csgenetics

SimpleCell 3’ Gene Expression Assay

Reference Guide

For Research Use Only

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Introduction

CS Genetics' highly parallel *single-cell sequencing* technology enables profiling thousands of single cells by utilizing the principle of *kinetic confinement*. It allows a rapid, solution-phase indexing reaction to tag the RNA from a large number of single cells simultaneously. The reaction is engineered to take place in a single tube that can be easily adopted. The workflow is compatible with Illumina® short-read sequencing technology and delivers a scalable instrument-free platform that produces libraries of high complexity, outstanding sensitivity, and specificity of transcript quantification. This document outlines the protocol for generating gene expression libraries from single cells.

Kit Components

Box	Component
Box 1 (**4°C**)	CPair (Cell Pairing Beads) DB (Dilution Buffer) WB (Wash Buffer B)* EA (Enrichment Mix)
Box 2 (**-20°C**)	CPM (Cell Pairing Mix - vacuum sealed bag under tube insert) KCB (Kinetic Confinement Buffer) WA (Wash Buffer A)* R1A (RT Mix 1A) R1B (RT Mix 1B) R1C (RT Mix 1C) R2 (RT Mix 2) R3 (RT Mix 3) H (RNase H) S1A (3S Mix 1A) S1B (3S Mix 1B) S2 (3S Mix 2) K (Proteinase K) A1 (Amplification Mix 1) A2 (Amplification PCR 1 Primers)
Accessory Starter kit (**-20°C**)	• I1-I8** UDI pairs (Illumina Adapters)
Accessory Starter kit (**Room Temp**)	• V-bottom Plate (Thermo Fisher, 277143) • Falcon Flat-bottom Plate (Falcon, 351172)

* x2 for 16-sample kits

** 1-16 for 16-sample kits

User Supplied Equipment, Reagents and Consumables

Equipment	Supplier	Lab Designation
Centrifuge with swinging bucket plate attachment		Pre PCR
Thermocycler	Recommended: MiniAMP Thermo Fisher cat no. A37834	Pre and Post PCR
Vortex Mixer		Pre and Post PCR
Minifuge with both 8-strip and 1.5 mL tube adapter		Pre and Post PCR
96-sample plate side magnet	Thermo Fisher cat no. 12331D or 12027	Pre and Post PCR
8-sample 1.5 mL tube magnet	Thermo Fisher cat no. 12321D	Pre PCR
Single-channel pipettes (p10, p20, p200 and p1000)		Pre and Post PCR
Multi-channel pipettes (for volumes 1–200 µL)		Pre and Post PCR

Chemicals/Reagents	Supplier	Lab Designation
Ethanol, Absolute Extra Pure (EtOH)	Sigma Aldrich, cat no. 1009861000	Pre and Post PCR
RNaseZap	Ambion, cat no. AM9780	Pre PCR
Nuclease-free water (nf H_2O) – not DEPC-Treated	Ambion, cat no. AM9937	Pre and Post PCR
DPBS, no calcium, no magnesium	Thermo Fisher, cat no. 14190144	Pre and Post PCR
Bovine Serum Albumin, 7.5%	Thermo Fisher, cat no. 15260037	Pre PCR
1M DTT, Molecular Biology Grade	Thermo Fisher, cat no. R0861	Pre and Post PCR
NGS Clean-up Beads	Recommendations	Pre and Post PCR
Unique Dual Indexes (UDIs)	Supplied with CS Genetics Accessory Starter kit, cat no. 1000396, 1000406 or self-supplied (UDI adaptors design guide)	Post PCR

Plastics/Consumables	Supplier	Lab Designation
Pipette tips, filtered (p10, p20, p200 and p1000)		Pre and Post PCR

Plastics/Consumables	Supplier	Lab Designation
DNA LoBind 1.5 mL microcentrifuge tubes, PCR clean	Eppendorf, cat no. 022431021	Pre and Post PCR
DNA LoBind 2.0 mL microcentrifuge tubes, PCR clean	Eppendorf, cat no. 022431048	Pre and Post PCR
8-strip PCR tubes, 0.2 mL with caps		Pre and Post PCR
50 mL conical tubes	Fisher Scientific, cat no. 14-432-22	Pre and Post PCR
PCR tube and microtube racks		Pre and Post PCR
Crushed Ice		Pre and Post PCR
Dry Ice (pellets or smaller)		Pre PCR
V-bottom Plate	Supplied with CS Genetics Accessory Starter kit, cat no. 1000396, 1000406, **or** Fisher Scientific, cat no. 277143	Pre PCR
Flat-bottom Plate	Supplied with CS Genetics Accessory Starter kit, cat no. 1000396, 1000406, **or** Falcon, cat no. 351172	Pre PCR

Instruments and Reagents set-up

- RNA-related work should be carried out in a specially designated cleanroom or in a laminar flow hood equipped with UV light for sterilization. Irradiate a PCR hood with UV for **15–20** min before experiment.
- All work surfaces, equipment, and plasticware should be decontaminated with DNA Away and RNAZap before and after the experiment.
- Thaw and thoroughly mix reagents before use.
- Keep the cell/reagent settling reactions covered to avoid evaporation and contamination.
- Use reverse pipetting technique or ergonomic positive displacement pipette when working with viscous reagents.
- Prepare **80% EtOH** freshly with **ddH_2O**.

Cell handling

- A single-cell contains a very low amount of RNA. Therefore, take extra effort to avoid sample degradation and contamination.
- Keep the cells in an RNase free environment chilled on ice or any other available cooling system during the preparation and reaction set up.
- We recommend filtering cells before cell priming as cells may tend to clump. This will greatly reduce the number of doublets seen in the assay.
- High cell viability is imperative for optimal performance within the assay. We recommend no less than 90% viable cell samples be processed.
- If sorting with MACS, use negative sorting. Positive sorting will interfere with the assay.

Library Overview

CS Genetics SimpleCell™ 3’ Gene Expression libraries are optimized for Illumina sequencers, with set up as follows:

- Read 1: 92 Cycles
- Index 1: 10 Cycles
- Index 2: 10 Cycles
- Read 2: 26 Cycles

Libraries can also be run on other manufacturer sequencers per their recommendations. Schematic of the libraries below:

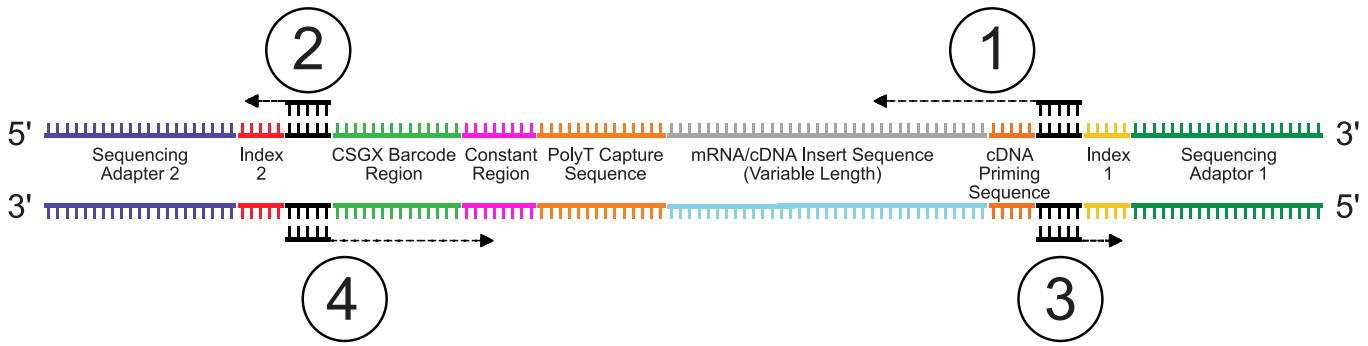


Figure 1: CS Genetics Library Structure (Numbers based on read order on a NextSeq 2000)

