Asteria™
Single-cell RNA-seq Benchtop Kit

Catalog no. 001-1000

USER GUIDE

For bench-top preparation of single-cell barcoded cDNA for RNA sequencing

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For Research Use Only. Not for use in clinical diagnostic procedures.

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Revision history:

Version no.	Publication Date	Description of modifications
1.5	2024/03/20	- Section II: Update video links
1.4.1	2023/12/04	- Section I.2: Addition of warning regarding -80°C gelation tube storage - Section II: Addition of warning regarding -80°C gelation tube storage - Section III.4: Addition of warning regarding -80°C gelation tube storage
1.4	2023/09/27	- Section I.4: Addition of steps timing and possible stopping points. - Section III.4: Extended stability of the first stopping point (in lysis buffer) to 35 days. - Section III.9: Stopping point added. - Section VI: Addition of expected DNA profile. - Section VI: Recommendations if multiple preparations from unique cell sample. - Section VII: Sequencing data QC recommendations added. - Minor text and phrasing adjustments.
1.3	2022/10/12	- Update on Cell input number: 5,000 to 15,000 input cells - Section I.2: Update on box 1 composition - Section I.3: Addition of missing references - Section III: Update on Dilution Reagent preparation - Section III.2: Input cell number: Former Annex1 integrated in protocol - Section VI (library preparation): contains additional informations - Section VII (sequencing conditions): contains additional informations
1.0	2022/05/04	First release

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I. PRODUCT INFORMATION

1. Product description

Scipio bioscience Asteria™ Single-Cell RNA-seq kit is a benchtop solution to prepare single cell 3' end cDNA libraries from cell suspensions. It contains the required materials for 4 samples of 5,000-15,000 input cells each.

This Asteria™ single-cell cDNA library must be converted into an Illumina-compatible cDNA library before sequencing.

The sequencing data obtained can be analyzed using Scipio bioscience **Cytonaut™ cloud-based software** (https://www.cytonaut-scipio.bio).

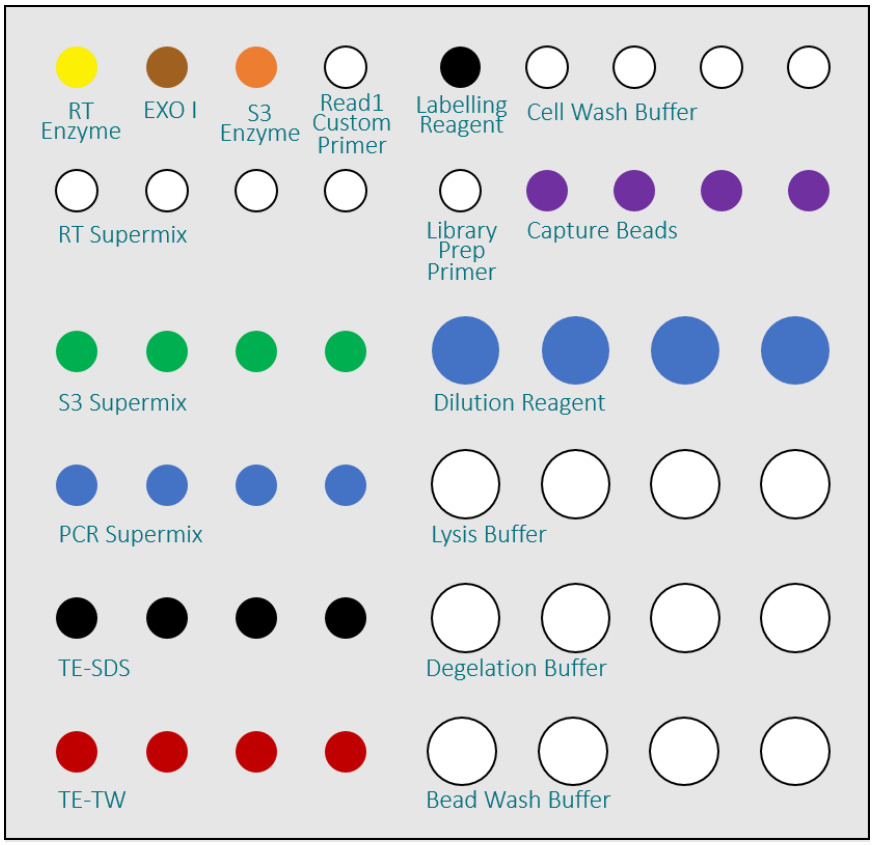
2. Kit content and storage

Box 1/2 - Reagents:

Kit Components	Reference	No. of tubes per kit	Volume per tube	Storage
LABELLING REAGENT	001-1001	1	27µL	-20°C
CELL WASH BUFFER	001-1002	4	900 µL	-20°C
CAPTURE BEADS	001-1003	4	120 µL	-20°C
DILUTION REAGENT	001-1004	4	2.4 mL	-20°C
LYSIS BUFFER	001-1006	4	8.3 mL	-20°C
DEGELATION BUFFER*	001-1007	4	4.7 mL	-20°C
BEAD WASH BUFFER	001-1008	4	7.9 mL	-20°C
RT SUPERMIX	001-1009	4	82.5 µL	-20°C
RT ENZYME	001-1010	1	27 µL	-20°C
EXO I ENZYME	001-1011	1	27 µL	-20°C
S3 SUPERMIX	001-1012	4	85 µL	-20°C
S3 ENZYME	001-1013	1	16 µL	-20°C
TE-SDS	001-1014	4	280 µL	-20°C
TE-TW	001-1015	4	1.38 mL	-20°C
PCR SUPERMIX	001-1016	4	264 µL	-20°C
LIBRARY PREP PRIMER	001-1018	1	77 µL	-20°C
READ1 CUSTOM PRIMER	001-1019	1	77 µL	-20°C

*DEGELATION BUFFER contains a chaotropic agent that is a strong irritant. Wear protective equipment (gloves/protective clothing/eye protection) when handling. See pages 8-9 for Safety Information.

Representation of the Box 1/2 tube rack:



Box 2/2 - Accessories:

Kit Components	Reference	Quantity	Storage
TUBE HOLDER	001-1017	4 units	Room Temp
GELATION KIT*	001-1005	4 units	Room Temp

* For storage at -80°C (for example during a stopping point), never use polystyrene or other tight racks that might damage the structural integrity of the Gelation Tube during the freezing process.

3. Required materials not supplied with this kit

- Items required for **section IV: SAMPLE CLEAN-UP**

Reagents and kits :	• SPRIselect™ reagent kit (B23317, B23318, B23319) *(SPRIselect™ beads amount necessary for 1 sample is approx.. 550 µl)* • Ethanol, absolute (>99%) • Molecular biology grade DNase/RNase-free water
Equipment :	• Magnetic rack for 1.5mL tube DynaMag™-2 Magnet (ThermoFisher, #12321D) • Magnetic rack for PCR tube DynaMag™-PCR Magnet (ThermoFisher, #492025)

- Items required for **section V: QUALITY CONTROL AND QUANTIFICATION**

Reagents and kits :	• Qubit™ **dsDNA HS** assay kit (ThermoFisher, #Q32851, or #Q33230) • High Sensitivity **D5000** ScreenTape (Agilent, 5067-5592) • High Sensitivity **D5000** Reagents (Agilent, 5067-5593)
Equipment :	• Equipment for DNA quantification (Qubit™ fluorometer from Thermo Fisher Scientific, or equivalent) • Equipment for DNA automated electrophoresis (Tapestation™ from Agilent or equivalent)

- Items required for **section VI: LIBRARY PREPARATION**

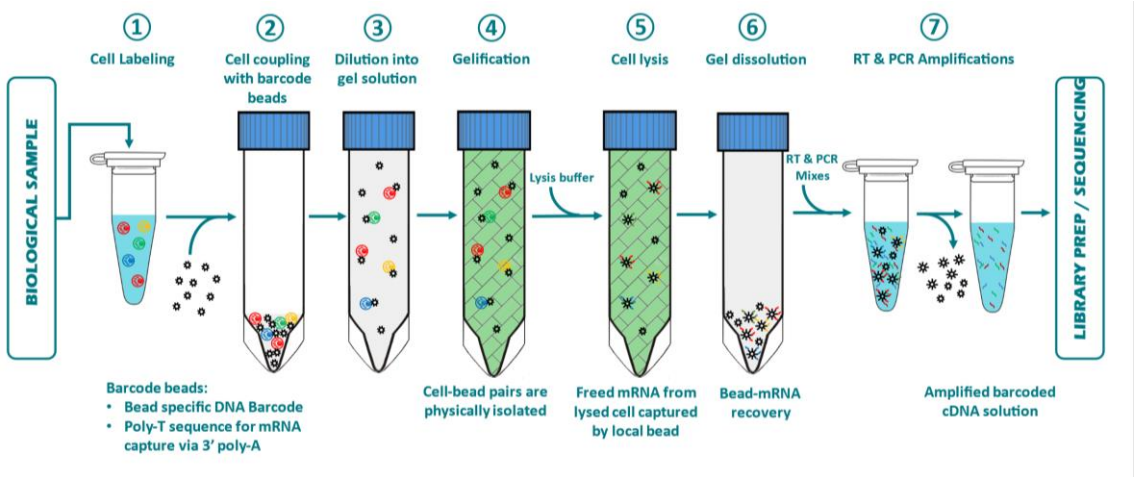
The kit mentioned below has been validated by Scipio bioscience and is strongly recommended (using an alternative kit may affect the library quality).

