STORM-seq (Single-cell Total RNA Miniaturized sequencing) Library Preparation Protocol for Illumina Sequencing

Basic Protocol Title

Automated STORM-seq (Single-cell Total RNA Miniaturized sequencing) Library Preparation Protocol for Illumina Sequencing

Materials

Reagents

Takara STORM-Seq® Kit 76 Rxns Cat Number 634751

Takara SMARTer® RNA Unique Dual Index Kit – 384 Sets A, B, C, D Cat Number 634752-5

ThermoFisher ERCC Spike-In Mix Cat Number 4456740

Beckman Coulter AMPure XP Beads 5mL Product No A63880

Invitrogen RNase Zap Part Number AM9780

Invitrogen UltraPure DNase/RNase-Free Distilled Water Catalog number 10977015

Teknova 10mM Tris-HCl, ph 8.0 CAT.No: T1173

100% Ethanol

Consumables

Thermo Scientific Snap Cap Low Retention Microcentrifuge Tubes 1.5mL Cat Num 3451PK

USA Scientific TEMPASSURE PCR FLEX-FREE 8-TUBE STRIPS Item #1402-4700

ArgosTechnologies™ Microcentrifuge Tubes - 5 mL

Eppendorf twin.tec PCR plate 384 LoBind, skirted, 45 µL, PCR clean Catalog No. 0030129547

USA Scientific Temp Plate EXT Sealing Foil Part Number 2998-7100

*384 well SPT Labtech Low Volume Serial Dilution Plate (P/N 4150-05829)

*Eppendorf twin.tec PCR Plate 96 LoBind, skirted, 150 µL, PCR clean Catalog No. 0030129512

** denotes Automated Protocol only*

Equipment

Benchtop centrifuge

Benchtop vortexer

BioRad Thermal Cycler (Deep Well)

Multichannel pipettes (10uL, 100uL, 200uL) with compatible filter tips

Pipettes (10uL, 100uL, 200uL, 1000uL) with compatible filter tips

Corning CoolRack® XT PCR384 Cooling Block Mfr # 432055

DynaMag™-96 Side Magnet Cat Number 12331D

DynaMag™-5 Magnet Cat Number 12303D

SPT Labtech Mosquito HV

Ice bucket

Timer

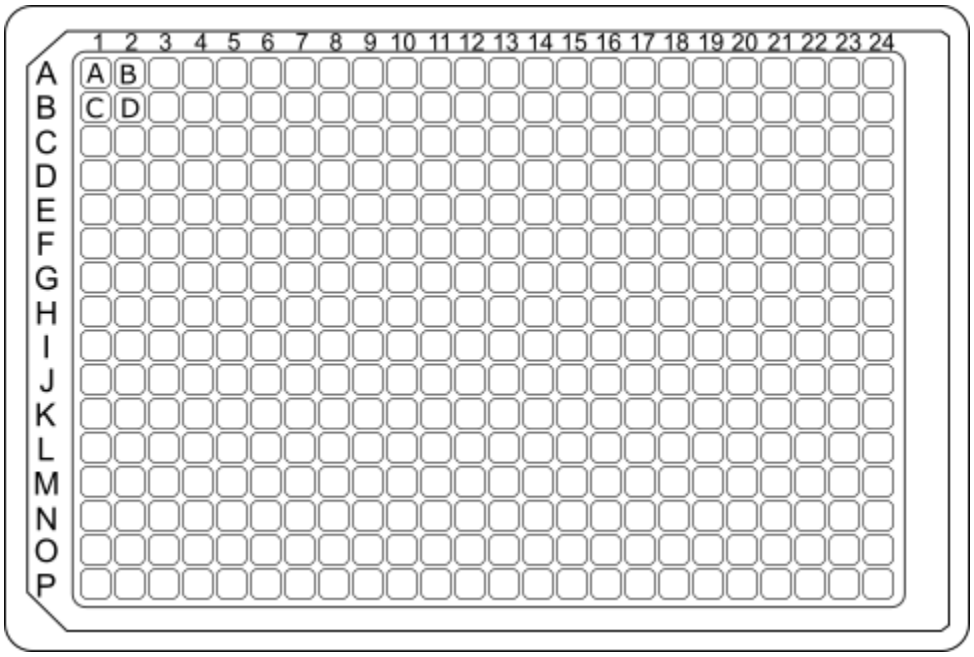
Tube racks

PCR racks

Prior to starting protocol:

Create 1:3 dilution plates of Takara SMARTer® RNA Unique Dual Index Kit Sets A, B, C and D in a 96 well Eppendorf plate using 10mM Tris-HCl pH 8. Combine dilution plates into a single 384 well Eppendorf plate as follows. Diluted UDI plates can be stored at -20°C until depleted.

The indexes will be added as follows:



Quadrant 1 – Set A

Quadrant 2 – Set B

Quadrant 3 - Set C

Quadrant 4 – Set D

Design Plate Layout for Cell Sorting: Leave two wells without a cell as a no template control. Leave two additional wells without a cell to spike in diluted positive control.

Procedure:

Dilute the Control Total RNA

1. Prepare RNase Inhibitor Water (RRI Water) by combining 398 µl of Nuclease-Free Water with 2 µl of RNase Inhibitor. Mix by vortexing and keep on ice until the next step.

