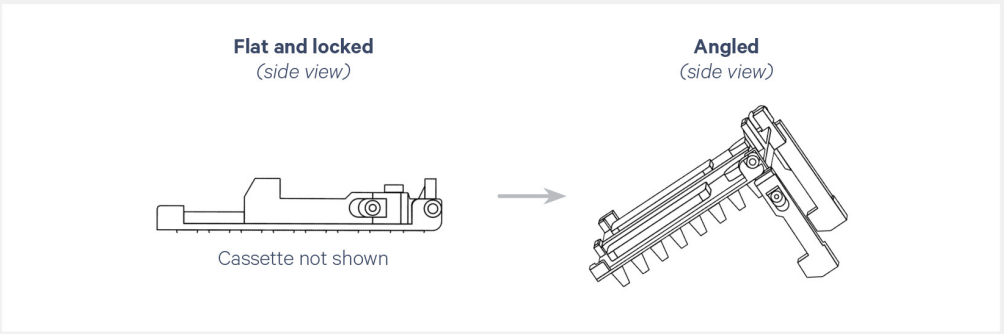
5.3 Signal Boost

DO NOT let tissue dry out during any wash steps. Add buffer back to the cassette as quickly as possible once buffer is removed.

a. Retrieve Atera Cassettes on Thermocycler Adaptors.

b. Remove Atera Cassette lids. **Save lids** for later steps.

c. Adjust Thermocycler Adaptors to angled position.



Three PBS-T Washes (PBS-T prepared in either the Atera Gene Expression - FFPE Tissue Preparation Handbook (CG001671) or FF Tissue Preparation Handbook (CG001670):

The insert will also be used for the second PBS-T wash. Do not remove insert until instructed.

d. **Wash 1:** Add **180 µl** PBS-T to each Atera Cassette Insert **injection** port. **Immediately** remove all displaced liquid from each **extraction** port. Incubate for **1 min** at **room temperature**.

e. **Wash 2:** Add **180 µl** PBS-T to each Atera Cassette Insert **injection** port. **Immediately** remove all displaced liquid from each **extraction** port. Incubate for **1 min** at **room temperature**.

f. **Wash 3:** Add **180 µl** PBS-T to each Atera Cassette Insert **injection** port. **Immediately** remove all displaced liquid from each **extraction** port. Incubate for **1 min** at **room temperature**.

g. Prepare Atera Signal Booster while samples are in PBS-T. Centrifuge briefly. Visually inspect the tube to ensure white powder is at the bottom of the tube before opening. Add **1,100 µl** 1X PBS. Pipette mix 5x and centrifuge for **5 sec**.

One Atera Signal Booster is good for two slides. Prepare two Atera Signal Booster tubes if working with four slides.

Atera Signal Booster will be added in two additions.

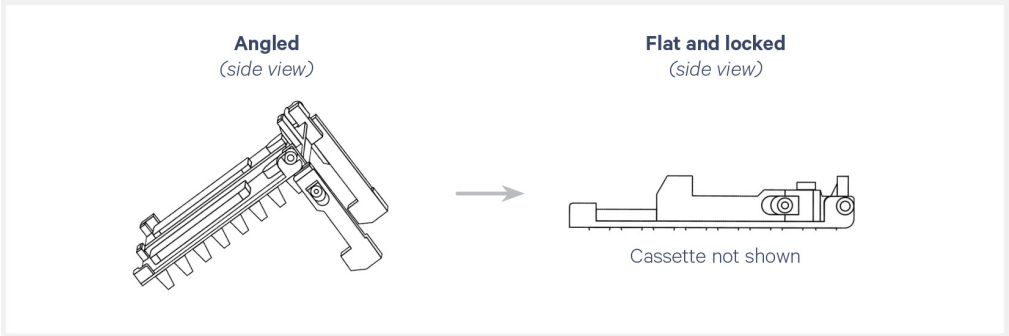
	Atera Signal Booster	10x PN	1X PBS (µl)
●	Atera Signal Booster	2001947	1,100

h. Add **180 µl** Atera Signal Booster into each Atera Cassette Insert **injection** port.

i. **Immediately** remove any displaced buffer from each Atera Cassette Insert **extraction** port.

Failure to remove remaining buffer may affect assay performance.

j. Adjust Thermocycler Adaptors to flat position and lock arms over cassettes.



k. Add **50 µl** Atera Signal Booster into each Atera Cassette Insert **injection** port.

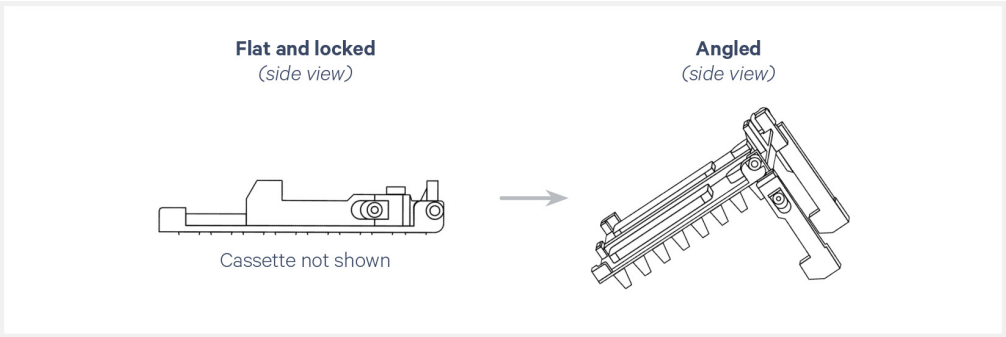
l. Open adaptor arms and apply Atera Cassette Lids to each cassette.

m. Inspect cassette at eye level and ensure all clips are secured. After inspection, lock adaptor arms over cassettes.

n. Incubate for **20 min** at **room temperature**.

o. Remove Atera Cassette lids. **Save lids** for later steps.

p. Adjust Thermocycler Adaptors to angled position.