Reagents and kits :	• NEBNext® Ultra™ II FS DNA Library Prep kit for Illumina® (NEB #E7805S) • NEBNext® Multiplex Oligos for Illumina® (Set 1, NEB #E7335) • High Sensitivity **D1000** ScreenTape (Agilent, 5067-5584) • High Sensitivity **D1000** Reagents (Agilent, 5067-5585)

- In addition to these section-specific requirements, this kit requires the following items:

Consumables and Reagents :	• Sterile serological pipets (5, 10 and 25 mL) • Sterile and nuclease-free micropipette filter tips (low-retention tips are critical for steps 5.8 and 10.3) • DNase/RNase-free 1.5 mL microtubes • DNase/RNase-free PCR strips • Molecular biology grade DNase/RNase-free water • 0.1M NaOH solution (freshly prepared from Molecular biology grade stock solution) • Parafilm® • RNaseZap or equivalent
Equipment :	• Heat block for 1.5 mL microtubes • Two heating devices compatible with 5 ml/15 ml tubes (water-bath, heat plate and beaker, heat block) • PCR thermocycler • A centrifuge for 50 mL tubes with swinging buckets • Cell counting equipment (microscope and C-chips from Nanotek are recommended) • Orbital shaker • Benchtop centrifuge for 1.5 mL microtubes • Mini-centrifuge for 1.5 mL microtubes • Rotator/wheel for 1.5 mL microtubes • Calibrated micropipettes (P10, P20, P200, P1000) • Vortex mixer

4. Workflow, timing and stopping points



- Asteria™ kit scRNA sample preparation overview:

Single-cell RNA sample preparation starts from a suspension of dissociated cells provided by the user. Cells are first labelled to allow interaction with the CAPTURE BEADS (1). Mixing labelled cells with CAPTURE BEADS generates cell-bead pairs (2). The suspension of cell-bead pairs is then diluted into the hydrogel solution and spread along the walls of the device by insertion of a piston to form a thin hydrogel solution layer (3). Upon incubation on ice, gelation occurs and immobilizes cell-bead pairs (4). Following cell lysis by the addition of lysis buffer, 3'-polyA$^+$ RNAs hybridize on the 3'-polyT extremities of the CAPTURE BEADS. RNA-loaded CAPTURE BEADS are then recovered following degelation (6) and transferred into 1.5 mL microtubes. Subsequent enzymatic steps include reverse transcription, exonuclease I treatment, second strand synthesis, and PCR to generate amplified barcoded cDNA molecules (7).
The resulting cDNA library must then be purified before proceeding to sequencing library preparation using the NEBNext Ultra II FS DNA Library Prep kit, followed by sequencing on an Illumina sequencer.

- Asteria™ protocol timing and stopping points:

Steps	Page	Estimated time*	Stopping point	stabilty
From cells preparation to gelation				
III.1 - Preliminary set-up	12	30 min		
III.2 - Cell labelling	13	45 min		
III.3 - Cell coupling and gelation	17	45 min		
From cell lysis and mRNA capture to degelation				
III.4 - Cell lysis	21	60 min	Possible stop**	35 days
III.5 - Degelation	23	45 min		
From reverse transcription to barcoded cDNA amplification				
III.6 - Reverse transcription	26	120 min	Possible stop	1 week
III.7 - ExoI treatment	27	60 min	Possible stop	1 week
III.8 - Denaturation	28	30 min		
III.9 - Second strand synthesis	29	80 min	Possible stop	1 week
III.10 - PCR	31	90 min	Possible stop	1 week
IV - cDNA clean-up using SPRIselect	32	45 min	Possible stop	3 months
V - Quality control and quantification	35	30 min		

*: Total time estimated for the preparation of 4 Asteria™ samples.
**: Proposed stopping point occurs right after the addition of the lysis buffer, before the lysis incubation (i.e. 2 hours after the beginning of the samples preparation).

5. Chemical safety

WARNING! GENERAL CHEMICAL HANDLING:
To minimize hazards, ensure laboratory personnel read and practice the general safety guidelines for chemical usage, storage, and waste provided below.

- Consult the SDS specific to this kit: https://scipio.bio/ressources/asteria-msds-safety-datasheet
- Read and understand the Safety Data Sheets (SDSs) provided by the chemical manufacturer before you store, handle, or work with any chemicals or hazardous materials. To obtain SDSs, see the “Documentation and Support” section of this document.
- Minimize contact with chemicals.
- Wear appropriate personal protective equipment when handling chemicals (for example, safety glasses, gloves, or protective clothing). Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with sufficient ventilation (for example, a fume hood).
- Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the manufacturers cleanup procedures as recommended in the SDS.
- Handle chemical wastes in a fume hood.
- Ensure use of primary and secondary waste containers. (A primary waste container

holds the immediate waste. A secondary container contains spills or leaks from the primary container. Both containers must be compatible with the waste material and meet federal, state, and local requirements for waste containers storage.)

- After emptying a waste container, seal it with the cap provided.
- Characterize (by analysis if needed) the waste generated by the particular applications, reagents, and substrates used in your laboratory.
- Ensure that the waste is stored, transferred, transported, and disposed of according to all local, state/provincial, and/or national regulations.

6. Biological hazard Safety

WARNING! BIOHAZARD.
Biological samples such as tissues, body fluids, infectious agents, and blood of humans and other animals have the potential to transmit infectious diseases. Conduct all work in properly equipped facilities with the appropriate safety equipment (for example, physical containment devices). Safety equipment can also include personal protection equipment, such as gloves, coats, gowns, shoe covers, boots, respirators, face shields, safety glasses, or goggles.

Individuals should be trained according to applicable regulatory and company/institution requirements before working with potentially biohazardous materials.

Follow all applicable local, state/provincial, and/or national regulations.

II. PROCEDURAL PRECAUTIONS & GUIDELINES

□ **Tutorial videos are available to clarify specific steps of the protocol if required. Viewing the following tutorial videos is critical to obtain optimal results:**

- Video 1: Coupling, dilution, and gelation procedures (steps 3.1 to 3.17)
- Video 2: GELATION PISTON removal and cell lysis (steps 4.1 to 4.6)
- Video 3: Degelation procedure (steps 5.1 to 5.17)

Concatenation of all 3 videos (steps 3.1 to 5.17) is available here.

□ **Ensure you are comfortable with cell-bead coupling.**

In particular, the pipetting speed during coupling should be performed around 90 bpm. A few tries with water and a metronome app could help to be more comfortable during your first use of the kit.

□ **Take RNase-free precautions.**

To maintain RNA integrity during sample preparation, be sure to work under RNase-free conditions: Work on a clean bench area, decontaminate bench, pipettes, and equipment using RNaseZap or equivalent, use RNase-free reagents and consumables, and filter pipette tips. Wear clean gloves at all times. Change gloves regularly.

□ **Note on initial cell suspension quality.**

The quality of your starting material is critical and will impact the quality of your results. Ensure to use an optimized method for tissue or cell dissociation that will provide a high-quality single-cell suspension; it is strongly recommended that samples have a cell viability above 85% and be as free of cellular debris and aggregates as possible.

□ **Use calibrated equipment.**

For optimal results, use calibrated pipettes to guarantee an accurate final concentration during mix assembly, a calibrated cell counting method, and calibrated thermocyclers for adequate amplification.

□ **Remove supernatants with extreme care.**

Any lost cell or bead is a lost datapoint!

Cell and bead pellets are easily destabilized. To minimize bead and cell loss during washing steps, collect the supernatant slowly and remove the volume indicated in the protocol. Aspirate the supernatant from the surface of the liquid, away from the pellet. Minimize penetration of the pipette tip into the solution.

□ **Reagents are ready-to-use, do not refreeze reagents once thawed.**

The only exceptions are ENZYME tubes (RT, S3, ExoI) and LABELLING REAGENT, which are provided for 4 reactions and with valid tolerance to repeated cycles of freezing and thawing, as well as BEAD WASH BUFFER and TE-TW (see details in the corresponding stopping points sections).

□ **Use good laboratory practices to minimize cross-contamination of products**.

If possible, perform the PCR step in an area or room that is free of amplicon contamination. Always change pipette tips between samples/reagents.

□ **Homogenize the reagent solutions after thawing and the bead or cell suspensions before pipetting.**

Unless stated otherwise, homogenization should be performed by pipetting half of the solution volume up and down a few times, with special care for viscous solutions.

□ **Pipetting viscous solutions** (such as CAPTURE BEADS, S3 SUPERMIX, and DILUTION REAGENT) must be done slowly to ensure proper volume aspiration and dispensing. Ensure that no liquid remains in the pipette tip.

□ **Cell suspension concentration should be measured with good precision.** (around +/- 10%).

Precise quantification requires counting at least 300 cells.

□ **Dilution reagent requires a careful preparation before use.**

Dilution reagent should be properly melted and homogenized to avoid manipulation problems and residual gel fragments.

The different preparation steps are listed below and indicated in the protocol:

1- Set a water-bath at 80°C (step 1.1)

2- Set a water-bath at 37°C (step 1.2)

3- Melt the DILUTION REAGENT at 80°C for at least 30 min (step 1.4).

4- Following the incubation at 80°C, homogenize the DILUTION REAGENT by vortexing and inverting the tube at least 10 times (ensure that solution is uniformly transparent) (step 2.18).

5- Transfer the DILUTION REAGENT at 37°C and incubate at least 15 min (step 2.18).

6- Homogenize the Dilution Reagent before use by inverting several times (step 3.6).