2. Dilute Takara Control Total RNA (human brain) to 50 ng/µl by mixing 38 µl of RRI Water with 2 µl of Control Total RNA (1 µg/µl) in a sterile microcentrifuge tube.

NOTE: Fresh dilutions should be made before each use.

3. Further dilute Control Total RNA to 5 ng/µl by mixing 45 µl of RRI Water with 5 µl of 50 ng/µl Control Total RNA in a sterile microcentrifuge tube.

4. Further dilute Control Total RNA to 0.25 ng/µl by mixing 95 µl of RRI Water with 5 µl of 5 ng/µl Control Total RNA in a sterile microcentrifuge tube.

5. Further dilute Control Total RNA to 0.125ng/ul by mixing 4ul of RRI Water with 4 ul of 0.25ng/ul Control Total RNA in a sterile microcentrifuge tube.

6. Keep on ice until Step 5.

Prepare dilution of ERCC spike-ins:

1. Dilute ERCC Spike-in Mix 1 by mixing 18 µl of Molecular Grade Water with 2 µl of stock ERCC Spike-in Mix 1 in a sterile microcentrifuge tube.

2. Further dilute ERCC Spike-in Mix 1 by mixing 38 µl of Molecular Grade Water with 2ul of ERCC Spike-in Dilution 1 in a sterile microcentrifuge tube.

3. Keep on ice until Step 8.

Sort/Fragmentation

1. Preheat the thermal cycler with the program “85-hold”.

2. Prepare a stock solution of 10X Lysis Mix by mixing 10X Lysis Buffer with RNase Inhibitor as indicated below. Mix by gently vortexing and keep on ice until Step 3.

73.2 µl 10X Lysis Buffer
3.85 µl RNase Inhibitor
77.05 µl Total volume

3. Prepare enough Sort/Fragmentation Master Mix for all reactions, by combining the following reagents on ice, in the order shown. Mix by gently vortexing.

538.0 ul 1X dPBS
77.0 µl 10X Lysis Mix (prepared above)
77.0 µl SMART scN6
308.0 µl scRT Buffer
1000 µl Total volume

4. Aliquot 62ul of Sort/Fragmentation Master Mix into wells A, C, E, G, I, K, M, O of columns 1 and 2 in an empty 384-well SPT LVSD plate. This will be the Reagent Plate. Place the Reagent Plate in position 2 on the Mosquito plate deck. Place a clean empty 384-well Eppendorf plate in position 3 on the Mosquito plate deck, this will be the Sample Plate. In the Mosquito software, run the protocol 'STORMSeq 384 Part 1 Setup Sort Plate'. 2.167ul of Sort/Fragmentation buffer will be delivered to each well of the 384 well plate.

5. Spike in 0.5ul controls to previously determined wells. Use RRI water as a negative control and dilute Control RNA as a positive control.

6. Seal with a foil seal and pulse centrifuge to 280 x rcf. Bring Sample Plate on cold block and cells on ice to FACS sorter. Sort a single cell into every well aside from pre-determined control wells. Seal Sample Plate with a fresh seal when sort is complete.

7. When sort is finished, take sealed plate back to bench on cold block on ice. Wipe sealed plate and ice bucket with a kimwipe sprayed with RNase Zap and pulse centrifuge plate to 280 x rcf.

SAFE STOP POINT. Plate can be frozen at -20C for up to 3 days.

NOTE: The next reaction steps (Steps 8–11) are critical for first-strand synthesis and should not be delayed after Step 8.

8. Prepare enough First-Strand Master Mix for all reactions, plus 10%, by combining the following reagents on ice, in the order shown:

357.6 µl SMART UMI-TSO Mix
39.1 µl RNase Inhibitor
<u>162.1 µl SMARTScribe II RT</u>
558.8 µl Total volume

Spike in 1ul of ERCC Spike-in Mix 1 Dilution 2 to First-Strand Master Mix

NOTE: The SMART UMI-TSO Mix is very viscous—it may be left at room temperature after thawing to facilitate accurate pipetting. Make sure to homogenize the First-Strand Master Mix very well by vortexing for ~5 seconds followed by a brief spin-down.

9. Aliquot 34.5 ul into wells A, C, E, G, I, K, M, O of columns 3 and 4 in the Reagent Plate. Store on ice until step 11.

10. Incubate the Sample Plate containing cells at 85°C in a preheated, hot-lid thermal cycler for 3 min then immediately place the plate on an ice-cold PCR chiller rack for 2 min.

NOTE: The samples should be taken out of the thermal cycler immediately after the time indicated to avoid over-fragmentation. Make sure to wait by the thermal cycler when the incubation time is over and immediately chill the samples. Then start the program “RT” and leave it on hold until Step 12.

11. Place the Reagent Plate in position 2 of Mosquito plate deck and Sample Plate (remove seal) in position 3 on the Mosquito plate deck. Start protocol ‘STORMSeq 384 Part 2b To Post PCR1 Pool v1.5’. The Mosquito will deliver 1.17ul per well. At the next pause in the program, place a fresh seal on the plate, vortex and spin down Sample Plate.

NOTE: The samples will be viscous—make sure to homogenize the contents of the plate very well.

12. Incubate the Sample Plate in a preheated hot-lid thermal cycler with the program “RT”:

42°C 180 min
70°C 10 min
4°C forever

13. Leave the samples in the thermal cycler at 4°C until the next step.

SAFE STOPPING POINT: Samples can be left overnight in the thermal cycler at 4°C. If not

processed the next day, freeze the cDNA at –20°C for up to 2 weeks.