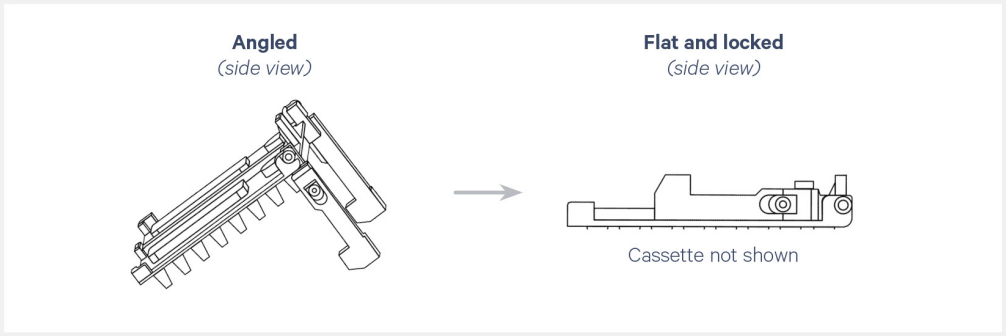
Three PBS-T Washes (PBS-T prepared in either the Atera Gene Expression - FFPE Tissue Preparation Handbook (CG001671) or FF Tissue Preparation Handbook (CG001670):

q. **Wash 1:** Add **180 µl** PBS-T to each Atera Cassette Insert **injection** port. **Immediately** remove all displaced liquid from each **extraction** port. Incubate for **1 min** at **room temperature**.

r. **Wash 2:** Add **180 µl** PBS-T to each Atera Cassette Insert **injection** port. **Immediately** remove all displaced liquid from each **extraction** port. Incubate for **1 min** at **room temperature**.

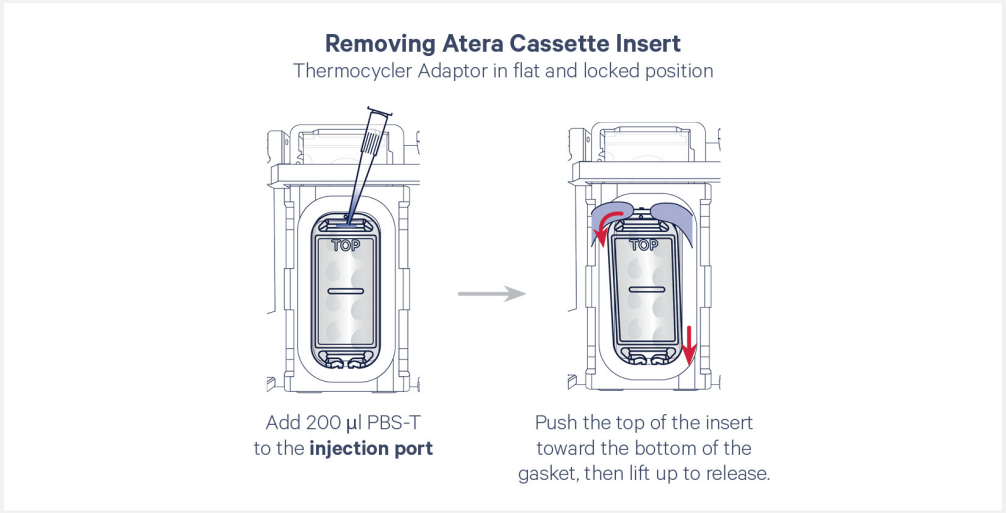
s. **Wash 3:** Add **180 µl** PBS-T to each Atera Cassette Insert **injection** port. **Immediately** remove all displaced liquid from each **extraction** port. Incubate for **1 min** at **room temperature**.

t. Adjust Thermocycler Adaptors to flat position and lock arms over cassettes.



u. Add **200 µl** PBS-T into each Atera Cassette Insert **injection** port.

v. Carefully remove Atera Cassette Inserts. **Discard** used Atera Cassette Inserts.



w. Immediately proceed to Ethanol Drying on page 64.

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Step 6:

Ethanol Drying

6.0 Get Started 65

6.1 Ethanol Drying 66

6.0 Get Started

Item		10x PN	Preparation & Handling	Storage
Obtain				
☐	Nuclease-free Water	-	-	Ambient
☐	100% Ethanol	-	-	Ambient
☐	PBS-T	-	Prepared in either the Atera Gene Expression - FFPE Tissue Preparation Handbook (CG001671) or FF Tissue Preparation Handbook (CG001670).	Ambient

6.1 Ethanol Drying

a. Prepare one thermal cycler for every two samples with the following incubation protocol and start the program.

Lid Temperature	Reaction Volume	Run Time
Off	100 µl	-
Step	**Temperature**	**Time** *hh:mm:ss*
Pre-equilibrate	37°C	Hold
Ethanol Drying	37°C	00:05:00

b. Prepare 70% ethanol shortly before use (if optional Cell Segmentation was performed, 70% ethanol has already been prepared). Add reagents in the order listed (some overage is included). Pipette mix 10x and centrifuge briefly.