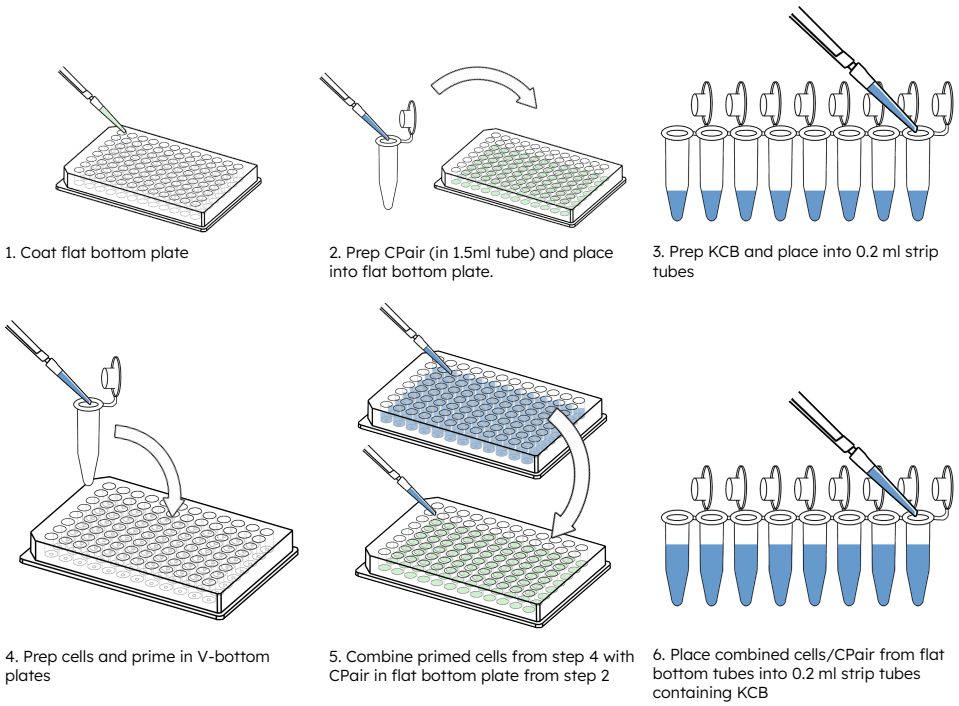


Figure 2: Cell Pairing & Kinetic Confinement Workflow

Protocol Steps and Timing

Pre-PCR:

Step #	Workflow Stage	Duration
1	Cell Priming and Pairing	**1 h 45 min**
2	Kinetic Confinement	**45 min**
3	Barcoding and Capture of Target RNA	**45 min**
4	Post-RNA Capture/Barcoding Bead Clean-up	**10 min**
5	Reverse Transcription (RT)	**45 min**
6	RNase H Treatment (RNAH)	**30 min**
7	Second Strand Synthesis (3S)	**30 min**
8	Proteinase K Treatment (PK)	**30 min**
9	Post Second Strand Synthesis Clean-up	**25 min**

Post-PCR:

Step #	Workflow Stage	Duration
10	Amplification PCR (PCR1)	**30 min**
11	Enrichment Bead Preparation	**10 min**
12	Post Amp PCR Size Selection and Library Enrichment	**1 h**
13	Indexing PCR (PCR2)	**20 min**
14	Post Indexing PCR Clean-up	**25 min**

Thermocycler Programming

Before starting the protocol, program the thermocycler with the following 7 programs:

Target RNA barcoding program (TRB)*

Volume	Time	Temperature
100 µL	**≈ 6-10 min**	Lid temperature at **105 °C** • **4°C** Hold • **95°C - 15 sec** • **55°C** to **40°C** - **90 sec** decreasing (**1°C/6 sec**) • **35°C** to **20°C** - for **135 sec** decreasing (**1°C/9 sec**) • **20°C** Hold

***Specific Thermal Cycler Ramping Speeds** (Link)

Reverse Transcription program (RT)

Volume	Time	Temperature
20 µL	**≈ 40 min**	Lid temperature at **105 °C** • **4°C hold** • **20°C – 2 min** • **30°C – 2 min** • **40°C – 2 min** • **50°C – 15 min** • **55°C – 15 min** • **98°C - 3 min** • **4°C** hold

RNase Treatment program (RNAH)

Volume	Time	Temperature
22 µL	**≈ 25 min**	Lid temperature at **105 °C** • **4°C** hold • **37°C - 20 min** • **65°C - 5 min** • **4°C** hold

Second Strand Synthesis program (3S)

Volume	Time	Temperature
32 µL	**≈ 25 min**	Lid temperature at **105 °C** • **4°C** hold • **75°C to 20°C – 22 min** decreasing (**2.5°C/1 min**) • **4°C** hold

Proteinase K program (PK)

Volume	Time	Temperature
33 µL	**≈ 25 min**	Lid temperature at **105 °C** • **4°C** hold • **37°C - 15 min** • **55°C - 10 min** • **4°C** hold

PCR Amplification program (PCR1)

Volume	Time	Temperature

40 µL	**≈ 25-30 min**	Lid temperature at **105°C**
		• **4°C** hold
		• **98°C - 45 sec**
		• 12 Cycles for Cell Lines or 13 Cycles for PBMCs
		  • **98°C - 15 sec**
		  • **60°C - 30 sec**
		  • **72°C - 30 sec**
		• **72°C - 1 min**
		• **4°C** hold

Indexing PCR program (PCR2)

Volume	Time	Temperature

40 µL	**≈ 15-20 min**	Lid temperature at **105°C**
		• **4°C** hold
		• **98°C - 45 sec**
		• 6 Cycles for Cell Lines or 7 Cycles for PBMCs
		  • **98°C - 15 sec**
		  • **60°C - 30 sec**
		  • **72°C - 30 sec**
		• **72°C - 1 min**
		• **4°C** hold