PCR1—Addition of Illumina Adapters and Indexes

NOTE: If library purification will be performed immediately following PCR1, remove aliquots of AMPure beads from the refrigerator to allow them to reach room temperature.

1. Preheat the thermal cycler with the program “PCR1”.

2. Prepare a PCR1 Master Mix for all reactions. Combine the following reagents in the order shown, then mix well and spin the tube briefly in a 2.0mL microcentrifuge tube:

134.4 µl Nuclease-Free Water
1708.8 µl SeqAmp CB PCR Buffer (2X)
69.1 µl SeqAmp DNA Polymerase
1912 µl Total volume

3. Aliquot 59 ul into wells A, C, E, G, I, K, M, O of columns 5, 6, 7, 8 in the Reagent Plate.

Place 384-well plates on the Mosquito plate deck as follows:

Position 1: Diluted Takara SMARTer RNA UDI 384 plate

Position 2: Reagent plate

Position 3: Sample plate

4. Resume the Mosquito protocol. 4.67ul of PCR1 Master Mix and 0.33ul of UDI will be delivered to each well.

5. When protocol reaches pause, put a fresh seal on the Sample Plate, gently vortex, then spin down briefly.

6. Place the tubes in a preheated hot-lid thermal cycler. Perform PCR using the program “PCR1”

94°C 1 min

10 cycles:

98°C 15 sec
55°C 15 sec
68°C 30 sec

68°C 2 min
4°C forever

SAFE STOPPING POINT: Samples can be left for up to 1 hr in the thermal cycler at 4°C. If not processed within the next hour, freeze the PCR products at –20°C for up to 2 weeks.

Pooling

1. Start protocol 'STORMSeq 384 Part 3 Post PCR1 Pool. Place plates on the Mosquito Plate Deck as follows:

Position 3: Sample Plate

Position 5: Pooled Sample Plate (new 96-well Eppendorf plate)

Continue Mosquito protocol. The quadrants will be pooled into the first four columns in the 96-well plate.

2. Pool columns by hand into a 5mL ArgosTechnologies™ Microcentrifuge Tubel.

SAFE STOPPING POINT: Samples can be left at –20°C for up to 2 weeks.

The rest of the protocol is performed by hand.

IMPORTANT: Do not start this protocol if you do not have enough time to perform all steps up to ribosomal depletion.

3. Allow AMPure beads to come to room temperature before use (~30 min). Make 15mL of 80% EtOH.

4. Add 2048 ul AMPure beads to pooled sample. The beads are viscous and even when using such a large volume, it is critical to pipette the entire volume up and then out very slowly.

5. Mix by vortexing gently.

6. Incubate at room temperature for 8 min to allow the DNA to bind to the beads.

7. Place the sample tube on the magnetic separation device for 10 min or longer, until the solution is completely clear.

NOTE: Because of the large volume involved, a very strong magnet is required. Make sure the solution is completely clear before moving on to the next step. Failure to recover all the beads will lead to low-yield and low-quality libraries.

8. While the tube is sitting on the magnetic separation device, pipette out the supernatant and discard.

9. Keeping the tube on the magnetic separation device, add 5mL of freshly made 80% ethanol to each sample—without disturbing the beads—to wash away contaminants. Wait for 1 min and carefully pipette out and discard the supernatant. cDNA will remain bound to the beads during the washing process.

10. Repeat Step 9 once.

11. Let the open sample tube rest at room temperature for 7-8 min until the pellets appear dry.

12. Once the beads are dry, add 201 µl of Nuclease-Free Water to the tube. Mix thoroughly by vortexing to resuspend all the beads. Note: Because of the large number of beads and low volume of water, vortexing the tube on its side allows for easier resuspension.

13. Incubate at room temperature for 5 min to rehydrate.

14. Place the sample tube on the magnetic separation device for 5 min or longer, until the solution is completely clear. The solution may appear clear after only 1 min, but still incubate for 5 min.

15. Pipette 200 µl of supernatant into a new 1.5mL Eppendorf tube.

16. Add 160 µl of AMPure beads to the sample and mix by gently vortexing.

NOTE: Accurate pipetting of AMPure beads with a well-calibrated pipet is critical.

17. Incubate at room temperature for 8 min to allow the DNA to bind to the beads. During the incubation time, proceed immediately to the next section.

Depletion of Ribosomal cDNA with scZapR and scR-Probes

1. Thaw scR-Probes and ZapR Buffer at room temperature. Place scR-Probes on ice as soon as it is thawed, but keep ZapR Buffer at room temperature. scZapR should be kept on ice at all times and returned to the freezer immediately after use.

2. Preheat the thermal cycler with the program “PreZap”.

3. Upon completion of the 8-min incubation (step 16), place the sample tube on the magnetic separation device for 5 min or longer, until the solution is completely clear.

NOTE: It is acceptable—and in some cases necessary—to leave the tube on the magnetic separation device for more than 5 min.

4. During the 5-min incubation time in Step 3, pipette 14 ul scR-Probes into a pre-chilled PCR tube. Keep the PCR tube containing scR-Probes on ice and immediately return the remaining unused scR-Probes to a –70°C freezer.