□ **Never use polystyrene or tight tube racks when storing Gelation Tubes at -80°C.**

Improper storage at -80°C during stopping points might damage the structural integrity of the Gelation Tube. Polystyrene-based or other tight containers might retract tighter than the tube itself (this also applies to generic Falcon tubes) and the pressure may cause cracks to appear on the tube wall with the risk of leaks from or into the Tube, potentially leading to loss of the sample and risk to operator

III. PREPARING cDNA FROM SINGLE CELLS

1. Preliminary setup

[Estimated step timing: 30 min]

□ Important notes:

- Carefully read the section “PROCEDURAL PRECAUTIONS & GUIDELINES” (page 10).
- RNase-free precautions:
  - Work on a clean bench area, decontaminate pipettes and equipment.
  - Use RNase-free reagents and consumables, and filter pipette tips.
  - Wear clean gloves at all times. Change gloves regularly.

1.1. Set a water-bath at 80°C (for step 1.4)

- *water-bath or any heating device compatible with 15 mL tubes*

1.2. Set a water-bath at 37°C (for steps 2.18)

- *water-bath or any heating device compatible with 15 mL tubes*

1.3. Set a heat block (compatible with 1.5 mL-tubes) at 37°C (for step 2.8).

1.4. Melt the DILUTION REAGENT at 80°C for at least 30 min.

1.5. Thaw the following reagent **on ice**:

- LABELLING REAGENT: 1 for up to 4 samples (spin down once thawed)

1.6. Thaw the following reagents **at room temperature**:

- CELL WASH BUFFER: 1 per sample (place on ice once thawed)
- LYSIS BUFFER: 1 per sample (place on ice once thawed)
- *DEGELATION BUFFER: 1 per sample (equilibrate at 25°C once thawed)
- *BEAD WASH BUFFER: 1 per sample (place on ice once thawed)

* **if plan to stop the experiment at step III.4 (table timing and stopping point in page8), thaw these two reagents when resuming the experiment (box at step III.4.3 in page 22.**

2. Cell labelling

[Estimated step timing: 45 min]

□ Important notes:

- Depending on the number of available cells, the cell labelling step can start from two different cell input quantities (see step 2.2):

- Option A: If the starting cell number is below 50,000 cells, label 5,000 to 15,000 cells.

- Option B: If the starting cell number is above 50,000 cells, label 50,000 cells. This option enables to count the labelled cells in order to start cell coupling (step 3.10) with 5,000 to 15,000 cells, which increases the final cell output number.

- Centrifugation parameters depend on your cell type of interest. **Using your usual cell centrifugation conditions (temperature, speed, duration) is recommended**.

- If needed, some centrifuge parameters are proposed in **Table 1**.

- If you have several samples, process them in parallel.

□ Items used in this step:

- LABELLING REAGENT: on ice (return to -20°C after use)

- CELL WASH BUFFER: on ice

- A heat block at 37°C

- A water-bath (or equivalent) at 37°C

Cell types	Centrifuge force	Duration
Large cells (Cardiomyocytes...)	200xg	3 min
Medium size cells (NIH, HEK...)	300xg	5 min
Small cells (PBMC...)	500xg	5 min

***Table 1**: Suggested centrifuge parameters for different cell types*

Cell labelling:

2.1. Count cells.

- During cell counting, ensure that the cells are properly dissociated.

2.2. Depending on the number of available cells (see “Important notes” above), transfer cells into a 1.5 mL microtube, according to one of the options below:

Option A:	**Option B:**
(Starting cell number below 50,000) - Transfer 5,000 to 15,000 freshly harvested and dissociated cells.	(Starting cell number above 50,000) - Transfer 50,000 freshly harvested and dissociated cells. (*If the cell suspension is used for several Asteria™ samples, load up to 200,000 cells in a single labelling reaction)*

2.3. Centrifuge cells at appropriate temperature, speed and duration (See **Table 1** if needed).

- Using your usual cell centrifugation conditions is recommended.

- If using fixed angle rotor, note the orientation of the microtube on the rotor to anticipate the future cell pellet location.

2.4. Prepare a tube of labelling mix containing:

- CELL WASH BUFFER (25 µL per sample)
- LABELLING REAGENT (2.8 µL per sample)

- Homogenize, spin down to collect liquid at the bottom, and keep on ice.

- Return LABELLING REAGENT to -20°C after use.

2.5. Following cell centrifugation, carefully remove supernatant leaving no more than 20 µL.

- Cell pellet may not be visible. When removing the supernatant, pipet at the opposite side of expected cell pellet location.

2.6. Add 25 µL of the labelling mix prepared in step 2.4.

2.7. Gently pipet up/down a few times to resuspend the cells.

2.8. Incubate for 5 min at 37°C.

2.9. Thaw the CAPTURE BEADS (1 per sample) at room temperature.

Cell Washes

2.10. Add 250 µL of cold CELL WASH BUFFER to your labelled cells.

2.11. Centrifuge cells at appropriate temperature, speed, and duration (See **Table 1** if needed).

- Using your usual cell centrifugation conditions is recommended.

- If using fixed angle rotor, note the orientation of the microtube on the rotor to anticipate the future cell pellet location.

2.12. Carefully remove 250 µL of supernatant.

- *Cell pellet may not be visible. When removing the supernatant, pipet at the opposite side of expected cell pellet location.*

2.13. Add 250 µL of cold CELL WASH BUFFER.

2.14. Gently pipet up/down a few times to resuspend the cells.

2.15. Centrifuge cells a second time.

- *Using your usual cell centrifugation conditions is recommended.*

- *If using fixed angle rotor, note the orientation of the microtube on the rotor to anticipate the future cell pellet location.*

2.16. Carefully remove 250 µL of supernatant.

- *Cell pellet may not be visible. When removing the supernatant, pipet at the opposite side of expected cell pellet location.*

2.17. Resuspend cell pellet using CELL WASH BUFFER, according to the option used in step 2.2:

If **Option A** followed in step 2.2:	If **Option B** followed in step 2.2:
- Adjust volume to 100 µL using cold CELL WASH BUFFER and resuspend cells. (We *recommend transferring 100 µL of CELL WASH BUFFER in a control tube and visually adjust the sample volume to the control volume)* - Keep cells on ice.	- Add 50 µL of cold CELL WASH BUFFER and resuspend cells. (*If more than 50,000 cells were used in step 2.2, increase your resuspension volume accordingly)* - Count labelled cells. - Keep cells on ice.

2.18. Proceed with the DILUTION REAGENT preparation:

- Following the incubation at 80°C, homogenize the DILUTION REAGENT by vortexing and inverting the tube at least 10 times (ensure that solution is uniformly transparent).

- Transfer the DILUTION REAGENT at 37°C (The DILUTION REAGENT should be incubated at least 15 minutes at 37°C before its use at step 3.13. Any shorter incubation could damage the cells, as the temperature of the DILUTION REAGENT could then be above 37°C).

2.19. Prepare labelled cell dilution, according to the option used in step 2.2:

If **Option A** followed in step 2.2:	If **Option B** followed in step 2.2:
Dilution is not necessary (the 100 µL cell mix prepared during step 2.17 will be used at step 3.10).	In a new 1.5 mL tube, prepare 120 µL of a labelled cell suspension at the appropriate concentration depending on the required input cell number: - 50 cells/µL (for 5,000 cell input) - 100 cells/µL (for 10,000 cell input) - 150 cells/µL (for 15,000 cell input) *- Use CELL WASH BUFFER to prepare your labelled cell suspension.* *- 100 µL of this cell mix will be used at step 3.10.*

2.20. Keep labelled cells on ice until coupling. We recommend using them within 30 min for the cell coupling step (following section).

3. Cell coupling & gelation

[Estimated step timing: 45 min]

□ Important notes:

- It is important to perform steps 3.8 to 3.17 one sample at a time, without breaks. Ensure having all the necessary equipment before starting (see list below).

- **Video 1 illustrates the procedure from step 3.1 to 3.17.**

□ Items used in this step:

- GELATION DEVICE. It contains 3 parts: a transparent GELATION TUBE, a blue GELATION PISTON, and a blue GELATION CAP (see **Figure 1**).

- CAPTURE BEADS: Equilibrated at room temperature

- DILUTION REAGENT: Equilibrated at 37°C (higher temperature could damage the cells during step 3.13)

- Clean 50 mL tubes, to store the GELATION PISTON

- A metronome set at 90 bpm

A. **B.**

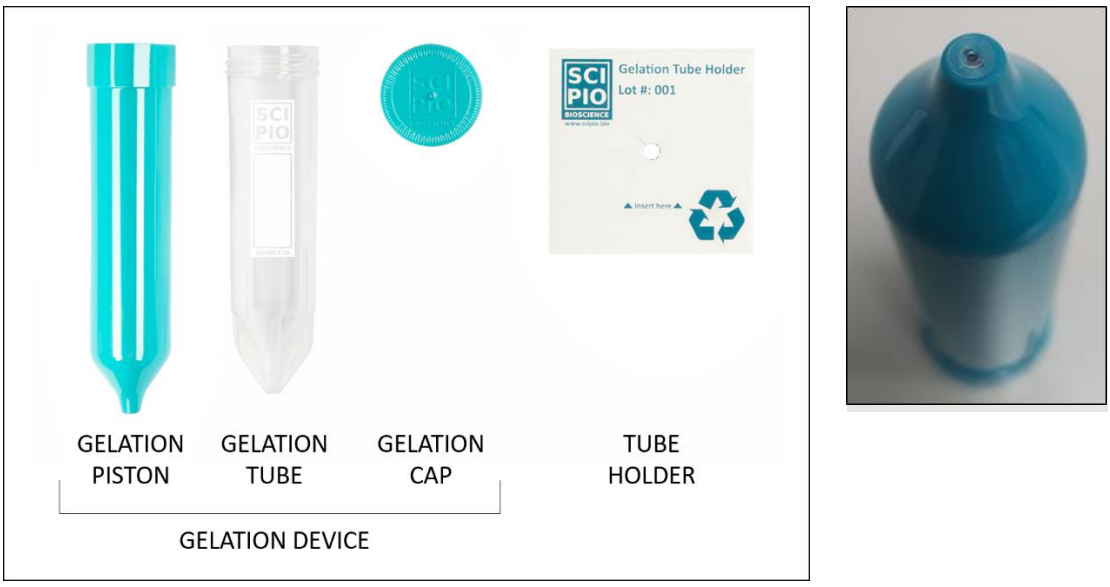


Figure 1: A: *Components of the GELATION DEVICE (used from step 3.1) and the accompanying TUBE HOLDER (used from step 4.4).* ***B:*** *A transparent valve is located at the extremity of the GELATION PISTON to facilitate its removal following gelation.*

CAPTURE BEADS preparation:

3.1. Prepare GELATION DEVICE before proceeding:

- Label the GELATION TUBE and place it vertically in a 50 mL tube stand.