Step 1. Cell Priming and Pairing

Item	Supplier	Kit Storage Condition	Instructions
Prepare **3.5 mL** (8 sample kit) or **7 mL** (16 sample kit) of **0.1% BSA** in **dPBS**	User		47 µL 7.5% BSA + 3,463 µL dPBS (8 Samples) Place on ice
Prepare **4 mL** (8 sample kit) or **8 mL** (16 sample kit) **3.75% BSA in dPBS**	User		2 mL 7.5% BSA + 2 mL dPBS (8 Samples) Place on ice
Prepare **1 mL** (8 sample kit) or **1 mL** (16 sample kit) **0.04% BSA** in **dPBS**	User		5 µL 7.5% BSA + 995 µL dPBS (8 Samples) Place on ice
20 mL dPBS	User		Place on ice
1 M DTT	User		**1 mL nf H_2O + 0.154 g DTT** powder. Fully reconstitute. Keep on ice.
CPair	CS Genetics	**4°C**	Remove from storage and place on ice
KCB	CS Genetics	**-20°C**	Remove from storage and place on ice
CPM (Remove insert inside the box to access the **CPM** tube)	CS Genetics	**-20°C**	Remove from storage **immediately before use** and place on ice
V-bottom Plate	CS Genetics	**Room Temp**	Place on ice
Falcon Flat-bottom Plate	CS Genetics	**Room Temp**	Place on ice
Centrifuge with swinging bucket plate attachment	User		Pre-chill to **4°C**

1 A. Coating of Flat-bottom Plates

1. Add **120 µL** of cold **dPBS + 0.1% BSA** to the required number of Flat-bottom wells (1 well per sample). Check that there are no bubbles at the bottom of the wells.
2. Incubate for at least **1 min** at **4°C** or on ice.
3. Set pipetter to **120 µL**, remove the **dPBS + 0.1% BSA** buffer from the Flat-bottom wells and discard.
4. Set pipetter to **10 µL**, remove the remaining **dPBS + 0.1% BSA** buffer from the bottom of the wells, as excess will interfere with the assay.
5. Store plates at **4°C** or on ice until use.

1 B. CPair Preparation

Note	Do not vortex or spin down **CPair** reagent. Tap the tube on the lab bench to get the liquid to the bottom of the tube. During the mixing you can use the solution to wash droplets from the side of the tube into the solution.

1. Set pipetter to **150 µL** and pipette **CPair** up and down **10x** to resuspend the solution without introducing bubbles. When solution is homogenous, proceed immediately.

Note	Avoid warming tube with your hands while performing this step. Do not let the solution sit for more than **30 sec** before performing next step.

2. Based on kit size, transfer the required volume of **CPair** into a **1.5 mL** microcentrifuge tube.

   – For 8 Sample kit, transfer **170 µL**
   – For 16 Sample kit, transfer **340 µL**

3. Place tube onto **1.5 mL** tube magnet at **room temperature** and incubate for **1 min.**

Note	Pellet is small and will slide down tube while liquid is being removed, so take care and avoid removing any beads. If pellet is disturbed, allow pellet to reattract to the magnet.

4. Based on kit size, slowly remove the majority of the supernatant, taking care not to disturb the pellet of beads attracted to the magnet.

   – For 8 Sample kit, remove **150 µL**
   – For 16 Sample kit, remove **300 µL**

5. Wash the **CPair** as follows:

   a. Remove the tube from the magnet and resuspend the pellet by adding **300 µL** of cold **dPBS**, pipetting up and down **5x**.
   – Place tube back on magnet and allow it to pellet for **1 min**.
   b. Remove and discard **300 µL** of the supernatant, being sure not to disturb the pellet.

c. Remove tube from magnet and resuspend the pellet by adding the following volumes of cold **dPBS** into the tube and pipetting up and down **10x**.
   - For 8 Sample kit, add **150 µL of cold dPBS**.
   - For 16 Sample kit, add **300 µL of cold dPBS**.

d. Resuspend the pellet by pipetting up and down **10x**.

Note	Unused washed **CPair** can be stored at **4°C** and used for **1 month** after washing. For use after one month, follow this link.

6. Using the washed **CPair** beads, briefly mix by pipetting up and down **10x** and place the following volumes into each of the pre-coated **0.2 mL** Flat-bottom wells
   - For PBMCs/Organoids/Primary Tissues: **20 µL of washed CPair beads**
   - For Cell Lines: **15 µL of washed CPair beads + 5 µL of cold dPBS**

Note	Make sure to mix the washed CPair beads before placing them into the Flat-bottom wells, as additional time will cause beads to settle and introduce variability in the results.

7. Cover tubes with the lid back on and set aside at **4°C** or on ice. **Take care not to freeze.**

1 C. Prepare Kinetic Confinement Master Mix (KCB MM)

1. Quick spin **KCB** and mix thoroughly using a pipette set to **600 µL (8 sample kit) or 900 µL (16 sample kit)** until the solution is completely homogenous.

Note	**KCB** is extremely viscous. Take care not to introduce bubbles by pipetting very slowly.

2. Prepare **KCB Master Mix** (**KCB MM**) by using appropriate volumes from the table below and then store on ice until use.

Note	Unused **KCB** and **DTT** can be stored at **-20°C** respectively for future reactions.

	1 Sample	1 Sample + 25% excess	8 Samples + 25% excess	16 Samples + 25% excess
KCB	**73.6 µL**	**92 µL**	**736 µL**	**1472 µL**
1M DTT	**6.4 µL**	**8 µL**	**64 µL**	**128 µL**

3. Thoroughly mix the **KCB MM** by pipette mixing **slowly**.

Note	The **KCB MM** is a viscous solution so slow pipetting is required as to not introduce bubbles. Ensure homogenous solution (no refraction should be visible) before continuing with the protocol.

4. Using reverse pipetting, aliquot **80 µL** of **KCB MM** per tube for each sample into a set of strip tubes and place directly on ice.

1 D. Cell Priming

Note	Cells should be counted after filtering and viability should be >90%. Refer to this link for guidance on cell prep.

1. Adjust cell volume to **20 µL** in **cold dPBS + 0.04% BSA** at the following concentration:
   - **500 cells/µL (10,000 cells total) for Organoids or Primary tissues**
   - **425 cells/µL (8,500 cells total) for PBMCs**
   - **250 cells/µL (5,000 cells total) for Cell lines**
2. Remove **CPM** from **-20°C** and place on ice.
3. Gently mix cells, ensuring they are homogeneous, aliquot **20 µL** of sample **(5000 cells for cell lines, 8500 cells for PBMCs or 10000 for Organoid/Primary Cells)** into the wells of the V-bottom plate.
4. Quick spin **CPM** reagent tube using a Minifuge to be sure all powder is at the bottom of the tube.
5. Resuspend **CPM** in **200 µL** of **ice cold dPBS** by vortexing for **30 s**. Be sure all powder is fully in solution and there is no particulate visible. Quick spin **CPM** tube using a Minifuge until all liquid is at the bottom of the tube. This would be **6X CPM**.