5. Incubate the PCR tube containing scR-Probes at 72°C in a preheated hot-lid thermal cycler using the program “PreZap”:

72°C 2 min
4°C forever

6. Leave the scR-Probes tube in the thermal cycler at 4°C for at least 2 min, but for no more than 10–15 min, before using it in the next step.

7. Once the 5-min incubation on the magnetic separation device is complete, pipette out the supernatant and discard, while keeping the tube sitting on the magnetic separation device.

8. Keeping the tube on the magnetic separation device, add 1000 ul of freshly made 80% ethanol to the sample—without disturbing the beads—to wash away contaminants. Wait for 1 min and carefully pipette out and discard the supernatant. cDNA will remain bound to the beads during the washing process.

9. Repeat Step 8 once

10. Allow beads to dry for 5 minutes.

11. While the beads are drying, prepare the scZapR Master Mix. Prepare enough Master Mix for all reactions by combining the following reagents at room temperature in the order shown.

Make sure to add the preheated and chilled scR-Probes last. Return scZapR to a –20°C freezer immediately after use. Mix the components well by vortexing gently and spin the tubes briefly in a microcentrifuge.

152.8 µl Nuclease-Free Water
20 µl 10X ZapR Buffer
13.6 µl scZapR
13.6 µl scR-Probes
200 µl Total volume per reaction

12. Once the beads are dry, move the sample off the magnet. Add 162ul Zap Master Mix to the tube and vortex to resuspend. Quickly spin down sample.

13. Incubate at room temperature for 5 min to rehydrate, place the tube on the magnetic separation device for 1 min or longer, until the solution is completely clear.

14. Pipette out 160ul of supernatant to a well of an 8 well strip tube. Spin down briefly.

15. Incubate the tubes in a preheated hot-lid thermal cycler using the program “Zap”:

37°C 60 min
72°C 10 min
4°C forever

NOTE: Samples can be left in the thermal cycler at 4°C for up to 1 hr. However, we recommend proceeding immediately to the next step.

NOTE: ENSURE THERMAL CYCLER VOLUME IS SET TO MAXIMUM VOLUME

PCR2—Final RNA-Seq Library Amplification

1. Preheat the thermal cycler with the program “PCR2”.

2. Prepare a PCR2 Master Mix for all reactions, by combining the following reagents in the order shown below. Then mix well and spin the tubes briefly in a microcentrifuge.

228.8 µl Nuclease-Free Water
440 µl SeqAmp CB PCR Buffer
17.6 µl PCR2 Primers
17.6 µl SeqAmp DNA Polymerase
704 µl Total volume

3. Add 640 uL of PCR2 Master Mix to a 1.5mL Eppendorf tube. Then add the 160ul volume from the depleted sample pool to the tube. Mix by vortexing gently, then spin down.

4. Divide the 800ul sample volume between eight PCR tubes (100 ul in each tube) (ensuring sample volume is within spec of thermal cycler - this is often 125 uL max for deep well reaction thermal cyclers).

5. Place the tubes in a preheated hot-lid thermal cycler. Perform PCR using the program “PCR2” and the recommended number of cycles according to Table I (13 cycles currently recommended, adjust as necessary):

94°C 1 min

10–14 cycles:

98°C 15 sec
55°C 15 sec
68°C 30 sec

4°C forever

SAFE STOPPING POINT: Samples can be left overnight in the thermal cycler at 4°C. If not processed within the next day, freeze the PCR products at –20°C for up to 2 weeks.

IMPORTANT: Recombine the individual pools from the split reactions done in 8 before proceeding to the next purification. Example: Sample pool was 800 uL and was split into 8 x 100

uL reactions for the PCR2 amplification. Recombine each 100 uL amplification into a single 800 uL pool again in a new 1.5mL eppendorf tube.

Purification of Final RNA-Seq Library Using AMPure Beads

1. Allow AMPure beads to come to room temperature before use (~30 min). Transfer each sample pool to a 1.5mL tube, recombining the split samples. Add 720 µl of AMPure beads to the sample pool and mix well by vortexing for 5 sec.

NOTE: Accurate pipetting of AMPure beads with a well-calibrated pipet is critical.

2. Incubate at room temperature for 8 min to let the cDNA bind to the beads.

3. Briefly spin the sample tube to collect the liquid at the bottom. Place the sample tube on the magnetic separation device for 10 min or longer, until the solution is completely clear.

4. While the tube is sitting on the magnetic separation device, pipette out the supernatant and discard.

5. Keep the tube on the magnetic separation device. Without disturbing the beads, add 1000 µl of freshly made 80% ethanol to the sample to wash away contaminants. Wait for 30 sec and carefully pipette out the supernatant. cDNA will remain bound to the beads during the washing process.

6. Repeat Step 5 once.

7. Perform a brief spin of the tube (~2,000g) to collect the remaining ethanol at the bottom of each tube. Place the tube on the magnetic separation device for 30 sec, then carefully remove all remaining ethanol with a pipette, without disturbing the beads.

8. Let the sample tube rest open at room temperature for 5-10 min until the pellets appear dry.

NOTE: You may see a tiny crack in each pellet. Do not overdry. Once the beads are dry, add 82 µl of Tris Buffer to cover the beads. Close the tube, remove from the magnetic separation device and mix thoroughly by vortexing until all the beads have been washed off the sides of the tube.