70% Ethanol	Stock	Final	1X (µl)	2X (µl)	4X (µl)
Nuclease-free Water	-	-	247.5	495.0	990.0
Ethanol	100%	70%	577.5	1,155.0	2,310.0
Total		**-**	**825.0**	**1,650.0**	**3,300.0**

c. Retrieve Atera Cassettes and if not already on Thermocycler Adaptors, place on the Thermocycler Adaptors and lock arms over cassettes.

d. If lids are present, remove the Atera Cassette Lids. **Save lids** for later steps.

e. Remove all PBS-T from each well.

f. **Immediately** add **750 µl** 70% ethanol to each well. Incubate for **1 min** at **room temperature**. Remove all 70% ethanol.

Two 100% Ethanol washes:

g. **Wash 1:** Add **750 µl** 100% ethanol to each well. Incubate for **1 min** at **room temperature**. Remove all 100% ethanol.

h. **Wash 2:** Add **750 µl** 100% ethanol to each well. Incubate for **1 min** at **room temperature**. Remove all 100% ethanol.

i. Place Thermocycler Adaptors with cassettes on the pre-heated thermal cyclers without the lids. Leave the thermal cycler lids open.

j. Skip Pre-equilibrate step to initiate Ethanol Drying.

k. Retrieve the Thermocycler Adaptors with cassettes and place on a flat, clean work surface.

Optional: photograph the slide against a black background. This image can

be used for comparison purposes to identify tissue detachment downstream in the workflow.

l. Add **1.5 ml** PBS-T to each well.

m. Incubate for **2 min** at **room temperature**.

n. Unlock Thermocycler Adaptor arms and remove cassettes from Thermocycler Adaptors.

o. Place lids on cassettes.

i. If loading the Atera Instrument, proceed to Instrument Loading Preparation on page 68.

Atera Plate and Pouch 4 thawing should be started the night before the Atera Instrument run. See Atera Plate Preparation on page 70 and Atera Pouch Preparation on page 71 for thawing information, including a quick thawing option for Pouch 4.



ii. If storing, place at **4°C** in the dark for up to **one week**.

If processing an FFPE sample and not performing the instrument run on the same day as the completion of this Ethanol Drying step, additional drying is required. Consult the Atera Instrument User Guide (CG001673) for instructions. This additional drying step is not required for FF samples.

If desired, clean the Thermocycler Adaptor by spraying with 70% isopropanol and wiping the adaptors with a lint-free laboratory wipe. Air dry.

Step 7:

Instrument Loading Preparation

7.0 Get Started 69

7.1 Atera Plate Preparation 70

7.2 Atera Pouch Preparation 71

7.0 Get Started

The required plates and Pouch 4 for the Atera Instrument should have been thawed the night before the instrument run. The required plates for each assay type are summarized below.

Assay	Required Plates	Preparation
Atera WTA, 2 rxn	Plate 1, 2 rxn AND Plate 2, 2 rxn	Keep plates in original packing and thaw **overnight (16–24 h)** at **4°C**
Atera WTA, 4 rxn	Plate 1, 4 rxn AND Plate 2, 4 rxn	
Atera Select, 2 rxn	Plate 1, 2 rxn	
Atera Select, 4 rxn	Plate 1, 4 rxn	

Items		10x PN	Preparation & Handling	Storage
Maintain on Ice				
☐	Atera Plate 1	2001614 or 2001616	-	–20°C
☐	Atera Plate 2 (if running WTA assay)	2001615 or 2001617	-	–20°C
Obtain				
☐	Atera Pouch 1 (Universal)	2001931	-	Ambient
☐	Atera Pouch 2 (Universal)	2001932	-	Ambient
☐	Atera Pouch 3 (Universal)	2001933	-	Ambient
☐	Atera Pouch 4 (Small or Large)	2001935 or 2001930	-	–20°C
☐	Deep-well, 96 well plate for counterbalancing	-	-	Ambient
☐	Centrifuge compatible with deep-well 96 well plates (~2 ml vol.) *(Allegra X-14 Series Benchtop centrifuge 120 V or equivalent)*	-	-	Ambient
☐	Serological Pipettes	-	-	Ambient
☐	Plate seal	-	-	Ambient
☐	Laboratory balance	-	-	Ambient

7.1 Atera Plate Preparation

*Keep plates in their original mylar packaging during storage at **–20°C** and during thaw at **4°C**. The initial thaw of Atera plates and Pouch 4 should have started the night before the instrument run.*

a. Thaw plates in original packaging at **4°C** for **16–72 h** or at **37°C** for **2.5 h**. Unopened plates in their original mylar packaging may be kept at **4°C** for up to **3 days**. Total time (thawing and time at 4°C) should not exceed **72 h**.

b. Equilibrate thawed plates at **room temperature** for **30 min**.

c. Open the mylar packaging to remove plates. Do not remove foil seal at any time. Inspect plates to ensure foil seals are intact.

Do not puncture foil seals. If foil seals are punctured, the affected plate cannot be used for the instrument run.

d. Mix by gently inverting the plate 20X without introducing bubbles. DO NOT vortex. Maintain at **room temperature**.

e. Prepare counterbalancing plates.