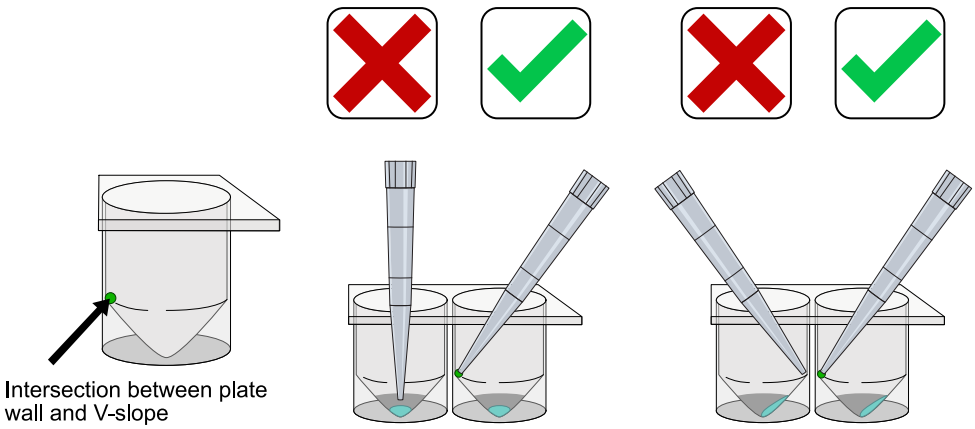
Note	If you don't plan on using the entire kit in one day, aliquot into **30 µL** portions in pre-chilled tubes and place at **-80°C** for later use.

6. Further dilute newly reconstituted **CPM** to achieve the concentration required for your sample type in pre-chilled **1.5 mL** tube.
   - **1X CPM** for cell lines and PBMCs **-** adding **20 µL 6X CPM** to **100 µL** of **ice cold dPBS**
   - **1.5X CPM** for organoids **-** adding **30 µL 6X CPM** to **90 µL** of **ice cold dPBS**
   - **2X CPM** for primary tissues **-** adding **40 µL 6X CPM** to **80 µL** of **ice cold dPBS**

Note	**CPM** in solution is unstable at temperatures above **4°C**. Make sure you resuspend in **cold dPBS** and keep on ice until used. Use aliquot within **15 min** of resuspending.

7. In the wells containing cells within the V-bottom plate, add **6 µL** of **diluted CPM** and mix with pipetter set to **20 µL**, mix by pipetting up and down **5x**.
8. Allow to incubate on ice for **20 min**.
9. Once the incubation is complete, add an additional **175 µL** of ice cold **dPBS + 3.75% BSA** to the **cell/CPM** mix. Set pipetter to **150 µL** and mix up and down **5x**.
10. Pellet cells by centrifuging at **500 x g** for **3 min** at **4°C**. If you are working with fragile cells, centrifuge at the highest possible speed while keeping cells viable.

11. Using a **200 µL** pipetter, remove and discard supernatant while avoiding the cell pellet (see below diagram).



Do not remove supernatant from the bottom of the plate. Supernatant must be removed from the intersection between the plate wall and the V-slope (green dot). This can be achieved by tilting the tip. If cell pellet forms a smear, remove the supernatant from the opposite side of the smear to ensure minimal cell loss.

Figure 3: Removing the Supernatant

12. Resuspend cells in **200 µL** of cold **dPBS + 3.75% BSA** by setting a pipetter to **150 µL** and mixing up and down **5x**.
13. Centrifuge cells immediately at **500 x g** (or previously used speed) for **3 min** at **4°C**.
14. Set pipetter to **200 µL**, remove and discard supernatant while avoiding the cell pellet.
15. Resuspend cells in **200 µL** of cold **dPBS (no BSA)** by setting a pipetter to **150 µL** and mixing up and down **5x**.
16. Centrifuge cells immediately at **500 x g** (or previously used speed) for **3 min** at **4°C**.
17. Using a **200 µL** pipette tip, remove and discard supernatant while avoiding the cell pellet. Using a **20 µL** pipette tip, remove and discard excess supernatant while avoiding the cell pellet.

Note	There should be minimal supernatant remaining in the plate without disturbing the pellet. Excessive supernatant will interfere with CPair/Cell binding.

18. Resuspend cells in **20 µL** of cold **dPBS** and mix up and down **10x**. Place plate on ice.
19. Transfer the total volume of suspended cells (approximately **20 µL–30 µL**) into the pre-coated Flat-bottom plates containing **CPair**. Immediately after the transfer, with a **200 µL** pipetter set to **20 µL**, pipette up and down **20x** to mix the cells and **CPair**.

Note	Be sure to fully resuspend the beads to allow for proper mixing. We recommend tilting the plate while aspirating from the corner and sides. Dispense from the top of the well to resuspend any beads from the surface.

20. Cover the wells to avoid evaporation and place the plate **flat** on ice for **40 min**.

Note	Plate needs to be kept in a vibration-free environment so that **CPair/Cells** can settle to the bottom of the well.

Step 2. Kinetic Confinement

Note	If processing >8 samples at a time (>1 strip tube), proceed through next four steps one strip tube at a time.

1. When incubation is complete, with a pipette set to **20 µL**, mix **20x <u>slowly</u>** using the technique described below. **<u>Proceed immediately to the next step</u>**.

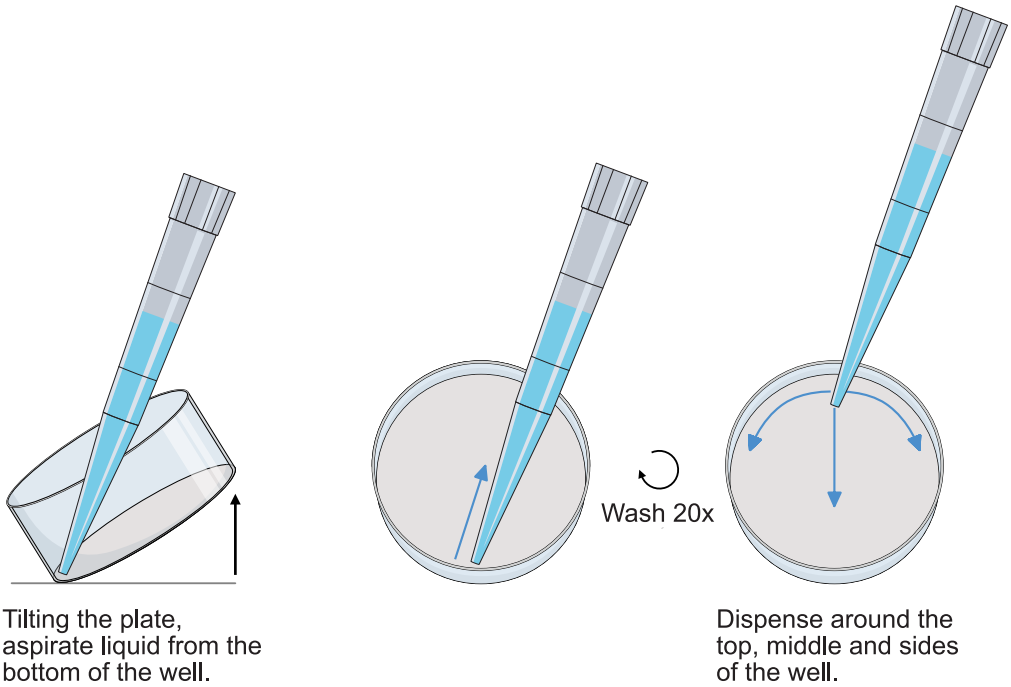


Figure 4: Disrupting cell-bead mix

2. Add the full volume **(40-50 µL)** of **Cells-CPair** to the pre-aliquoted **KCB MM**.
3. Set pipetter to **80 µL**. **<u>Slowly</u>** mix by pipetting up and down **15x**, using the following diagram as a guide, aspirate from the bottom of the liquid and dispense with the tips raised out of the liquid.