9. Incubate at room temperature for 5 min to rehydrate.

10. Briefly spin the sample tube. Place the sample tube on the magnetic separation device for 5 min or longer, until the solution is completely clear.

11. Transfer the supernatants (80 µl) to a new 1.5mL Eppendorf tube.

12. Perform a second bead clean-up by adding 72 µl of AMPure beads to the sample. Mix by vortexing for 5 sec.

NOTE: Accurate pipetting of AMPure beads with a well-calibrated pipet is critical.

13. Incubate at room temperature for 8 min to let the DNA bind to the beads.

14. Briefly spin the sample tube to collect the liquid from the side of the wall. Place the sample tube on the magnetic separation device for 5 min or longer, until the solution is completely clear.

15. While the tube is sitting on the magnetic stand, pipette out the supernatant.

16. Keep the tube on the magnetic stand. Add 1000 µl of freshly made 80% ethanol to each sample, without disturbing the beads, to wash away contaminants. Wait for 30 sec and carefully pipette out the supernatant. DNA will remain bound to the beads during the washing process.

17. Repeat Step 16 one more time.

18. Perform a brief spin of the tubes (~2,000g) to collect the remaining ethanol at the bottom of each tube. Place the tube on the magnetic stand for 30 sec, then remove all the remaining ethanol with a pipette.

19. Let the sample tube rest open at room temperature for 3-5 min until the pellet appears dry. You may see a tiny crack in the pellet.

20. Once the beads are dry, add 16 µl of Tris Buffer to cover the beads. Close the tube, remove the tube from the magnetic separation device and mix thoroughly by vortexing until all the beads have been washed off the sides of the tube.

21. Incubate at room temperature for 5 min to rehydrate.

22. Briefly spin the sample tube. Place the sample tube on the magnetic separation device for 2 min or longer, until the solution is completely clear.

23. Transfer the supernatants (15 µl) to non-sticky low-bind tube.

24. Proceed immediately to validation or store at –20°C.

Alternate Protocol Title

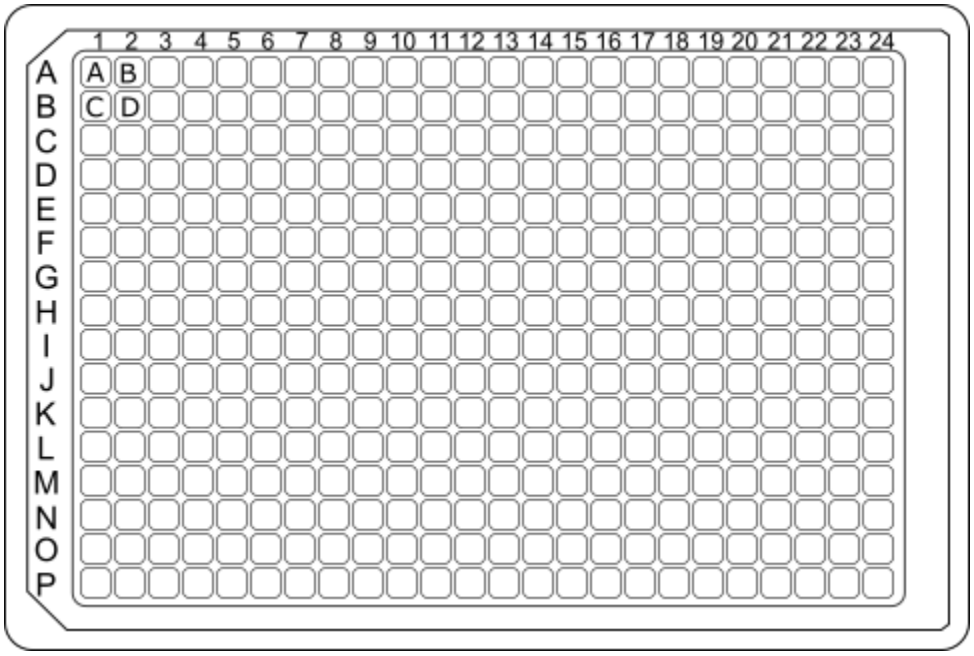
Manual STORM-seq (Single-cell Total RNA Miniaturized sequencing) Library Preparation Protocol for Illumina Sequencing

Protocol Steps

Prior to starting protocol:

Create 1:6 dilution plates of Takara SMARTer® RNA Unique Dual Index Kit Sets A, B, C and D in a 96 well Eppendorf plate using 10mM Tris-HCl pH 8. Combine dilution plates into a single 384 well Eppendorf plate as follows. Diluted UDI plates can be stored at -20°C until depleted.

The indexes will be added as follows:



Quadrant 1 – Set A

Quadrant 2 – Set B

Quadrant 3 - Set C

Quadrant 4 – Set D

Design Plate Layout for Cell Sorting: Leave two wells without a cell as a no template control. Leave two additional wells without a cell to spike in diluted positive control.

Procedure:

Dilute the Control Total RNA

1. Prepare RNase Inhibitor Water (RRI Water) by combining 398 µl of Nuclease-Free Water with 2 µl of RNase Inhibitor. Mix by vortexing and keep on ice until the next step.

2. Dilute Takara Control Total RNA (human brain) to 50 ng/µl by mixing 38 µl of RRI Water with 2 µl of Control Total RNA (1 µg/µl) in a sterile microcentrifuge tube.