- Atera Plates 1 and 2 do not weigh the same and should be counterbalanced separately. The plate reaction size also affects plate weight.
- Weigh the Atera Plate. (example: 190 g).
- Place the empty counterbalancing deep-well 96 well plate on the weighing balance. Pipette (multichannel/serological) water to the plate wells until the total weight is equal to the Atera Plate ± 1 g (example: counterbalancing plate with water=189.6 g).
- Remove the counterbalancing plate from the weighing balance, add a seal to it, and use for counterbalancing the appropriate Atera plate.

f. Place reagent and counterbalancing plates in a swinging bucket centrifuge. Centrifuge at **300 rcf** for **1 min** at **room temperature**.

g. Remove from centrifuge and leave plate at **room temperature** until ready to load. DO NOT invert the plate after centrifugation.

7.2 Atera Pouch Preparation

Pouch 1–3

a. Pouches 1–3 are shipped and stored at ambient temperature. No further preparation is needed.

DO NOT *remove cap until immediately before loading.*



Excessive agitation of pouches may result in a decrease in assay performance due to the presence of surfactants in the buffer.

Pouch 4

a. Thaw Pouch 4 **overnight (16–24 h)** at **room temperature**.

This thaw should be started the night before the Atera Instrument run. If Pouch 4 was not thawed overnight, Pouch 4 may be thawed for ***3 h*** *in a* ***37°C*** *water bath. Water bath must be at least 7 L. Pouch should be upright with water covering most of the pouch except for the cap. DO NOT submerge the cap.*

b. Invert pouch **30X** before loading onto the instrument.

DO NOT *remove cap until immediately before loading.*



Failure to invert pouch sufficiently may result in a decrease in assay performance.

c. Proceed to Atera Instrument User Guide (CG001673) for further instructions on instrument operation and loading.

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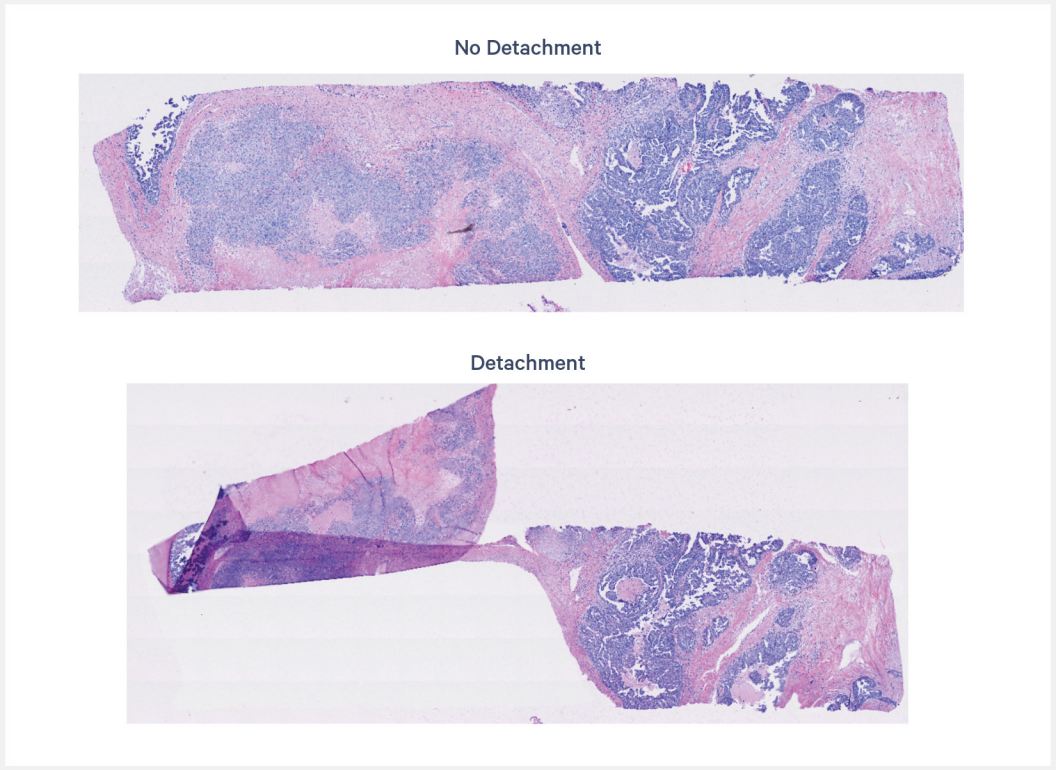
Troubleshooting

Tissue Detachment

Tissue detachment may result in a lack of decodable data in the region where detachment occurred. If the tissue has folded on itself, this may also cause elevated signal in the overlapping areas. To keep track of possible detachment, photograph the slides after ethanol drying.

If detachment is observed during the workflow (>30% tissue detachment), 10x Genomics does not recommend proceeding with the workflow. To mitigate detachment, place difficult tissues (low quality samples, TMAs, etc.) on Schott Nexterion Slide H - 3D Hydrogel Coated Slides (Schott North America, PN-1800434) or Tissue Adhesion Slides (10x Genomics, PN-1001228).