Note	Solution needs to be homogeneous before proceeding to next step, which can be determined when refraction can no longer be observed in the tube. Avoid introducing bubbles.

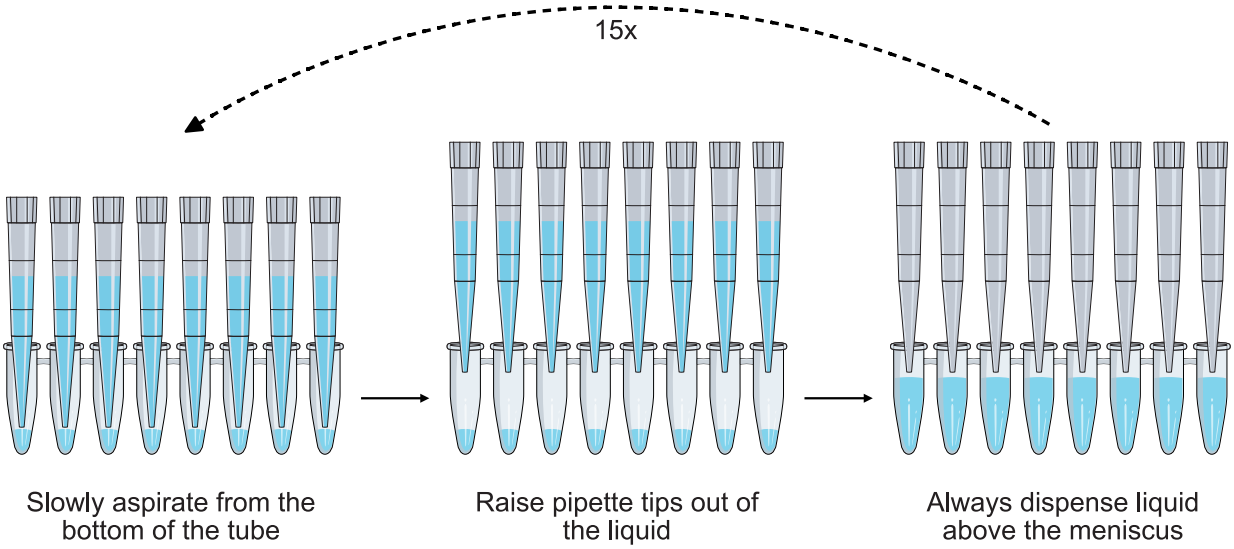


Figure 5: Mixing KCB 15x.

4. Seal tubes with lids and **immediately fully submerge** in dry ice for a minimum of **40 min**.

Safe Stopping Point	The samples can stay on dry ice for up to **24 h**. For long-term storage (up to **4 weeks**), transfer tubes to **-80°C** freezer after a minimum of **40 min** in dry ice. Do not allow samples to thaw during transfer.

Step 3: Barcoding and Capture of Target RNA

Item	Supplier	Kit Storage Condition	Instructions
1 M DTT	User		**1 mL nf H_2O + 0.154 g DTT** powder. Fully reconstitute. Keep on ice.
DB	CS Genetics	**4°C**	Place on ice
R1A, R1B, R1C, **R2**, and **R3**	CS Genetics	**-20°C**	Place on ice
WA	CS Genetics	**-20°C**	Place at **room temperature**
WB	CS Genetics	**4°C**	Place at **room temperature**

1. Start the Target RNA barcoding program (TRB) on the thermocycler.
2. Prepare **DB**, **WA** (addition of **DTT**), and **RT MM** solutions before processing your samples.

Note	Use freshly made **1 M DTT** solution or the frozen **1 M DTT** solution from Step 1.

3. Quick spin **DB** reagent and mix thoroughly with a pipette **10x** so that the beads are homogenous. Do not vortex.
4. Prepare the **DB Master Mix** as described below. Adjust volumes based on number of samples being processed. Store on ice until use. Place unused reagents in noted storage conditions.

	1 Sample	1 Sample + 25% excess	8 Samples + 25% excess	16 Samples + 25% excess
DB Reagent	**75.6 µL**	**94.5 µL**	**756 µL**	**1512 µL**
1 M DTT	**6.4 µL**	**8 µL**	**64 µL**	**128 µL**

5. Ensure precipitate has dissolved by vortexing the **WA** reagent. Quick spin and prepare the **WA Master Mix** as described below. Adjust volumes based on number of samples being processed. Store at **room temperature** until use. Place unused reagents in noted storage conditions.

	1 Sample	1 Sample + 25% excess	8 Samples + 25% excess	16 Samples + 25% excess
WA Reagent	**153.6 µL**	**192 µL**	**1536 µL**	**3072 µL**
1 M DTT	**6.4 µL**	**8 µL**	**64 µL**	**128 µL**

6. Mix both tubes by pipetting up and down **10x**.
7. Prepare **166 µM** DTT as follows:

   – Add **2 µL** of **1 M DTT** to **998 µL** of **nf H_2O** to make **2 mM DTT.** Mix well.
   – Add **83 µL** of the **2 mM DTT** solution to **917 µL** of **nf H_2O** for a final concentration of **166 µM**. Mix well.

8. Quick spin **R1A, R1B, R1C**, **R2**, and **R3** reagents. Prepare the **RT Master Mix** as described below. Adjust volumes based on number of samples being processed. Store on ice until use. Place unused reagents in noted storage conditions.

Note	Vortex **R1A, R1B** and **R1C** reagents before use to ensure that they are well mixed. Do not vortex **R2** and **R3** reagents.

	1 Sample	1 Sample + 10% excess	8 Samples + 10% excess	16 Samples + 10% excess
R1A Reagent	**4 µL**	**4.4 µL**	**35.2 µL**	**70.4µL**
R1B Reagent	**2 µL**	**2.2 µL**	**17.6 µL**	**35.2 µL**
R1C Reagent	**10 µL**	**11 µL**	**88 µL**	**176 µL**
R2 Reagent	**0.75 µL**	**0.8 µL**	**6.6 µL**	**13.2 µL**
R3 Reagent	**0.25 µL**	**0.3 µL**	**2.2 µL**	**4.4 µL**
166 µM DTT	**3 µL**	**3.3 µL**	**26.4 µL**	**52.8 µL**
Total volume	**20 µL**	**22 µL**	**176 µL**	**352 µL**

9. Mix **RT MM** by pipetting up and down **10x**.
10. Transfer the frozen **Cells-CPair/KCB MM** directly from dry ice to the thermocycler and run the pre-set **Target RNA barcoding program (TRB)** – reaction volume = **100 µL**, approximate run time **≈ 6–10 min**.
11. When program is finished, remove tubes from thermocycler and proceed immediately to the next step. Keep samples at **room temperature** for the following steps.
12. Mix prepared **DB Master Mix** by pipetting **10x** to fully resuspend beads.
13. Add **82 µL** of **DB Master Mix** to each sample in 8-strip tube.