- Wearing gloves, transfer the GELATION PISTON into a clean RNase-free 50 mL tube (until use at step 3.15).

3.2. Briefly spin down the CAPTURE BEADS.

3.3. Thoroughly homogenize CAPTURE BEADS suspension by gentle pipetting.

- CAPTURE BEADS are in a viscous solution. Be sure to pipet gently to prevent bubble formation.

- Visually confirm that CAPTURE BEADS suspension is homogenous before proceeding.

3.4. Transfer 100 µL of the CAPTURE BEADS into the GELATION TUBE.

Cells coupling with CAPTURE BEADS and gelation

3.5. Prepare all necessary reagents/equipment for steps 3.8 to 3.17 (See list “Items used in this step”).

- It is critical that this sequence is performed one sample at a time, without breaks.

- Gather a metronome, a P200 pipette (set at 100 µL), serological 5 mL and 10 mL pipettes and pipette controller.

3.6. Homogenize the DILUTION REAGENT by inverting several times. Keep at 37°C.

- Reminder: Prior to step 3.13, the DILUTION REAGENT should be incubated at least 15 minutes at 37°C (see step 2.18).

3.7. Start metronome at 90 bpm (for step 3.12).

3.8. Homogenize labelled cells suspension by gentle pipetting.

3.9. Thoroughly homogenize the CAPTURE BEADS suspension by gentle pipetting. Proceed immediately to next step to prevent bead sedimentation.

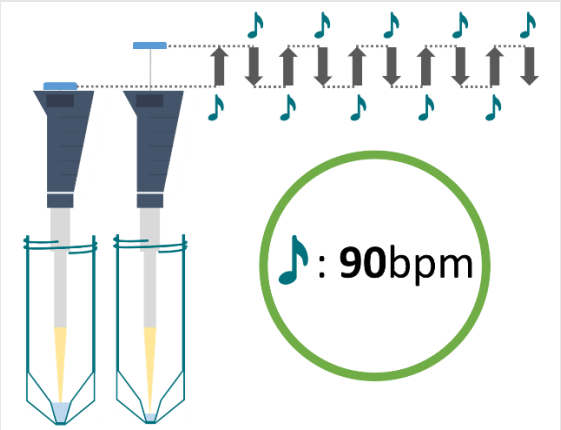
- CAPTURE BEADS are in a viscous solution. Be sure to pipet gently to prevent bubble formation.

- Visually confirm that CAPTURE BEADS suspension is homogenous before proceeding.

3.10. Transfer 100 µL of the labelled cell suspension prepared in step 2.19 into the middle height of the CAPTURE BEADS suspension.

3.11. Once cells are dispensed, place tip 3-5 mm from the bottom of the tube.

3.12. Immediately perform 5 up & down cycles of pipetting with a P200 pipette set at 100 µL, moving at 90 bpm: pipet up on the first beat, pipet down on the second beat and continue for 4 additional up & down cycles.



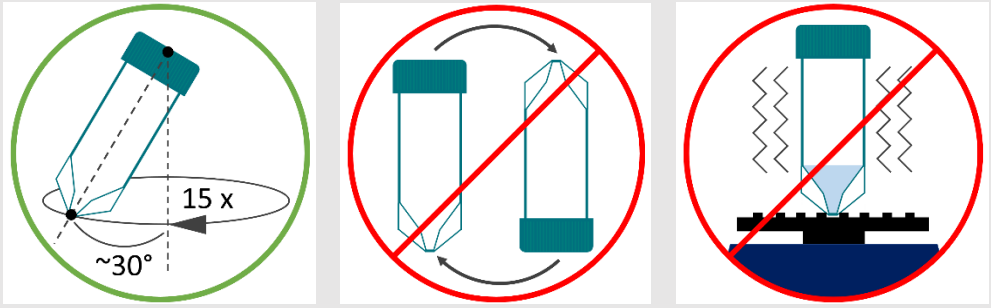
3.13. Add 2 mL of the DILUTION REAGENT at the bottom of the GELATION TUBE using a serological pipette, as illustrated in the Video 1.

- Dilution reagent is a viscous solution. Pipet slowly and ensure that no liquid remains in the pipette.

3.14. Close the GELATION TUBE with the GELATION CAP and homogenize by rotating the tube 15 times at an angle of approximately 30° for 10 seconds, as demonstrated in the Video 1.

- Do not flip the GELATION TUBE upside down

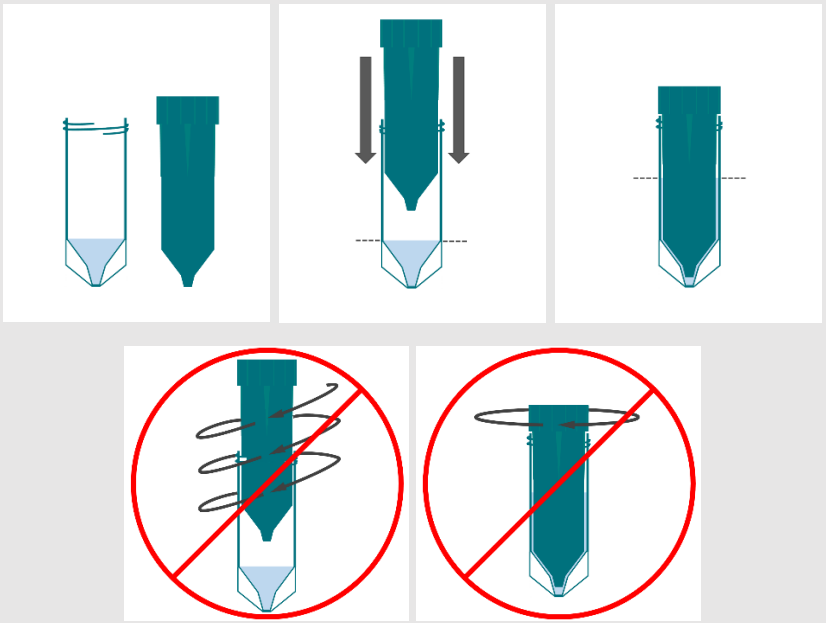
- Do not vortex



3.15. Remove the cap and place the tube vertically (at room temperature). Insert the GELATION PISTON in the GELATION TUBE and slowly press it down to the bottom of the GELATION TUBE while keeping both vertical (see Video 1 and **Figure 2**).

- Do not tilt, rotate, or try to remove the piston during this step.

- The dashed line in the following pictograms represents the level of the gel solution before and after GELATION PISTON insertion.



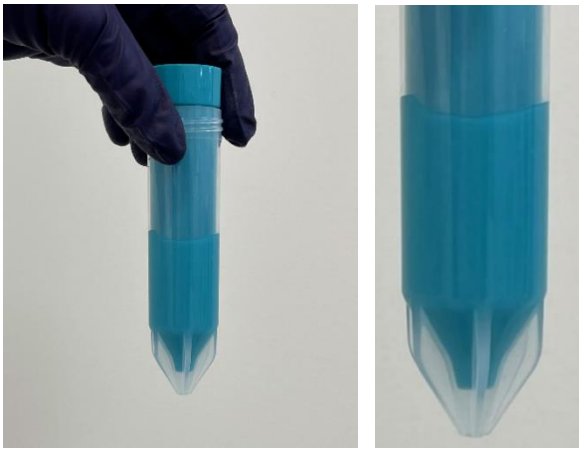


Figure 2: *After inserting the GELATION PISTON, the hydrogel solution should be approximately level around the piston.*

3.16. Wait 1 min with GELATION PISTON inserted.

3.17. Place the assembled GELATION DEVICE vertically in ice and incubate for 20 min to allow for complete gelation.

- Ensure that the gel layer inside the GELATION DEVICE is fully surrounded by ice.

4. Cell lysis

[Estimated step timing: 60 min]

□ Important notes:

- Video 2 illustrates the procedure from step 4.1 to 4.6.

- **If performing the stopping point at step 4**, samples are stored in GELATION DEVICE at -80°C after step 4.3. Be careful not to store the GELATION DEVICE in a polystyrene or other tight container that might damage the wall of the tube during the freezing process. When resuming the preparation, thaw samples at 25°C (water-bath) for 15 minutes. In addition, thaw BEAD WASH BUFFER and DEGELATION BUFFER at 25°C. Place the BEAD WASH BUFFER on ice once thawed (DEGELATION BUFFER remains at 25°C).

□ Items used in this step:

- LYSIS BUFFER: ensure complete thawing.
- Orbital shaker

□ Items to prepare for following steps:

If performing the stopping point at steps 6,7 or 9:

- DEGELATION BUFFER: Equilibrated at 25°C (using water-bath)
- BEAD WASH BUFFER: Thaw on ice
- A centrifuge for 50 mL tubes (swinging buckets) set at room temperature

4.1. Slowly pull out the GELATION PISTON from the GELATION TUBE (see Video 2).

- Do not rotate the piston during this step.