NOTE: Fresh dilutions should be made before each use.

3. Further dilute Control Total RNA to 5 ng/µl by mixing 45 µl of RRI Water with 5 µl of 50 ng/µl Control Total RNA in a sterile microcentrifuge tube.

4. Further dilute Control Total RNA to 0.25 ng/µl by mixing 95 µl of RRI Water with 5 µl of 5 ng/µl Control Total RNA in a sterile microcentrifuge tube.

5. Further dilute Control Total RNA to 0.125ng/ul by mixing 4ul of RRI Water with 4 ul of 0.25ng/ul Control Total RNA in a sterile microcentrifuge tube.

6. Keep on ice until Step 5.

Prepare dilution of ERCC spike-ins:

1. Dilute ERCC Spike-in Mix 1 by mixing 18 µl of Molecular Grade Water with 2 µl of stock ERCC Spike-in Mix 1 in a sterile microcentrifuge tube.

2. Further dilute ERCC Spike-in Mix 1 by mixing 38 µl of Molecular Grade Water with 2ul of ERCC Spike-in Dilution 1 in a sterile microcentrifuge tube.

3. Keep on ice until Step 8.

Sort/Fragmentation

1. Preheat the thermal cycler with the program “85-hold”.

2. Prepare a stock solution of 10X Lysis Mix by mixing 10X Lysis Buffer with RNase Inhibitor as indicated below. Mix by gently vortexing and keep on ice until Step 3.

73.2 µl 10X Lysis Buffer
3.85 µl RNase Inhibitor
77.05 µl Total volume

3. Prepare enough Sort/Fragmentation Master Mix for all reactions, by combining the following reagents on ice, in the order shown. Mix by gently vortexing.

538.0 ul 1X dPBS
77.0 µl 10X Lysis Mix (prepared above)
77.0 µl SMART scN6
308.0 µl scRT Buffer
1000 µl Total volume

4. Aliquot 124ul of Sort/Fragmentation Master Mix into wells of a clean PCR strip tube. Use a multichannel pipette to add 2.167ul of Sort/Fragmentation buffer to each well of the 384 well plate (Sample Plate).

5. Spike in 0.5ul controls to previously determined wells. Use RRI water as a negative control and dilute Control RNA as a positive control.

6. Seal with a foil seal and pulse centrifuge to 280 x rcf. Bring Sample Plate on cold block and cells on ice to FACS sorter. Sort a single cell into every well aside from pre-determined control wells. Seal Sample Plate with a fresh seal when sort is complete.

7. When sort is finished, take sealed plate back to bench on cold block on ice. Wipe sealed plate and ice bucket with a kimwipe sprayed with RNase Zap and pulse centrifuge plate to 280 x rcf.

SAFE STOP POINT. Plate can be frozen at -20C for up to 3 days.

NOTE: The next reaction steps (Steps 8–11) are critical for first-strand synthesis and should not be delayed after Step 8.

8. Prepare enough First-Strand Master Mix for all reactions, plus 10%, by combining the following reagents on ice, in the order shown:

357.6 µl SMART UMI-TSO Mix
39.1 µl RNase Inhibitor
162.1 µl SMARTScribe II RT
558.8 µl Total volume

Spike in 1ul of ERCC Spike-in Mix 1 Dilution 2 to First-Strand Master Mix

NOTE: The SMART UMI-TSO Mix is very viscous—it may be left at room temperature after thawing to facilitate accurate pipetting. Make sure to homogenize the First-Strand Master Mix very well by vortexing for ~5 seconds followed by a brief spin-down.

9. Aliquot 69 ul into wells of a clean PCR strip tube. Close caps, spin briefly and store on ice until step 11.

10. Incubate the Sample Plate containing cells at 85°C in a preheated, hot-lid thermal cycler for 3 min then immediately place the plate on an ice-cold PCR chiller rack for 2 min.

NOTE: The samples should be taken out of the thermal cycler immediately after the time indicated to avoid over-fragmentation. Make sure to wait by the thermal cycler when the incubation time is over and immediately chill the samples. Then start the program “RT” and leave it on hold until Step 12.

11. Use a multichannel pipette to deliver 1.17ul of First-Strand Master Mix to each well of the Sample Plate. Place a fresh seal on the plate, vortex and spin down the Sample Plate.

NOTE: The samples will be viscous—make sure to homogenize the contents of the plate very well.

12. Incubate the Sample Plate in a preheated hot-lid thermal cycler with the program “RT”:

42°C 180 min
70°C 10 min
4°C forever

13. Leave the samples in the thermal cycler at 4°C until the next step.

SAFE STOPPING POINT: Samples can be left overnight in the thermal cycler at 4°C. If not processed the next day, freeze the cDNA at –20°C for up to 2 weeks.

PCR1—Addition of Illumina Adapters and Indexes

NOTE: If library purification will be performed immediately following PCR1, remove aliquots of AMPure beads from the refrigerator to allow them to reach room temperature.