14. Set pipetter to **120 µL** and mix the samples up and down **10x**. Always aspirate from the bottom of the liquid and dispense with the tips raised out of the liquid. Be sure solution looks homogeneous before proceeding to the next step.

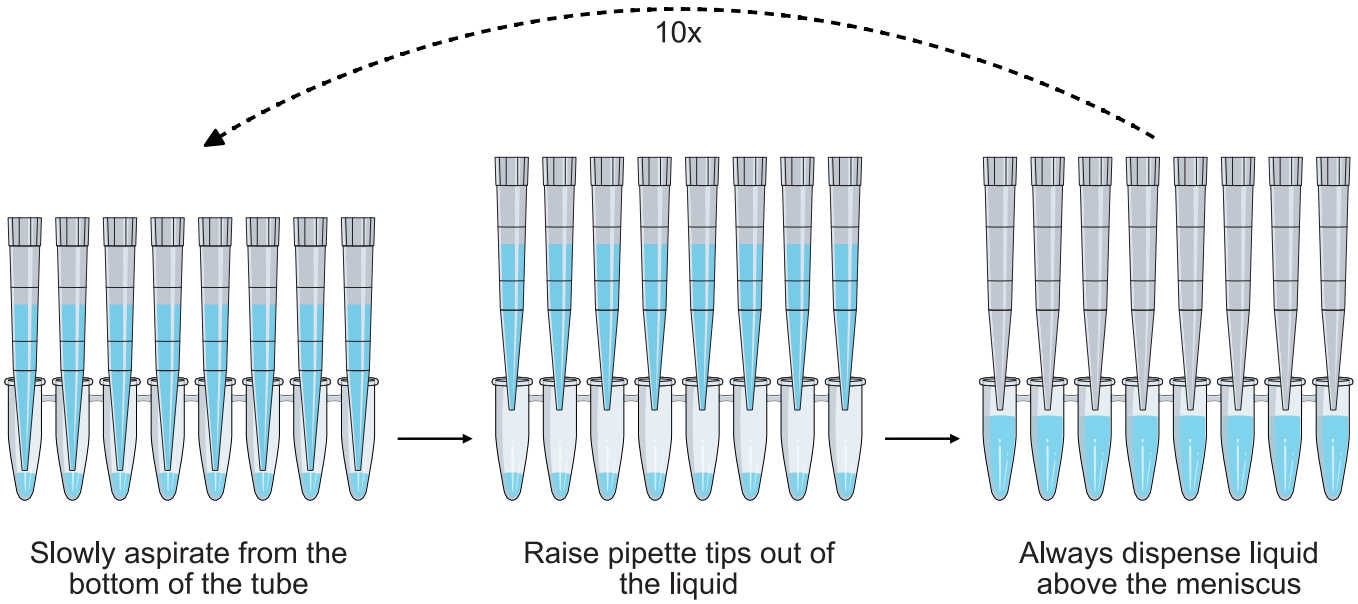


Figure 6: Mixing KCB and DB 10x.

15. Place tubes on magnet for **5 min** until the solution is visually clear and the beads have pulled to the magnet. During this time, start the **RT** program (Reverse Transcription) on the thermocycler.

Step 4. Post-RNA Capture/Barcoding Bead Clean-up

1. While keeping the tubes on the magnet, set the pipetter to **180 µL** and carefully remove the supernatant without disturbing or touching the beads and discard the supernatant. Using a **20 µL** multi-channel pipette set to **20 µL**, remove the remaining supernatant.
2. Remove the tubes from the magnet, add **160 µL** of **WA** to the tube. Set the pipetter to **120 µL**, mix up and down **10x** to resuspend beads without introducing bubbles.

Note	Some clumping might be noticed in this step. Be sure to completely resuspend beads before proceeding.

3. Put the tubes onto a magnet for **2 min** or until the solution is visually clear.
4. Remove **160 µL** of supernatant and discard. Using a **10 µL** or **20 µL** pipette set to **10 µL**, carefully remove the remaining supernatant without touching the beads.
5. Keeping the tubes on the magnet, add **180 µL** of **WB**.
6. Incubate for **30 sec** then remove **180 µL** of the supernatant. Using a **10 µL** or **20 µL** pipette set to **10 µL**, remove the remaining supernatant without disturbing the beads.
7. Repeat steps 5 and 6, for a total of 2 washes with **WB**.

Note	Do not let the beads dry out. Proceed immediately to next step.

Step 5. Reverse Transcription (RT)

1. Remove tube from magnet and place on ice or cold block. Add **20 µL** of **RT MM** into each tube with washed beads. Set pipetter to **15 µL** and thoroughly resuspend beads by pipetting up and down **15x**, being careful not to introduce bubbles.

Note	Some bead clumping may be present at this step, even after resuspension. Mix another **10-20x** to break up the clumps.

2. Seal the tubes with lids, transfer to the thermocycler and continue the Reverse transcription program (RT). Reaction volume = **20 µL**. Run time **≈ 40 min**.

Step 6. RNaseH Treatment (RNAH)

Item	Supplier	Instructions
Remove **RNaseH (H)** from **-20°C**	CS Genetics	Place on ice

1. Remove samples from thermocycler and place on ice.
2. Start the RNAH program on the thermocycler.
3. Quick spin samples on a benchtop centrifuge.
4. Add **1.5 µL** of **H** to each sample. There is no need to mix your samples.
5. Seal the tubes with lids, transfer to the thermocycler and continue the **RNaseH treatment program (RNAH).** Reaction volume = **22 µL. Run time ≈ 25 min**.

Step 7. Second Strand Synthesis (3S)

Item	Supplier	Kit Storage Condition	Instructions
S1A, S1B, S2	CS Genetics	**-20°C**	Place on ice
DNA Purification beads	User		Place at **Room Temperature**

1. Quick spin **S1A, S1B** and **S2** reagents. Prepare **3S Master Mix (3S MM)** as described below. Adjust amount to be used per number of samples you will be preparing. Store on ice until use. Place unused reagents in noted storage conditions.

Note	Vortex **S1A** and **S1B** reagents to ensure that they are well mixed. **S1A** reagent precipitates during storage so ensure that the precipitate fully dissolves before use. Do not vortex **S2** reagent.

	1 Sample	1 Sample + 10% excess	8 Samples + 10% excess	16 Samples + 10% excess
S1A Reagent	**6.7 µL**	**7.4 µL**	**59 µL**	**118 µL**
S1B Reagent	**2.7 µL**	**3 µL**	**23.8 µL**	**47.5 µL**
S2 Reagent	**0.6 µL**	**0.7 µL**	**5.3 µL**	**10.6 µL**
Total Volume	**10 µL**	**11 µL**	**88 µL**	**176 µL**

2. Mix **3S MM** by pipetting up and down **10x**.
3. Remove samples from thermocycler and place on ice.
4. Start the 3S program on the thermocycler.
5. Quick spin samples on a benchtop centrifuge.
6. On ice or a cold block, add **10 µL** of **3S MM** to the post-RT sample. Set pipetter to **20 µl** and mix up and down **15x**.