- The gel will stay inside the tube. The piston should come out clean.

4.2. Homogenize LYSIS BUFFER by pipetting.

4.3. Add 7.5 mL of LYSIS BUFFER to each sample and close the GELATION TUBE with the GELATION CAP. Seal the tube cap with Parafilm to prevent leakage.

- The gel will collapse to the bottom of the tube.

- Minor leakages may occur during lysis or degelation, without consequence on performance.

Possible stopping point

- Place GELATION DEVICE vertically in -80°C freezer.

- Samples can be stored at -80°C for up to 35 days.

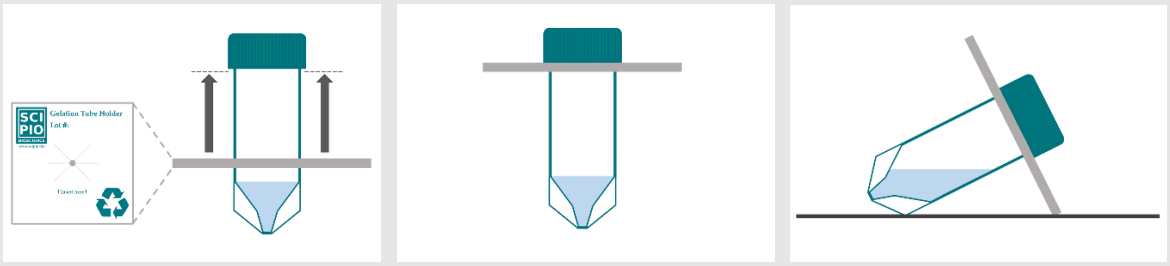
- For further information, see the stopping point stability results available at this link.

- When resuming the preparation later day, thaw at 25°C (water-bath) for 15 min before proceeding to the next steps (do not Vortex) as well

4.4. Insert the GELATION DEVICE into the TUBE HOLDER.

- The TUBE HOLDER should be pushed up to the GELATION CAP.

- Ensure that the gel is fully immersed in the LYSIS BUFFER.



4.5. Put the tubes on an orbital shaker at around 60 rpm.

4.6. Keep the tubes on agitation for 60 min at room temperature.

- Once lysis incubation has started, ensure that DEGELATION BUFFER is at 25°C.

5. Degelation

[Estimated step timing: 45 min]

□ Important notes:

- Following lysis incubation, proceed immediately to degelation (**Do not put sample on ice)**.

- Process all samples simultaneously.

- Video 3 illustrates the procedure from step 5.1 to 5.17.

□ Items used in this step:

- DEGELATION BUFFER: Equilibrated at 25°C (using water-bath)

- BEAD WASH BUFFER: On ice

- A centrifuge for 50 mL tubes (swinging buckets) set at room temperature

□ Items to prepare for following steps:

- RT SUPERMIX (1 per sample): Thaw at room temperature (spin and place on ice once thawed)

- A heat block at 25°C

Hydrogel degelation:

5.1. Vortex the DEGELATION BUFFER for 15 sec.

5.2. Add 3.75 mL of DEGELATION BUFFER to each GELATION TUBE (see Video 3).

- If processing multiple samples, dispense DEGELATION BUFFER in all samples before proceeding.

5.3. Close tube and seal the cap with Parafilm.

5.4. Vortex for 5 min at maximum speed.

- Vortex all samples together

CAPTURE BEADS wash:

5.5. Remove Parafilm from GELATION DEVICE.

5.6. Spin down (3 min, 1000xg, room temperature) in a swinging bucket centrifuge to pellet the CAPTURE BEADS.

- If working with an odd number of samples, the centrifuge can be balanced using a 50 mL tube containing 12.5 mL of water.

5.7. Remove supernatant carefully with a 25 ml serological pipet. Leave around 500 µL.

*- Please refer to **Figure 3** to estimate the 500 µL in the Gelation Device.*

- DEGELATION BUFFER contains a chaotropic agent that is a strong irritant. Be sure to discard it in the appropriate chemical waste.

***Figure 3:** Example of 500 µL in the GELATION TUBE (DMEM is used for instruction purpose)*

5.8. Pipet up and down 5 times using a P1000 set at 250 µL with low retention tip.

- Gel fragments can be detected during pipetting (clogging). If gel fragments are still present, keep pipetting until complete degelation.

- Using low retention tip decreases the risk of losing beads at this step.

5.9. Add 5 mL of ice-cold BEAD WASH BUFFER directly on the bead pellet.

- Do not pipet for homogenization. Beads are resuspended upon buffer addition.

5.10. Spin down (3 min, 1000xg, room temperature) in a swinging bucket centrifuge to pellet the CAPTURE BEADS.

- If working with an odd number of samples, the centrifuge can be balanced using a 50 mL tube containing 4 mL of water.

5.11. Remove supernatant carefully using a 5 mL serological pipette, leave around 500 µL.

*- Please refer to **Figure 3** to estimate the 500 µL in the Gelation Device.*

CAPTURE BEADS transfer to microtubes:

5.12. Using a P1000 micropipette, transfer the CAPTURE BEADS suspension into a DNase/RNase-free 1.5 mL microtube**.**

5.13. Rinse the GELATION TUBE with 500 µL of BEAD WASH BUFFER, to collect any remaining CAPTURE BEADS, and transfer the solution into the sample tube.

*- **During bead washing hereafter, do not homogenize after wash buffer dispense**. CAPTURE BEADS are resuspended upon buffer addition.*

5.14. Spin 60 sec in a mini-centrifuge.

5.15. Remove 500 µL of supernatant carefully.

5.16. Spin 30 sec in a mini-centrifuge.

5.17. Remove supernatant carefully.

- Beads pellets are unstable. To prevent loss of CAPTURE BEADS, we recommend leaving about 50 µL of supernatant.

5.18. Add 250 µL of BEAD WASH BUFFER to each tube. Do not homogenize by pipetting.

5.19. Spin 30 sec in a mini-centrifuge.

5.20. Remove supernatant carefully.

- Beads pellets are unstable. To prevent loss of CAPTURE BEADS, we recommend leaving about 50 µL of supernatant.

5.21. Repeat steps 5.18 to 5.20.

5.22. Adjust total volume to 20 µL (including bead pellet) with BEAD WASH BUFFER.

- We recommend transferring 20 µL of BEAD WASH BUFFER into a control tube and visually adjust the sample volume to the control volume

- Don't discard the remaining BEAD WASH BUFFER. It will be used in steps 8.12 and 8.15.

6. Reverse transcription

[Estimated step timing: 120 min]

□ Items used in this step:

- RT SUPERMIX: On ice
- RT ENZYME: (1 tube for up to 4 samples):
  - Spin before use
  - Keep on ice briefly during pipetting and immediately return to -20°C after use
- A heat block at 25°C

If possible, prepare the following program: 10 min at 25°C, then 90 min at 42°C.

6.1. Spin RT SUPERMIX and RT ENZYME.

6.2. Homogenize RT SUPERMIX.

6.3. Add 75 µL of RT SUPERMIX to the sample tube (20µL of bead suspension).

6.4. Add 5 µL of RT ENZYME.

6.5. Homogenize reaction mix by pipetting.

6.6. Transfer your sample into the heat block and incubate the samples as follows:

- 10 min at 25°C
- 90 min at 42°C

6.7. Place the sample tube on ice for 2 min and then spin briefly.

6.8. Set the heat block at 37°C.

Possible stopping point

- Store samples up to one week at -20°C
- Freeze the previously used BEAD WASH BUFFER (will be used in steps 8.12 and 8.15)
- Thaw on ice before proceeding to the next steps.

7. Exonuclease I treatment

[Estimated step timing: 60 min]

□ Items used in this step:

- EXO I ENZYME (1 tube for up to 4 samples):
  - Spin before use
  - Keep on ice until use and immediately return to -20°C after use
- TE-SDS (1 per sample): Thaw and equilibrate at room temperature.
- A heat block set at 37°C
- **If performing stopping point at step 7**: TE-TW (1 per sample): Thaw at room temperature and place on ice once thawed. Return to -20°C after use.

□ Items to prepare for following steps:

- TE-TW (1 per sample): Thaw at room temperature and place on ice once thawed.

Exonuclease I digestion:

7.1. Briefly spin samples in a mini-centrifuge.

7.2. Add 5 µL of EXO I ENZYME.

7.3. Homogenize samples by pipetting.

7.4. Incubate samples 50 min at 37°C.

Exonuclease I inactivation and washes:

7.5. Spin samples for 20 sec in a mini-centrifuge.

7.6. Carefully remove 80 µL of supernatant.

7.7. Add 250 µL of TE-SDS.

7.8. Spin samples for 20 sec in a mini-centrifuge.

7.9. Carefully remove 250 µL of supernatant.

Possible stopping point

- Perform 2 additional washes using 250 µL of TE-TW.
- Store samples up to one week at -20°C
- Freeze the previously used BEAD WASH BUFFER (will be used in steps 8.12 and 8.15) and TE-TW (will be used in steps 8.9, 9.10 and 9.13)
- Thaw on ice before proceeding to the next step.

8. Denaturation

[Estimated step timing: 30 min]

□ Items used in this step:

- TE-TW: on ice
- BEAD WASH BUFFER: on ice (re-use from Degelation step)
- 0.1M NaOH in RNase-free water (150 µL required per sample)
- a microtube rotator/wheel

□ Items to prepare for following steps:

- S3 SUPERMIX (1 per sample): Thaw at room temperature
- A heat block (or water-bath) set at 70°C

8.1. At this point, your CAPTURE BEADS should be in approximately 20 µL of total volume (beads included). If you notice a much larger volume, adjust it to 20 µL.