1. Preheat the thermal cycler with the program “PCR1”.

2. Prepare a PCR1 Master Mix for all reactions. Combine the following reagents in the order shown, then mix well and spin the tube briefly in a 2.0mL microcentrifuge tube:

134.4 µl Nuclease-Free Water
1708.8 µl SeqAmp CB PCR Buffer (2X)
69.1 µl SeqAmp DNA Polymerase
1912 µl Total volume

3. Aliquot 118 ul into wells of two clean PCR strip tubes and spin down briefly.

4. Using a multichannel pipette, add 4.67ul of PCR1 Master Mix and 1.0ul of UDI to each well.

5. Put a fresh seal on the Sample Plate, gently vortex, then spin down briefly.

6. Place the tubes in a preheated hot-lid thermal cycler. Perform PCR using the program “PCR1”

94°C 1 min

10 cycles:

98°C 15 sec
55°C 15 sec
68°C 30 sec

68°C 2 min
4°C forever

SAFE STOPPING POINT: Samples can be left for up to 1 hr in the thermal cycler at 4°C. If not processed within the next hour, freeze the PCR products at –20°C for up to 2 weeks.

Pooling

1. Pool sample plate into 4 clean PCR strip tubes, one quadrant per strip tube.

2. Combine two of the strip tubes samples into a 5mL ArgosTechnologies™ Microcentrifuge Tube. Combine the remaining two strip tube samples into a second 5mL tube.

SAFE STOPPING POINT: Samples can be left at –20°C for up to 2 weeks.

IMPORTANT: Do not start this protocol if you do not have enough time to perform all steps up to ribosomal depletion.

1. Allow AMPure beads to come to room temperature before use (~30 min). Make 15mL of 80% EtOH.

4. Add 1083 ul AMPure beads to EACH pooled sample. The beads are viscous and even when using such a large volume, it is critical to pipette the entire volume up and then out very slowly.

5. Mix by vortexing gently.

6. Incubate at room temperature for 8 min to allow the DNA to bind to the beads.

7. Place the sample tubes on the magnetic separation device for 10 min or longer, until the solution is completely clear.

NOTE: Because of the large volume involved, a very strong magnet is required. Make sure the solution is completely clear before moving on to the next step. Failure to recover all the beads will lead to low-yield and low-quality libraries.

8. While the tubes are sitting on the magnetic separation device, pipette out the supernatant and discard.

9. Keeping the tubes on the magnetic separation device, add 5mL of freshly made 80% ethanol to each sample—without disturbing the beads—to wash away contaminants. Wait for 1 min and carefully pipette out and discard the supernatant. cDNA will remain bound to the beads during the washing process.

10. Repeat Step 9 once.

11. Let the open sample tubes rest at room temperature for 7-8 min until the pellets appear dry.

12. Once the beads are dry, add 101 µl of Nuclease-Free Water to cover the beads. Mix thoroughly by vortexing until all the beads have been washed off the sides of the tubes. Note: Because of the large number of beads and low volume of water, vortexing the tube on its side allows for easier resuspension.

13. Incubate at room temperature for 5 min to rehydrate.

14. Place the sample tube on the magnetic separation device for 5 min or longer, until the solution is completely clear. The solution may appear clear after only 1 min, but still incubate for 5 min.

15. Pipette 100 µl of EACH supernatant into the SAME new 1.5mL Eppendorf tube to form one pool of 200ul.

16. Add 160 µl of AMPure beads to the sample and mix by gently vortexing.

NOTE: Accurate pipetting of AMPure beads with a well-calibrated pipet is critical.

17. Incubate at room temperature for 8 min to allow the DNA to bind to the beads. During the incubation time, proceed immediately to the next section.

Depletion of Ribosomal cDNA with scZapR and scR-Probes

1. Thaw scR-Probes and ZapR Buffer at room temperature. Place scR-Probes on ice as soon as it is thawed, but keep ZapR Buffer at room temperature. scZapR should be kept on ice at all times and returned to the freezer immediately after use.

2. Preheat the thermal cycler with the program “PreZap”.

3. Upon completion of the 8-min incubation (step 16), place the sample tube on the magnetic separation device for 5 min or longer, until the solution is completely clear.

NOTE: It is acceptable—and in some cases necessary—to leave the tube on the magnetic separation device for more than 5 min.

4. During the 5-min incubation time in Step 3, pipette 14 ul scR-Probes into a pre-chilled PCR tube. Keep the PCR tube containing scR-Probes on ice and immediately return the remaining unused scR-Probes to a –70°C freezer.

5. Incubate the PCR tube containing scR-Probes at 72°C in a preheated hot-lid thermal cycler using the program “PreZap”:

72°C 2 min
4°C forever

6. Leave the scR-Probes tube in the thermal cycler at 4°C for at least 2 min, but for no more than 10–15 min, before using it in the next step.

7. Once the 5-min incubation on the magnetic separation device is complete, pipette out the supernatant and discard, while keeping the tube sitting on the magnetic separation device.

8. Keeping the tube on the magnetic separation device, add 1000 ul of freshly made 80% ethanol to the sample—without disturbing the beads—to wash away contaminants. Wait for 1 min and carefully pipette out and discard the supernatant. cDNA will remain bound to the beads during the washing process.

9. Repeat Step 8 once

10. Allow beads to dry for 5 minutes.

11. While the beads are drying, prepare the scZapR Master Mix. Prepare enough Master Mix for all reactions by combining the following reagents at room temperature in the order shown.

Make sure to add the preheated and chilled scR-Probes last. Return scZapR to a –20°C freezer immediately after use. Mix the components well by vortexing gently and spin the tubes briefly in a microcentrifuge.