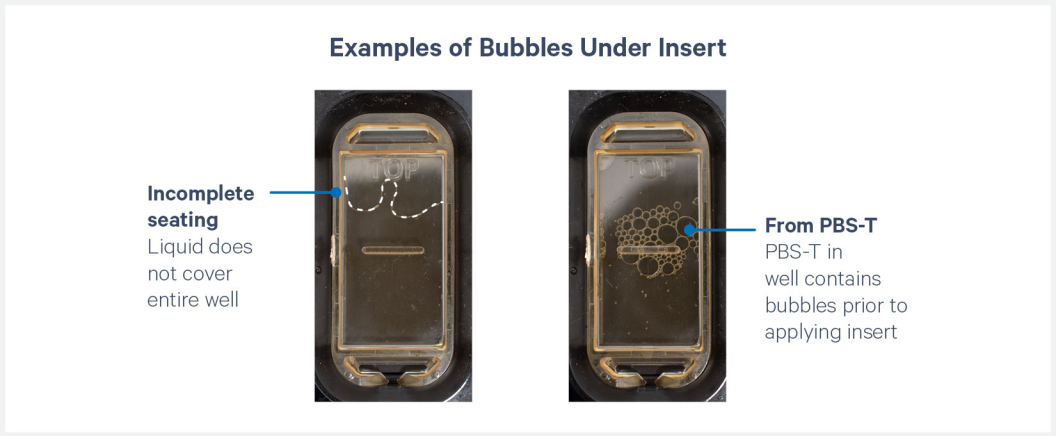
Bubbles During Workflow

Bubbles may occur throughout the workflow, including during PBS-T washes. Bubbles floating on the surface of the slide are unlikely to compromise assay performance. However, bubbles that are in contact with the tissue during the assay may result in a lack of decodable data in the tissue area where the bubbles occurred.

- Avoid generating bubbles during reagent dispensing by pipetting slowly and avoiding expelling air from the pipette tip.
- Bubbles that occur during Probe Hybridization after lid addition may be due to an incorrect amount of Probe Hybridization Mix added to the cassette; however, some bubble formation is normal. Verify that the correct volume was added to the cassette.
- Gently tap or rock the cassette after reagent dispensing and inspect the cassette carefully to ensure liquid is fully covering the tissue.
- Carefully pop bubbles with a P20 pipette tip.
- Ensure there are no bubbles on the cassette well before loading it into the Atera Instrument.

Bubbles During Atera Cassette Insert Placement

Bubbles may occur during Atera Cassette Insert placement. If bubbles are observed, remove the insert and reapply to expel bubbles.



Insufficient Washes

All washes in this protocol are critical for assay performance. Failure to perform the correct number of washes or use the correct buffer can reduce the fraction of usable decodable data. A similar effect is observed when washing for less than the recommended time, or when reagent is carried over during the washes. Remove all liquid from the well when washing, and see User Guide step for correct number of washes and incubation times.

Samples Dry Out

Drying of tissue samples may lead to decreased decoding efficiency and unusable data and will impact overall assay performance and sensitivity. Work quickly and ensure reagents are dispensed evenly across tissues during incubation and wash steps throughout the workflow to prevent drying out of tissues. Ensure tissue sections are always covered with reagent in between removal and addition steps.

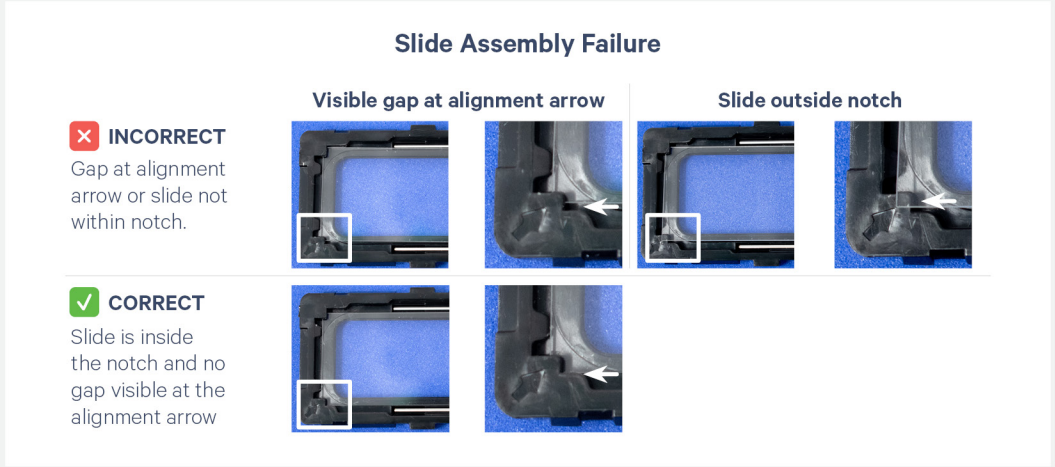
Evaporation in the Atera Cassette may occur after probe hybridization if water is not added on top of the Atera Cassette Insert. Ensure that water is added on top of the insert as called for during the protocol before incubation.

Cassette Assembly Failure

Incorrect assembly of the Atera cassettes can negatively impact assay performance. Inspect the slide carefully to ensure it is seated fully within the cassette before assembly. Example scenarios that may indicate incorrect cassette assembly are described below:

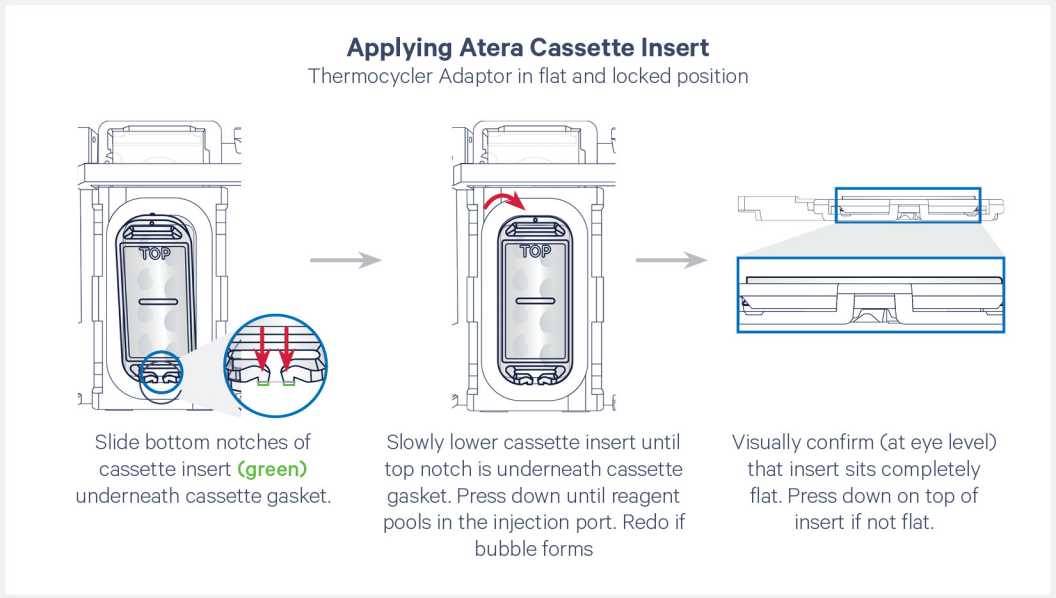
- The cassette does not click shut or appears domed after assembly.
- Slide is not aligned to the long edge of the cassette.

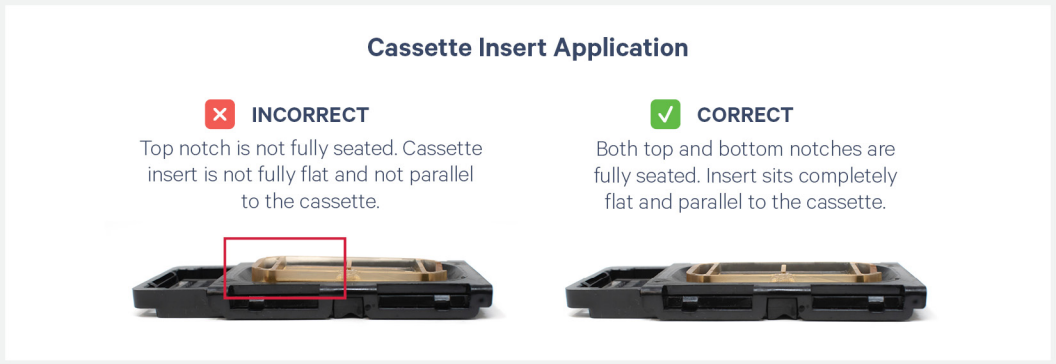




Improper Insert Placement

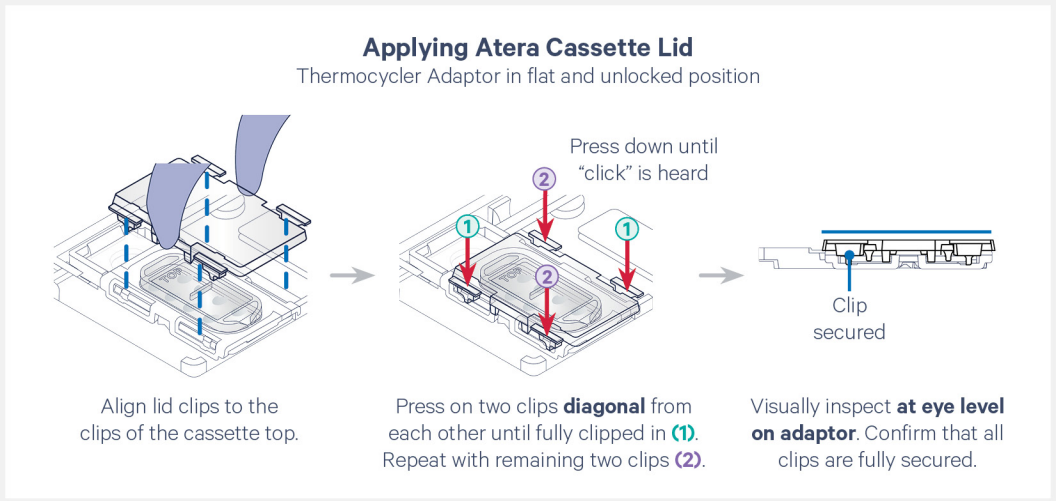
Improper insert placement into the Atera Cassette can negatively impact assay performance. Ensure that no gaps or bubbles are present after assembly. If necessary, remove the insert and reattempt assembly. Ensure that insert is fully placed under cassette notches.





Improper Lid Placement

Improper lid placement onto the Atera Cassette can negatively impact assay performance. Ensure the lid is fully placed onto the cassette. Though audible clicks are heard upon lid application, this does not guarantee that the lid is seated properly. Inspect the lid at eye level to ensure all clips are engaged.