We recommend transferring 20 µL of TE-SDS to a control tube for reference and visually adjusting the sample volume to the control volume.

Alkaline denaturation:

8.2. Add 100 µL of 0.1M NaOH solution.

8.3. Spin samples for 20 sec in a mini-centrifuge.

8.4. Remove 100 µL of supernatant.

8.5. Add 50 µL of 0.1M NaOH solution.

8.6. Rotate samples for 5 min on a microtube rotator/wheel.

CAPTURE BEADS wash:

- CAPTURE BEADS pellets are unstable. To prevent loss of CAPTURE BEADS, we recommend removing indicated volumes of supernatants.

8.7. Spin samples for 20 sec in a mini-centrifuge.

8.8. Remove 50 µL supernatant.

8.9. Add 250 µL of TE-TW (Do not homogenize by pipetting).

8.10. Spin samples for 20 sec in a mini-centrifuge.

8.11. Remove 200 µl of supernatant.

8.12. Add 250 µL of BEAD WASH BUFFER (Do not homogenize by pipetting).

8.13. Spin samples for 20 sec in a mini-centrifuge.

8.14. Remove 250 µL of supernatant.

8.15. Adjust the residual total volume to 20 µL (including bead pellet) using BEAD WASH BUFFER.

- We recommend transferring 20 µL of BEAD WASH BUFFER to a control tube for reference and visually adjusting the sample volume to the control volume.

9. Second strand synthesis

[Estimated step timing: 80 min]

□ Items used in this step:

- A heat block (or water-bath) set at 70°C
- A heat block set at 37°C
- S3 SUPERMIX
- S3 ENZYME (1 tube for up to 4 samples):
  - Spin before use
  - Keep on ice briefly during pipetting and immediately return to -20°C after use
- TE-TW: (on ice, reuse from previous section)
- nuclease-free water

□ Items to prepare for following steps:

- PCR SUPERMIX (1 per sample): Thaw on ice

S3 SUPERMIX preparation:

9.1. Incubate the S3 SUPERMIX for 5 min at 70°C.

9.2. Immediately incubate on ice for 1 min.

9.3. Spin S3 SUPERMIX briefly and homogenize by pipetting.

Second strand synthesis:

9.4. Add 77.5 µL of S3 SUPERMIX to sample tube.

9.5. Add 2.5 µL of S3 ENZYME to sample tube.

9.6. Thoroughly homogenize the sample by pipetting.

9.7. Incubate 1 hour at 37°C.

Post-Second strand synthesis washes:

- CAPTURE BEADS pellets are unstable. To prevent loss of CAPTURE BEADS, we recommend removing indicated volumes of supernatants.

9.8. Spin samples for 20 sec in a mini-centrifuge.

9.9. Remove 60 µL of supernatant.

9.10. Add 250 µL of TE-TW.

9.11. Spin samples for 20 sec in a mini-centrifuge.

9.12. Carefully remove 250 µL of supernatant.

9.13. Repeat steps 9.10 to 9.12.

Possible stopping point

- Store samples up to one week at -20°C

- Thaw on ice before proceeding to the next steps.

9.14. Add 250 µL of nuclease-free water.

9.15. Spin samples for 20 sec in a mini-centrifuge.

9.16. Carefully remove 140 µL of supernatant.

9.17. Adjust total volume to 160 µL (including bead pellet) with nuclease-free water.

- Adjusting the volume to 160 µl at this step is important for the following PCR step. We recommend checking the volume with a P200 pipette set to 160µl (use low retention filter tips).

10. Polymerase Chain Reaction

[Estimated step timing: 90 min]

□ Important notes:

- Reminder: The PCR SUPERMIX contains the enzyme.

□ Items used in this step:

- PCR SUPERMIX (on ice)
- PCR strips tubes and caps (1 per sample)
- A PCR thermocycler with the following program:

Step		Cycles	Temperature	Duration
Step 1	Initial denaturation	-	95°C	3 min
Step 2	Denaturation	4	98°C	20 sec
	Annealing		65°C	45 sec
	Extension		72°C	3 min
Step 3	Denaturation	9	98°C	20 sec
	Annealing		67°C	20 sec
	Extension		72°C	3 min
Step 4	Final extension	-	72°C	5 min
Step 5	Store	-	4°C	∞

10.1. Spin PCR SUPERMIX briefly and homogenize by pipetting.

10.2. Add 240 µL PCR SUPERMIX to each sample.

10.3. Homogenize beads by pipetting and split into 8 PCR tubes, with 50 µL in each.

- Accurate pipetting is critical (Use low retention filter tips).

- CAPTURE BEADS sediment very rapidly. Ensure homogeneous bead distribution by homogenizing with a P1000 pipette between each dispensing.

10.4. Close tubes tightly.

10.5. Transfer tubes to thermocycler.

10.6. Run the PCR program (see above for parameters).

Possible stopping point

- Store overnight up to one week at -20°C
- Thaw on ice before proceeding.

IV. SAMPLE CLEAN-UP USING SPRIselect

[Estimated step timing: 45 min]

□ Preliminary considerations:

The following protocol is to purify the amplified cDNA from a single pool of all split PCR reactions (see step IV.2).

Alternative options (and their protocol adaptations) are described in Annex 1: WORKING WITH SAMPLE SUBFRACTIONS (Page 41) to gain flexibility by keeping PCR reactions split and performing SPRIselect clean-up reactions on each sample subfractions. This allows to sequence a sample subfraction to ensure the overall sample quality, to sequence deeper a sample subfraction (from a reduced number of cells), or to correct a heterogenous beads distribution resulting from step 10.3.

□ Important notes:

- The following protocol is adapted from the SPRIselect user guide: https://research.fredhutch.org/content/dam/stripe/hahn/methods/mol_biol/SPRIselect%20User%20Guide.pdf
- For optimal quality of amplified barcoded DNA, we recommend performing two SPRIselect clean-up rounds (included in the protocol detailed below).
- Care should be taken not to aspirate more than a trace amount of SPRIselect beads during the different steps, as the desired library is associated with the SPRIselect beads. Significant SPRIselect beads loss will result in reduced yield.

□ Items used in this step:

- SPRIselect beads. Ensure that the SPRIselect suspension is homogeneous (thoroughly shake the bottle).
- Magnetic stands for 1.5 mL and 0.2 mL microtubes
- 85% Ethanol (freshly prepared using molecular biology grade nuclease-free water)
- Molecular biology grade nuclease-free water

1. Spin down PCR strip tubes for 20 sec using a mini-centrifuge.

2. For each sample, pool the 45 µL of CAPTURE BEADS-free supernatant from each of the 8 PCR reaction tubes into a clean 1.5 mL microtube.

3. Add 216 µL of SPRIselect beads (0.6X of PCR reaction volume) to each pool.

- Ensure that SPRIselect bead slurry is homogeneous and use low retention tip for accurate pipetting (critical for appropriate size selection).

- The 0.6X ratio of SPRIselect beads is crucial for proper size selection. If the post-PCR pooling volume is different from 360 µL (8 x 45 µL), please adjust the volume of added SPRI beads accordingly.

4. Mix by pipetting 10 times and incubate at room temperature for 1 min.

- Insufficient mixing of sample and SPRIselect beads will lead to inconsistent size selection results.

5. Place the samples on an appropriate magnetic stand and allow the SPRIselect beads to settle to the magnet.

6. Remove and discard the cleared supernatant.

7. Add 1 mL of 85% ethanol to each tube and incubate at room temperature for 30 sec.

- Do not resuspend the magnetic SPRIselect beads.

8. Remove and discard the ethanol supernatant.

9. Repeat steps 7 to 8.

10. Ensure no residual ethanol droplets remain.

11. Dry the magnetic beads at room temperature (maximum 3 min).

- Over-drying the beads may alter DNA recovery during elution.

12. Remove the microtubes from the magnet.

13. Add 102 µL of Nuclease-free water to each sample and resuspend SPRIselect beads by pipetting 20 times.

14. Incubate at room temperature for 1 min.

15. Place the samples on an appropriate magnetic stand and allow the SPRIselect beads to settle to the magnet.

16. Transfer 100 µL of the cleared solution (1st elution) to a nuclease-free 0.2 mL tube, being careful not to aspirate any magnetic SPRIselect beads.

17. Add 60 µL of SPRIselect beads (0.6X of PCR reaction volume) to each sample.

- Ensure that SPRI select bead slurry is well homogeneous and use low retention tip for accurate pipetting that is critical for appropriate size selection.

18. Mix by pipetting 10 times and incubate at room temperature for 1 min.

- Insufficient mixing of sample and SPRIselect beads will lead to inconsistent size selection results.

19. Place the samples on an appropriate magnetic rack and allow the SPRIselect beads to settle to the magnet.

20. Remove and discard the cleared supernatant.

21. Add 200µL of 85% ethanol to each tube and incubate at room temperature for 30 sec.

- *Do not resuspend the magnetic SPRIselect beads.*

22. Remove and discard the ethanol supernatant.

23. Repeat steps 21 and 22.

24. Ensure no residual ethanol droplets remain.

25. Dry the magnetic beads at room temperature (maximum 3min).

- *Over-drying the beads may alter DNA recovery during elution*

26. Remove the microtubes from the magnetic rack.

27. Add 32 µL of nuclease-free water to each microtube and resuspend the beads by pipetting 20 times.

28. Incubate at room temperature for 1 min.

29. Place the microtubes on the magnetic rack and allow the beads to settle to the magnet.

30. Transfer 30 µL of the 2nd elution solution to a clean 1.5 mL microtube, being careful not to aspirate any magnetic SPRIselect beads.