Note	When the **3S MM** is added, be sure to disrupt the beads from the bottom of the tube until the solution is homogenous.

7. Seal the tubes with lids, transfer to the thermocycler and run pre-set **3S program**. Reaction volume = **32 µL**. Run time ≈ **22–25 min**.

Step 8. Proteinase K Treatment (PK)

Item	Supplier	Kit Storage Condition	Instructions
Proteinase K (K)	CS Genetics	**-20°C**	Place on ice

1. After the **3S** program has finished, remove samples from thermocycler place on cold block/ice.
2. Start the PK program on the thermocycler.
3. Add **1 µL** of **Proteinase K (K)**.
4. Set pipetter to **20 µL** and mix up and down **10x**.
5. Run the PK program on the thermocycler. Reaction volume = **33 µL**. Run time **≈ 25 min**.

Step 9. Post Second Strand Synthesis Clean-up

Item	Supplier	Kit Storage Condition	Instructions
15 mL (8 samples) or **30 mL** (16 samples) of freshly prepared **80% EtOH**	User		
A1 and **A2**	CS Genetics	**-20°C**	Place on ice

The following steps should be performed at **room temperature**. Ensure that the **DNA Purification beads** are well mixed before use.

1. Add **20 µL** of nf **H_2O** to the sample from the **PK** step**.**
2. Add **75 µL** of **DNA Purification beads** to each sample and mix well by pipetting up and down **10x**.
3. Incubate for **5 min** at **room temperature.**
4. Put the tube on the magnet for **2 min** or until the supernatant is clear.
5. Set pipetter to **105 µL** and carefully remove most of the supernatant and discard.
6. Add **180 µL** of **80% EtOH** to the sample while on the magnet without touching the beads.
7. Wait **1 min** and remove all of the supernatant.
8. Repeat steps 6 and 7 twice more, 3 washes in total.
9. After the final **EtOH** wash, take a smaller pipette set to **10 µL–20 µL** and remove any residual **EtOH**.
10. Dry the pellet until it is matte (not glossy). Approximately **2–5 min**. Take care to not overdry the pellet.
11. Remove the tubes from magnet, add **30 µL** of **nf H_2O** and resuspend the bead pellet by pipetting.
12. Incubate for **5 min** at **room temperature**.

Note	During this incubation, you can prepare the **Amplification PCR MM** as described in next section.

13. Following the incubation, quick spin the samples and place the tube on the magnet for **2 min**.
14. Transfer **28 µL** of supernatant to a new **0.2 mL** tube. Avoid taking the beads up with the sample. The beads can be discarded.

Step 10. Amplification PCR (PCR1)

1. Set the Amplification PCR program (PCR1) on the thermocycler.

Note	If using cells lines, use **12 cycles** of PCR. If using primary cells, use **13 cycles** of PCR.

2. Quick spin **A1** and **A2** reagents. Prepare **Amplification PCR Master Mix (Amplification PCR MM)** as described below. Adjust volumes based on the number of samples being processed. Store on ice until use. Place unused reagents in noted storage conditions.

	1 Sample	1 Sample + 10% excess	8 Samples + 10% excess	16 Samples + 10% excess
A1 Reagent	**10 µL**	**11 µL**	**88 µL**	**176 µL**
A2 Reagent	**2 µL**	**2.2 µL**	**17.6 µL**	**35.2 µL**

Note	Do not discard the **A1** tube, as you will be using it in a later step. Place back at **-20°C**.

3. Mix **Amplification PCR MM** by pipetting up and down **15x** without introducing bubbles.
4. On ice or a cold block, add **12 µL** of Amplification PCR MM to the **28 µL** of eluate from **3S** wash step and mix well by pipetting up and down **5x**.
5. Seal the tubes with lids, transfer to the thermocycler and run pre-set Amplification PCR program (PCR1). Reaction volume = **40 µL. Run time ≈ 25-30 min**

Note	While samples are in the thermocycler for PCR1, prepare Library Enrichment beads in the next step if continuing the same day.

Safe Stopping Point	After the Amplification PCR program, the samples can be kept at **-20°C** for up to 3 days.

Step 11. Enrichment Beads Preparation

Item	Supplier	Kit Storage Condition	Instructions
EA	CS Genetics	**4°C**	Place at **room temperature**

1. Ensure **EA** is fully homogenous by vortexing and mixing up and down **10x** and transfer the appropriate volume of **EA** based on the number of samples to a new **1.5 mL** microcentrifuge tube.

	1 Sample	1 Sample + 10% excess	8 Samples + 10% excess	16 Samples + 10% excess
EA Reagent	**10 µL**	**11 µL**	**88 µL**	**176 µL**
dPBS	**10 µL**	**11 µL**	**88 µL**	**176 µL**

2. Mix by pipetting up and down **10x**.
3. Add **17 µL** of **diluted EA** beads to a new set of strip tubes (**17 µL** per sample) and set aside at **room temperature**.

Step 12. Post Amplification PCR Size Selection and Library Enrichment

The following steps should be performed at **room temperature**. Ensure that the **DNA Purification beads** are well mixed before use.

1. Add **60 µL** of **nf H_2O** to the sample from the Amplication PCR step.
2. Add **50 µL** of **DNA Purification beads** to the sample and mix well by pipetting up and down **10x**.
3. Incubate for **5 min** at **room temperature**.
4. Put the tube on the magnet for **2 min** or until the supernatant is clear.

Important	**DO NOT DISCARD SUPERNATANT ON NEXT STEP**.

5. Set pipetter to **145 µL** and carefully draw out the supernatant and transfer to new PCR tubes
6. Off magnet, add **20 µL** of DNA Purification beads to the **145 µL** SUPERNATANT and mix well by pipetting.
7. Incubate for **5 min** at **room temperature**.
8. Put the tube on the magnet for **2 min** or until the supernatant is clear.
9. Set pipetter to **150 µL** and carefully remove and discard most of the supernatant.
10. Add **180 µL** of **80% EtOH** to the sample while on the magnet without disturbing the beads.
11. Wait for **1 min** and remove all of the supernatant.
12. Repeat steps 10 and 11 twice more, 3 washes in total.
13. Take smaller pipette set to **10 µL–20 µL**, remove the rest of the **EtOH**.
14. Dry the pellet until it is matte (not glossy). Approximately **1-3 min,** take care to not overdry the pellet.
15. Remove the tubes from magnet and add **19 µL** of **nf H_2O** and resuspend the bead pellet.
16. Incubate for **5 min** at **room temperature**.
17. Following the incubation, quick spin the samples and place the tube on the magnet for **2 min**.
18. Transfer **17 µL** of eluate to the tube containing the **17 µL** of diluted **EA** and mix by pipetting up and down **10x**.
19. Incubate for **15 min** at **room temperature**, mixing **10x** every **5 min**.
20. Add **116 µL of dPBS** and mix **10x**
21. Put the tube on the magnet for **2 min** or until the supernatant is clear.
22. Set pipette to **150 µL** and carefully remove and discard all supernatant.
23. Keeping the tube on the magnet, add **150 µL** of **dPBS.**
24. Wait for **1 min** and remove **150 µL of dPBS,** taking care to not remove any beads. Remove and discard all supernatant.
25. Repeat steps 23 & 24 once more, 2 washes in total.
26. Remove the tubes from magnet and add **27 µL** of **nf H_2O** and resuspend the bead pellet containing the Enriched Library.