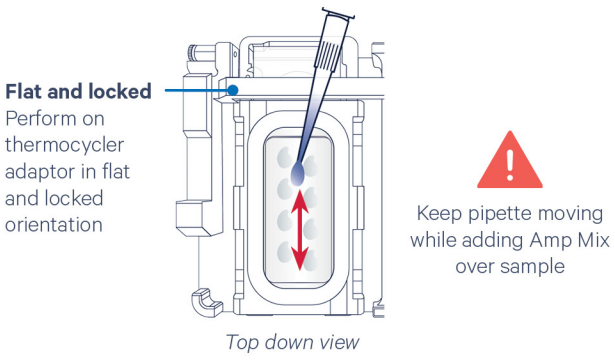
Cassette & Lid: Cleaning & Reuse

- Atera Cassettes, Inserts, and Lids are single use items and are to be discarded after use (unless specified in the protocol step that the lid should be saved and reused). Repeated use of the Atera Cassette beyond a single workflow results in lower cassette performance.
- Only one Atera Cassette and one Atera Cassette Lid are provided. Do not discard until the workflow is complete.
- PBS-T washes DO NOT require sealing of the cassette.
- Cassettes (before cassette assembly) or lids that are accidentally dropped may be reused after thorough cleaning.
    - Rinse the lid under running Milli-Q Water
    - Spray with 70% isopropanol
    - Rinse under running Milli-Q Water
    - Repeat spray and rinse one time.
    - Air dry

Insufficient Amp Mix Pipetting

During Amplification, failure to pipette Amp Mix 1 and 2 evenly across the sample may result in non-uniform assay performance. DO NOT add Amplification Mix along the side of the cassette.

This is the only step where pipetting over the sample is required. Pipetting directly onto the sample during other steps should be avoided, as it increases the risk of tissue detachment.



10x GENOMICS

Appendix

Workflow Checklist

Workflow Information				
Assay Workflow Date:				
Instrument Run Date:				
Run Name:				
	First Cassette	**Second Cassette**	**Third Cassette**	**Fourth Cassette**
Sample Name:				
Slide ID:				
Fixation Method:				
Tissue Type:				
Panel Name or Custom Panel ID:				

Always reference the detailed step by step instructions provided in this user guide when executing the workflow.

Day 1	
1.0 Probe Hybridization on page 27	**Notes**
☐ Thaw or obtain Probe Hybridization reagents (see Get Started on page 28)	
☐ Start Probe Hybridization thermal cycler program	
☐ Prepare Probe Hybridization Mix	
☐ Obtain Atera Cassette Insert package	
☐ Remove all buffer	
☐ Add: PBS-T **750 µl**	
☐ Remove all buffer	
☐ Add: PBS-T **300 µl**	
☐ Remove Atera Cassette Inserts from package and apply to each cassette	
☐ Add cassettes to Thermocycler Adaptor and adjust to angled position	
☐ Remove all buffer from extraction port	
☐ Add: Probe Hybridization Mix 1 **170 µl** to injection port	
☐ Remove all buffer from extraction port	
☐ Adjust Thermocycler Adaptor to flat position	
☐ Add: Probe Hybridization Mix 2 **60 µl** to injection port	
☐ Add: Nuclease-free water **200 µl** on top of insert	
☐ Apply lid, place adaptors with cassettes onto thermal cyclers	
☐ Skip Pre-equilibrate step in thermal cycler programs to start Probe Hybridization	
☐ If loading Atera Instrument the next day, start thawing relevant plates and Pouch 4.	

Day 2	
2.0 Post Hybridization Wash on page 38	**Notes**
☐ Thaw or obtain Post Hybridization Wash reagents (see Get Started on page 39)	
☐ Retrieve cassettes	
☐ Add: PBS-T **200 µl** to injection port	
☐ Remove inserts	
☐ Wash 1: PBS-T **750 µl**, **1 min**	
☐ Wash 2: PBS-T **750 µl**	
☐ Prepare thermal cycler programs and start hold	
☐ Remove **750 µl** PBS-T to complete Wash 2	
☐ Add: Post Hybridization Wash **750 µl** (apply lid, place adaptors with cassettes onto thermal cyclers)	
☐ Skip Pre-equilibrate step in thermal cycler programs to start Post Hybridization Wash	
3.0 Ligation on page 42	**Notes**
☐ Thaw or obtain Ligation reagents (see Get Started on page 43)	
☐ Retrieve cassettes	
☐ Remove all buffer	
☐ Wash 1: PBS-T **750 µl**, **1 min**	
☐ Wash 2: PBS-T **750 µl**, **1 min**	
☐ Wash 3: PBS-T **750 µl**	
☐ Prepare Ligation Mix	
☐ Prepare thermal cycler programs and start hold	
☐ Remove **750 µl** PBS-T to complete Wash 3	
☐ Add: Ligation Mix **750 µl** (apply lid, place adaptors with cassettes onto thermal cyclers)	
☐ Skip Pre-equilibrate step in thermal cycler programs to start Ligation	
4.0 Amplification on page 46	**Notes**
☐ Thaw or obtain Amplification reagents (see Get Started on page 47)	
☐ Retrieve cassettes	
☐ Remove all buffer	
☐ Wash 1: PBS-T **750 µl**, **1 min**	
☐ Wash 2: PBS-T **750 µl**, **1 min**	
☐ Wash 3: PBS-T **750 µl**	
☐ Prepare thermal cycler programs and start hold	
☐ Prepare Amplification Enhancer Mix	
☐ Remove **750 µl** PBS-T to complete Wash 3	
☐ Add: Amplification Enhancer Mix **750 µl** (apply lid, place adaptors with	