Possible stopping point:

Store samples at -20°C for at least 3 months

Thaw on ice before proceeding to next steps.

V. QUALITY CONTROL AND QUANTIFICATION OF PURIFIED cDNA

[Estimated step timing: 30 min]

Quantity and quality of the cleaned-up amplified barcoded DNA must be evaluated before preparing an Illumina-compatible library.

For cDNA concentrations measurements, we recommend Qubit dsDNA HS assay kit. **Table 2** indicates a range of typical yield of amplified cDNA after 2 rounds of SPRIselect clean-up. cDNA yields depend on cell input number and cell type.

Cell types	Initial cell number for coupling	Typical total yields
NIH, HEK	10,000	50-500 ng
PBMC	10,000	5-50 ng

Table 2: *Typical DNA concentration yields obtained from Asteria™ kit preparation using the indicated cell types and input number.*

For controlling sample quality, we recommend running your amplified barcoded DNA on Agilent TapeStation to control for amplified DNA size distribution. If the DNA concentration measured by Qubit is above the detection limit of a High Sensitivity (HS) D5000 ScreenTape (from 10 to 1,000 pg/µL), we recommend using a sample fraction to prepare a dilution compatible with the HSD5000 ScreenTape. Typical profile shows a distribution ranging from 250 to 3000 bp, centered around 600-900 bp (See **Figure 4**).

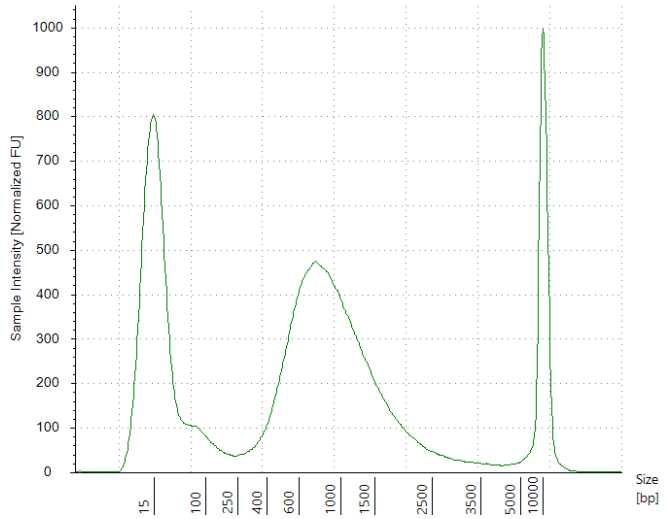


Figure 4: *Typical size distribution of purified cDNA (obtained using Agilent TapeStation High sensitivity D5000).*

VI. LIBRARY PREPARATION

□ Preliminary considerations:

In order to avoid cross-contaminations between samples, we recommend using filter tips and not letting more than one sample tube open at the same time.

- **Recommended cDNA input quantity:**

Any cDNA yield within the range indicated in **Table 2** (previous section) is suitable for library preparation.

- We recommend not starting with more than 20 ng (for 10,000 input cells) or 30 ng (for 15,000 input cells) of barcoded amplified cDNA.

- We strongly recommend not using more than half of your barcoded cDNA (the spared fraction of your sample can be stored at -20°C, to generate additional libraries if needed).

- **Custom oligos for library amplification and read1 sequencing:**

In order to selectively amplify the DNA fragments with cell barcode sequence, it is necessary to use the LIBRARY PREP PRIMER provided in Asteria™ kit to generate the library. This ensures that all DNA in the library have cell barcode sequence at the P5 side, and 3' end of the captured mRNA at the P7 side.

It is recommended to use NEBNext Ultra II FS DNA Library Prep kit for Illumina (NEB #E7805S) with NEBNext Multiplex Oligos for Illumina (Set 1, NEB #E7335), as library PCR primers are provided separately, which enables to replace the NEB kit "Universal PCR primer/i5" by Asteria™'s LIBRARY PREP PRIMER.

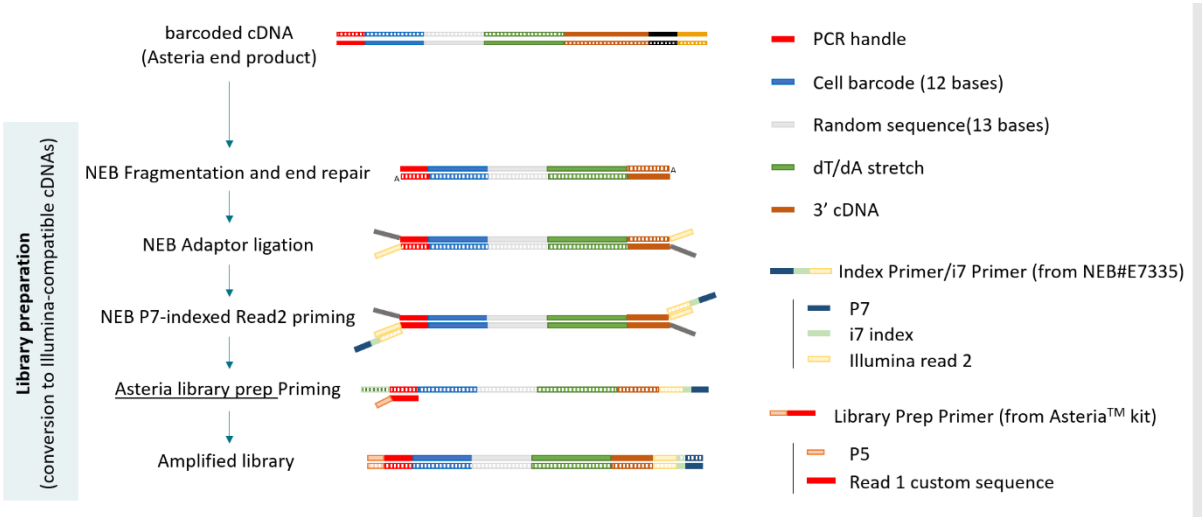


Figure 5: *Schematic representation of the sequences modifications from single cell barcoded cDNA library (Asteria™ end product) to Illumina-compatible cDNA library (using recommended NEBNext kit with Asteria's custom LIBRARY PREP PRIMER).*

- **Library preparation protocol:**

In case one cell sample is split into multiple Asteria™ preparations to increase cell output number, it is still necessary to use a unique library index for each Asteria™ preparation. The Cytonaut™ cloud software will process separately each indexed raw sequencing data and will later allow count matrices pooling in order to reconstitute the initial cell sample (for detailed procedure, see the Cytonaut™ user guide: https://www.cytonaut-scipio.bio/documentation).

The associated protocol for library preparation is available here: Protocol for FS DNA Library Prep Kit (E7805, E6177) with Inputs ≤100 ng | NEB, and the specific modifications required for starting Illumina-compatible library preparation starting from Asteria™ cDNA are listed below:

- Step 1.1.5:
  - Fragmentation step is 5 min at 37°C.

- Step 1.4.1A:
  - Replace “Universal PCR Primer/i5 Primer” by LIBRARY PREP PRIMER (included in the kit; ref:001-1018; use 5µL/PCR).
  - For multiple samples in one sequencing run, use unique index Primer for each library.

- Step 1.4.3:
  - Use 10 cycles of PCR for 5-30ng of cDNA input. When less than 5ng, use 12 cycles of PCR.

- **Quality control and quantification of cDNA libraries:**

Following the final SPRIselect clean-up (at 0.9X), a typical library profile shows a distribution centered around 300-400 bp (**Figure 6**).

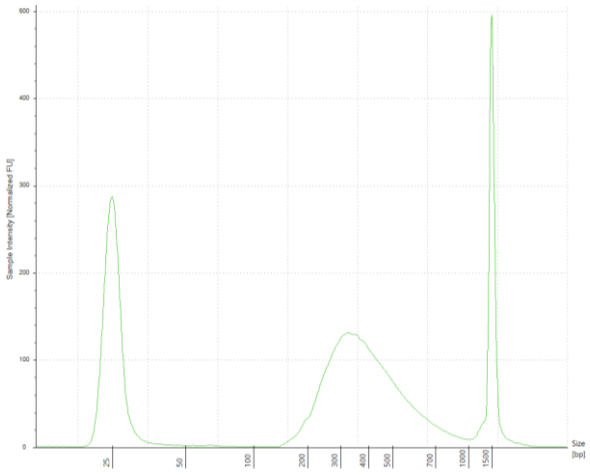


Figure 6: *Example of typical size distribution of Illumina-compatible barcoded cDNA library (Tapestation HSD1000).*

VII. SEQUENCING RECOMMENDATIONS

- **Custom read1 primer**

It is necessary to use READ1 CUSTOM PRIMER (included in Asteria™ kit) for sequencing of Asteria™ library. Concentration of the READ1 CUSTOM PRIMER is 100µM.

To spike-in the READ1 CUSTOM PRIMER into Illumina sequencing primers, please refer to Illumina's guide for spiking custom primer (detail depending on a model of sequencer and kit version):

https://support.illumina.com/bulletins/2016/04/spiking-custom-primers-into-the-illumina-sequencing-primers-.html

When sequencing with NextSeq 1000/2000; it is necessary to prepare Read1 Custom Primer by spiking into HP21 read primer mix of NextSeq 1000/2000 read primer kit. For details, please refer to illumina's NextSeq 1000 and 2000 Custom Primers Guide.

https://emea.support.illumina.com/downloads/nextseq-1000-2000-custom-primers-guide.html

It is strongly recommended to check this detail with your sequencing service provider.