Step 13. Indexing PCR (PCR2)

Item	Supplier	Kit Storage Condition	Instructions
A1	CS Genetics	**-20°C**	Place on ice
Illumina UDI's (Index 1-16)	CS Genetics	**-20°C**	Place on ice

1. Start the Indexing PCR program on the thermocycler.

Note	If using cells lines, use **6 cycles** of PCR. If using Primary cells, use **7 cycles** of PCR.

2. Quick spin **Illumina UDIs**. Determine which index will be associated with each sample and add **3 µL** of **Illumina Index** to the **27 µL** of Enriched Library Bead mixture and record the ID. Place the samples on ice or a cold block.
3. Quick spin **A1** reagent. Add **10 µL** of **A1** to each Index/Enriched Library tube and set pipette to **30 µL** and mix well up and down **15x**.
4. Cover the tubes with lids, transfer to the thermocycler and run pre-set **Indexing PCR program (PCR2)**. Reaction volume = **40 µL.** Run time **≈ 15-20 min**.

Step 14. Post Indexing PCR Clean-up

The following steps should be performed at **room temperature**. Ensure that the **DNA Purification beads** are well mixed before use.

1. Add **10 µL** of **nf H_2O** to the Post-Indexing PCR reaction.
2. Add **40 µL** of **DNA Purification beads** to the sample and mix well by pipetting.
3. Incubate for **5 min** at **room temperature**.
4. Put the tube on the magnet for **2 min** or until the supernatant is clear.
5. Set pipette to **70 µL** and carefully remove most of the supernatant.
6. Add **100 µL** of **80% EtOH** to the sample while on the magnet without disturbing the beads.
7. Wait for **1 min** and remove all of the supernatant.
8. Repeat steps 6 and 7 twice more, 3 washes in total.
9. Take smaller pipette set to **10 µL–20 µL**, remove the rest of the **EtOH**.
10. Dry the pellet until it is matte (not glossy). Approximately **2–5 min,** take care to not overdry the pellet.
11. Remove the tubes from magnet and add **20 µL** of **nf H_2O** and resuspend the bead pellet.
12. Incubate for **5 min** at **room temperature**.
13. Place the tube on the magnet for **2 min**.
14. Transfer **18 µL** to the new tube. Avoid taking the beads up with the supernatant. The beads can be discarded.
15. Cover the tubes of the ready to sequence libraries and store at **4°C** for **24 hrs** or **-20°C** in the freezer for long-term storage.

Library QC

Run **1 µL** library or pooled libraries on one of the following instruments:

- Agilent Advanced Analytical Fragment Analyzer

  OR

- Agilent 4200 Tapestation with a High Sensitivity DNA kit.

The following figures show typical library size profiles with an average fragment size of **600–800 bp** when analyzed with a size range of **150–1500 bp**.

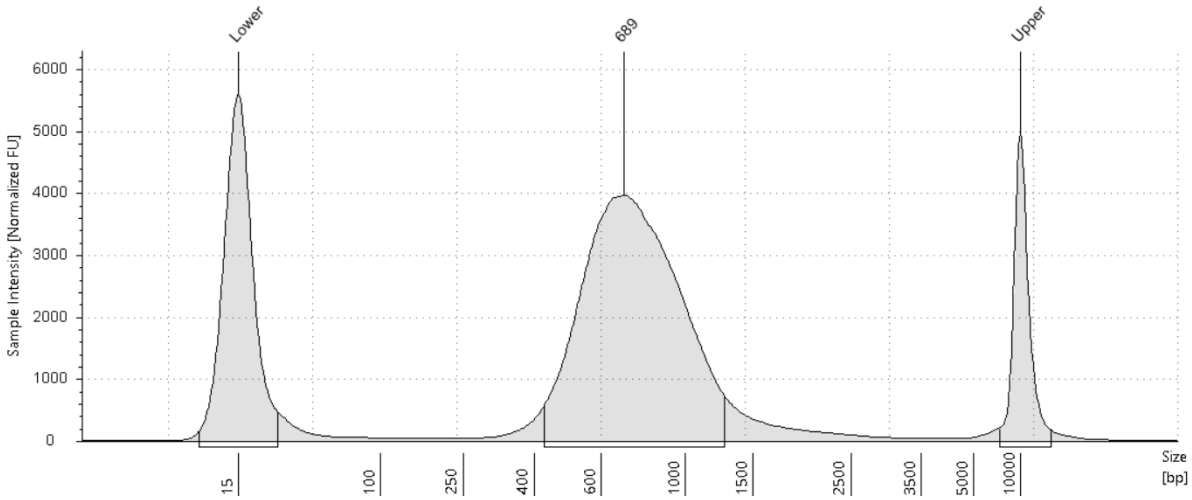


Figure 7: Example library trace with Agilent 4200 Tapestation with a D5000 kit.

Sequencing Recommendation

1. Recommended read depth is 30,000 reads/cell.
2. Recommended procedure for quantification is qPCR with an Illumina Library QC quantification kit.
3. Pool and dilute libraries according to the table below using an appropriate buffer.

Sequencer	Loading Concentration
AVITI FS	**600 pM**
NextSeq 2000	**1.4 nM**
NovaSeq 6000	**300 pM**
NovaSeq X	**150 pM**

4. Add **2% PhiX** as a sequencing control.
5. Sequencing on Illumina sequencers should be run as follows:

   - Read 1: 92 cycles
   - Index 1: 10 cycles
   - Index 2: 10 cycles
   - Read 2: 26 cycles

Informatics output is Count Matrix file and Assay QC file.

Index Pair Name (NextSeq 1000/2000 config)	Index Sequence 1 (i7)	Index Sequence 2 (i5) in Forward Orientation
Illumina UDI 1	GAACTGAGCG	TCGTGGAGCG
Illumina UDI 2	AGGTCAGATA	CTACAAGATA
Illumina UDI 3	CGTCTCATAT	TATAGTAGCT
Illumina UDI 4	ATTCCATAAG	TGCCTGGTGG
Illumina UDI 5	GACGAGATTA	ACATTATCCT
Illumina UDI 6	AACATCGCGC	GTCCACTTGT
Illumina UDI 7	CTAGTGCTCT	TGGAACAGTA
Illumina UDI 8	GATCAAGGCA	CCTTGTTAAT
Illumina UDI 9	GACTGAGTAG	GTTGATAGTG
Illumina UDI 10	AGTCAGACGA	ACCAGCGACA
Illumina UDI 11	CCGTATGTTC	CATACACTGT
Illumina UDI 12	GAGTCATAGG	GTGTGGCGCT
Illumina UDI 13	CTTGCCATTA	ATCACGAAGG
Illumina UDI 14	GAAGCGGCAC	CGGCTCTACT
Illumina UDI 15	TCCATTGCCG	GAATGCACGA
Illumina UDI 16	CGGTTACGGC	AAGACTATAG