Day 2		
	cassettes onto thermal cyclers)	
☐	Skip Pre-equilibrate step in thermal cycler programs to start Amplification Enhancement	
☐	Retrieve cassettes	
☐	Remove all buffer	
☐	Add: Amplification Enhancer Mix **750 µl**, **1 min**	
☐	Add: Atera Amp Mix 1 **500 µl**, **1 min**	
☐	Add: Atera Amp Mix 2 **500 µl**	
☐	Prepare thermal cycler programs and start hold	
☐	Apply lid, place adaptors with cassettes onto thermal cyclers	
☐	Skip Pre-equilibrate step in thermal cycler programs to start Amplification	
☐	Retrieve cassettes	
☐	Remove all buffer	
☐	Wash 1: TE Buffer, pH 8.0 **750 µl**, **1 min**	
☐	Wash 2: TE Buffer, pH 8.0 **750 µl**, **1 min**	
☐	Wash 3: TE Buffer, pH 8.0 **750 µl**, **1 min**	
☐	Wash 1: PBS-T **750 µl**, **1 min**	
☐	Wash 2: PBS-T **750 µl**, **1 min**	
☐	Wash 3: PBS-T **750 µl**	
☐	Prep Atera Signal Booster; Centrifuge briefly then add: 1X PBS **1,100 µl** to tube	
☐	Add: Atera Signal Booster **500 µl**	
☐	Apply lids and incubate **20 min**.	
☐	Remove all Atera Signal Booster	
☐	Wash 1: PBS-T **750 µl**, **1 min**	
☐	Wash 2: PBS-T **750 µl**, **1 min**	
☐	Wash 3: PBS-T **750 µl**	
☐	Proceed to either Optional - Cell Segmentation Staining on page 54 or Ethanol Drying on page 64.	
5.0 Optional - Cell Segmentation Staining on page 54		**Notes**
☐	Thaw or obtain Cell Segmentation reagents (see Get Started on page 55). If loading Atera Instrument the next day, start thawing relevant plates and Pouch 4.	
☐	Prepare buffers (see Buffer Preparation on page 56)	
☐	Retrieve cassettes	
☐	Remove all buffer	
☐	Wash 1: 70% Ethanol **750 µl**, **2 min**	
☐	Wash 2: 100% Ethanol **750 µl**, **2 min**	

Day 2	
☐	Wash 3: 100% Ethanol **750 µl**, **2 min**
☐	Wash 4: 70% Ethanol **750 µl**, **2 min**
☐	Add: PBS-T **750 µl**
☐	Remove all buffer
☐	Add: 1X Diluted Atera Block and Stain Buffer **750 µl**, **1 h**
☐	Prep Atera Multi-Tissue Stain; Add: 1X Diluted Atera Block and Stain Buffer **250 µl** to tube
☐	Pipette mix Atera Multi-Tissue Stain **30 min**
☐	Centrifuge Atera Multi-Tissue Stain for **10 min** at **14,000 rcf** at **4°C**
☐	Retrieve Atera Cassette Inserts and remove from packaging
☐	Remove **450 µl** buffer, leave **300 µl** in each cassette
☐	Apply Atera Cassette Insert and adjust thermocycler adaptors to angled position
☐	Remove all buffer from extraction port
☐	Add: Atera Multi-Tissue Stain **170 µl** to injection port
☐	Remove all buffer from extraction port and adjust thermocycler adaptors to flat position
☐	Add: Atera Multi-Tissue Stain **60 µl** to injection port
☐	Apply lids
☐	Incubate **overnight (16–24 h)** at **4°C**.
☐	Retrieve cassettes
☐	Wash 1: PBS-T **180 µl**, **1 min**
☐	Wash 2: PBS-T **180 µl**, **1 min**
☐	Wash 3: PBS-T **180 µl**, **1 min**
☐	Prep Atera Signal Booster; Centrifuge briefly then add: 1X PBS **1,100 µl** to tube
☐	Add: Atera Signal Booster **180 µl** to injection port
☐	Remove all buffer and adjust Thermocycler Adaptors to flat position
☐	Add: Atera Signal Booster **50 µl** to injection port
☐	Apply lids and incubate **20 min**
☐	Adjust Thermocycler Adaptors to angled position and remove lids
☐	Wash 1: PBS-T **180 µl**, **1 min**
☐	Wash 2: PBS-T **180 µl**, **1 min**
☐	Wash 3: PBS-T **180 µl**, **1 min**
☐	Adjust Thermocycler Adaptors to flat position
☐	Add: PBS-T **200 µl** to injection port
☐	Remove Atera Cassette Inserts

Day 2		
6.0 Ethanol Drying on page 64		**Notes**
☐	Obtain Ethanol Drying reagents (see Get Started on page 65)	
☐	Prepare thermal cycler programs and start hold	
☐	Prepare 70% Ethanol	
☐	Retrieve cassettes	
☐	Remove all buffer	
☐	Add: PBS-T **750 µl**, **1 min**	
☐	Remove all buffer	
☐	Add: 70% Ethanol **750 µl**, **1 min**	
☐	Wash 1: 100% Ethanol **750 µl**, **1 min**	
☐	Wash 2: 100% Ethanol **750 µl**, **1 min** (apply lid, place adaptors with cassettes onto thermal cyclers)	
☐	Skip Pre-equilibrate step in thermal cycler programs to start Ethanol Drying	
☐	Add: PBS-T **1.5 ml**, **2 min**	
☐	If proceeding: place lids on cassettes and proceed to Instrument Loading Preparation on page 68.	
☐	If not proceeding: place lids on cassettes and store at **4°C** in the dark for up to **one week**.	

Additional Notes