- **Reads per cell**

Required number of raw reads per analyzed cell is largely dependent on cell type and on required depth of information (specific to each project). Following table shows typical median number of transcripts and gene diversity per analyzed cell at given average number of raw reads per analyzed cell for HEK293 and NIH3T3 samples prepared with the Asteria™ kit:

	HEK293	NIH3T3
Median number of transcripts at 20,000 reads per cell	8,000	5,900
Median number of gene diversity at 20,000 reads per cell	3,600	3,000

Example: 20,000 raw reads per analyzed cell are needed for a sample starting with 10,000 NIH/HEK cells:

First, the number of analyzed cells detected as cell barcodes in the sequencing data is a product of input cell number and cell capture rate. In this example (10,000 input NIH/HEK cells), typical cell capture rate is 45%.

Then we have to take into account the proportion of raw reads that are associated with the detected cell barcodes. In this example (10,000 input NIH/HEK cells), the typical proportion of raw reads associated with detected cell barcodes is ~70%.

Thus, the total number of raw reads needed to be sequenced in this example is:

10,000 (input cells) x 0.45 (cell capture rate) x 20,000 (needed average number of raw reads per analyzed cell) / 0.7 (proportion of raw reads associated with detected cell barcodes) = 128 million raw reads.

Please contact Scipio bioscience support team (support@scipio.bio) if further help is needed to estimate the optimal read number required for your samples.

- **Read length**

Recommended sequence length for each read type is as follows:

Read type	Number of cycles	Sequencing information
Read1	25*	- Initial 12 bases for cell barcode - 13th to 25th base for random sequence
Index1 (i7)	6**	Only in case of multiple samples per sequencing run
Read2	50 to 75	3' region of polyA-tailed cellular RNA

* We do not recommend exceeding 25 bases for read1, as it would sequence the part of oligoT in the original mRNA capture oligo, which would significantly decrease base complexity and may result in highly compromised sequence quality.
** When using index primers from the recommended primer kit NEBNext Multiplex Oligos for Illumina (NEB #E7335).

- **PhiX sequencing control**

It is highly recommended to use 1% spike-in PhiX control to sequence your Asteria™ libraries in order to ensure correct read quality control.

- **Compatible sequencer models and sequencing kits**

Here is a list of sequencer instruments and associated kits compatible with Asteria™ kit:

Sequencer Model	Sequencing kit	Reads Capacity	Possible number of samples
NextSeq 500/550	NextSeq500 HighOutput v2.5 (75 cycles)	400 M	2-3
NovaSeq 6000	NovaSeq 6000 SP Reagent Kit (100 cycles)	800 M	4-6
NovaSeq 6000	NovaSeq 6000 S1 Reagent Kit (100 cycles)	1600 M	> 6
NextSeq 1000/2000*	NextSeq 1000/2000 P2 Reagents (100 cycles) v3	400 M	2-3

* for sequencing with NextSeq 1000/2000, HP21 read primer mix is necessary. Please read the Custom Read1 Primer part of the section VII in the previous page.

- **Sequencing data quality control**

It is strongly advised to check the quality of the raw sequencing data. When using Cytonaut™ cloud software, FASTQC files are generated after pre-processing analysis along with basic metrics related to single-cell RNA sequencing data analysis. The sequencing platform could also provide the FASTQC files together with the FASTQ files. For QC of sequencing data, please see Section II.5 of the Cytonaut™ User Guide.

VIII. DATA ANALYSIS USING CYTONAUT™

Cytonaut™ is a software-as-a-service application provided by Scipio bioscience for the analysis of single-cell RNA sequencing data of samples prepared with Scipio bioscience Asteria™ kit, using a simple website: https://www.cytonaut-scipio.bio.

A Cytonaut™ specific user guide is available following this link: https://scipio.bio/ressources/cytonaut-user-guide-v1-8-current.

IX. DOCUMENTATION AND SUPPORT

Customer technical support

Visit http://www.scipio.bio to access the latest service and support information from Scipio bioscience or our nearest representative for your country.

• For any support related to ordering, delivery, invoicing, contact us at sales@scipio.bio.
• For any support related to using your Asteria™ kit, contact us at support@scipio.bio.
• For any support related to using Cytonaut™ software, contact us at support@cytonaut-scipio.bio.

• For accessing your Asteria™ kit documentation, proceed to our website at https://scipio.bio/products/asteria/.
– User guides, protocols, training tutorials and literature
– FAQs
– Certificates of Analysis
– Material Safety Data Sheets (MSDSs)
Note that for MSDSs for reagents and chemicals not supplied with the Asteria™ kit, contact the corresponding manufacturer.

Limited product warranty

Scipio bioscience warrants its products as set forth in **Scipio bioscience**'s General Terms and Conditions of Sale at https://scipio.bio/legal-notices

If you have any questions, please contact us at support@scipio.bio.

ANNEX 1: WORKING WITH SAMPLE SUBFRACTIONS (SPLIT PCRs)

Corresponding to sections IV to VI.

1. Preliminary considerations:

Keeping PCR reactions separated (8 clean-up reactions/sample) instead of pooling them (1 clean-up reaction/sample) allows an additional degree of flexibility:

- Sequencing a sample subfraction:

  Sequencing only 1 (out of 8) PCR reaction allows to check sample quality prior to full sample sequencing (e.g., integrity of cells after dissociation procedure, optimal sequencing parameters), or to zoom in on a reduced number of cells (1/8th) to obtain more detailed transcript information.

- Correcting uneven bead distribution among PCR tubes:

  If it is suspected that beads were not evenly distributed between PCR tubes (during step 10.3), we recommend adjusting the cDNA quantity from each subfraction before library preparation.

Several pooling strategies are possible (described in **Table 3**), from keeping all PCR reactions separated to pooling 2, 4 or 8 PCR reactions (Note: Pooling the 8 PCR reactions is the option described in the main protocol).

When working with lower RNA content cells (PBMC, primary cells, …), cDNA concentrations may be below the detection limit of Tapestation High Sensitivity D5000 for size evaluation. In that case, keeping 8 separate PCR tubes is not recommended.

Initial cell number for coupling	Nb of PCR reactions per sample	Pooled PCR tubes per clean-up reaction	Nb of clean-up reactions per sample	Input cell nb Per clean-up reaction
5,000	8	1 (no pool)	8	625
5,000	8	2	4	1,250
5,000	8	4	2	2,500
5,000	8	8	1	5,000
10,000	8	1 (no pool)	8	1,250
10,000	8	2	4	2,500
10,000	8	4	2	5,000
10,000	8	8	1	10,000
15,000	8	1 (no pool)	8	1,875
15,000	8	2	4	3,750
15,000	8	4	2	7,500
15,000	8	8	1	15,000

***Table 3**: Input cell number per subfraction depending on the pooling strategy.*

2. Optimizations for SPRIselect samples clean-up

Table 4 indicates the volumes required for SPRI purifications depending on the number of cDNA PCR reactions to pool (volumes to apply to the protocol indicated in section IV):

Clean-up Round	SPRI clean-up protocol steps	PCR pooling options			
		No pool (8 Clean-up samples)	PCR reactions **pooled by 2** (4 Clean-up samples)	PCR reactions **pooled by 4** (2 Clean-up samples)	PCR reactions **pooled by 8** (1 Clean-up sample)
1st	Microtube to use (step 2)	0.2mL	1.5mL	1.5mL	1.5mL
	Sample volume (step 2)	45µL	90µL	180µL	360µL
	0.6X SPRIselect volume (step 3)	27µL	54µL	108µL	216µL
	85% ethanol volume/wash (step 7)	200µL	1mL	1mL	1mL
	1st elution volume (step 13)	52µL	52µL	102µL	102µL
2nd	Transferred eluted DNA solution (step 16)	50µL	50µL	100µL	100µL
	Microtube to use (step 16)	0.2mL	0.2mL	0.2mL	0.2mL
	0.6X SPRIselect volume (step 17)	30µL	30µL	60µL	60µL
	85% ethanol volume/wash (step 21)	200µL	200µL	200µL	200µL
	Final elution volume (step 27)	32µL	32µL	32µL	32µL

***Table 4**: Required volumes during SPRIselect clean-up procedure, according to PCR pooling strategy.*

3. Quality control of sample subfractions:

Table 5 provides examples of typical ranges of yield obtained from individual PCR tubes (using indicated pooling options):

Cell types	Initial cell number for coupling	Nb of PCR reactions per SPRIselect clean-up	Nb of subfractions	Input cell nb per subfraction	Typical yield per subfraction
NIH, HEK	10,000	1 (no pool)	8	1,250	5-50ng
PBMC	10,000	2	4	2,500	1-10ng

***Table 5**: Typical DNA concentration yields obtained from Asteria™ kit preparation using the indicated cell types, input number and pooling strategies.*

4. Optimizations of library preparation

- **Recommended cDNA input quantity:**

cDNAs from sample subfractions can be pooled for preparing a single sequencing library. In any case, you should pool equal DNA quantities from every sample subfraction. Nevertheless, the pooling details depends on the yield of each subfraction compared to the optimal DNA quantity:

$$Q_{opt} = \frac{Number\ of\ input\ cells}{Number\ of\ subfractions} * 2 \quad picograms$$

where Q_{opt} is each subfraction optimal cDNA quantity to add in the library preparation mix.

If the yield of each subfraction is larger than Q_{opt}, mix this DNA quantity (Q_{opt}) from each subfraction to obtain your pool. For example, for 10,000 cell sample, the final pool contains 20 ng of DNA.

If one of the subfraction yields is smaller than Q_{opt}, mix equal DNA quantities from each sample subfractions. You will then be limited by the available DNA quantity in the subfraction of smallest yield.

- **Library preparation kit recommendation:**

See the section VI of the main protocol.