152.8 µl Nuclease-Free Water
20 µl 10X ZapR Buffer
13.6 µl scZapR
13.6 µl scR-Probes
200 µl Total volume per reaction

12. Once the beads are dry, move the sample off the magnet. Add 162ul Zap Master Mix to the tube. Vortex to resuspend, then briefly spin down.

13. Incubate at room temperature for 5 min to rehydrate, place the tube on the magnetic separation device for 1 min or longer, until the solution is completely clear.

14. Pipette out 160ul of supernatant to a well of an 8 well strip tube. Spin down briefly.

15. Incubate the tubes in a preheated hot-lid thermal cycler using the program “Zap”:

37°C 60 min
72°C 10 min
4°C forever

NOTE: Samples can be left in the thermal cycler at 4°C for up to 1 hr. However, we recommend proceeding immediately to the next step.

NOTE: ENSURE THERMAL CYCLER VOLUME IS SET TO MAXIMUM VOLUME

PCR2—Final RNA-Seq Library Amplification

1. Preheat the thermal cycler with the program “PCR2”.

2. Prepare a PCR2 Master Mix for all reactions, by combining the following reagents in the order shown below. Then mix well and spin the tubes briefly in a microcentrifuge.

228.8 µl Nuclease-Free Water
440 µl SeqAmp CB PCR Buffer
17.6 µl PCR2 Primers
17.6 µl SeqAmp DNA Polymerase
704 µl Total volume

3. Add 640 uL of PCR2 Master Mix to a 1.5mL Eppendorf tube. Then add the 160ul volume from the depleted sample pool to the tube. Mix by vortexing gently, then spin down.

4. Divide the 800ul sample volume between eight PCR tubes (100 ul in each tube) (ensuring sample volume is within spec of thermal cycler - this is often 125 uL max for deep well reaction thermal cyclers).

5. Place the tubes in a preheated hot-lid thermal cycler. Perform PCR using the program "PCR2" and the recommended number of cycles according to Table I (13 cycles currently recommended, adjust as necessary):

94°C 1 min

10–14 cycles:

98°C 15 sec
55°C 15 sec
68°C 30 sec

4°C forever

SAFE STOPPING POINT: Samples can be left overnight in the thermal cycler at 4°C. If not processed within the next day, freeze the PCR products at –20°C for up to 2 weeks.

IMPORTANT: Recombine the individual pools from the split reactions done in 8 before proceeding to the next purification. Example: Sample pool was 800 uL and was split into 8 x 100 uL reactions for the PCR2 amplification. Recombine each 100 uL amplification into a single 800 uL pool again in a new 1.5mL eppendorf tube.

Purification of Final RNA-Seq Library Using AMPure Beads

1. Allow AMPure beads to come to room temperature before use (~30 min). Transfer each sample pool to a 1.5mL tube, recombining the split samples. Add 720 µl of AMPure beads to the sample pool and mix well by vortexing for 5 sec.

NOTE: Accurate pipetting of AMPure beads with a well-calibrated pipet is critical.

2. Incubate at room temperature for 8 min to let the cDNA bind to the beads.

3. Briefly spin the sample tube to collect the liquid at the bottom. Place the sample tube on the magnetic separation device for 10 min or longer, until the solution is completely clear.

4. While the tube is sitting on the magnetic separation device, pipette out the supernatant and discard.

5. Keep the tube on the magnetic separation device. Without disturbing the beads, add 1000 µl of freshly made 80% ethanol to the sample to wash away contaminants. Wait for 30 sec and carefully pipette out the supernatant. cDNA will remain bound to the beads during the washing process.

6. Repeat Step 5 once.

7. Perform a brief spin of the tube (~2,000g) to collect the remaining ethanol at the bottom of each tube. Place the tube on the magnetic separation device for 30 sec, then carefully remove all remaining ethanol with a pipette, without disturbing the beads.

8. Let the sample tube rest open at room temperature for 5-10 min until the pellets appear dry.

NOTE: You may see a tiny crack in each pellet. Do not overdry. Once the beads are dry, add 82 µl of Tris Buffer to cover the beads. Close the tube, remove from the magnetic separation device and mix thoroughly by vortexing until all the beads have been washed off the sides of the tube.

9. Incubate at room temperature for 5 min to rehydrate.

10. Briefly spin the sample tube. Place the sample tube on the magnetic separation device for 5 min or longer, until the solution is completely clear.

11. Transfer the supernatants (80 µl) to a new 1.5mL Eppendorf tube.

12. Perform a second bead clean-up by adding 72 µl of AMPure beads to the sample. Mix by vortexing for 5 sec.

NOTE: Accurate pipetting of AMPure beads with a well-calibrated pipet is critical.

13. Incubate at room temperature for 8 min to let the DNA bind to the beads.

14. Briefly spin the sample tube to collect the liquid from the side of the wall. Place the sample tube on the magnetic separation device for 5 min or longer, until the solution is completely clear.

15. While the tube is sitting on the magnetic stand, pipette out the supernatant.

16. Keep the tube on the magnetic stand. Add 1000 µl of freshly made 80% ethanol to each sample, without disturbing the beads, to wash away contaminants. Wait for 30 sec and carefully pipette out the supernatant. DNA will remain bound to the beads during the washing process.

17. Repeat Step 16 one more time.

18. Perform a brief spin of the tubes (~2,000g) to collect the remaining ethanol at the bottom of each tube. Place the tube on the magnetic stand for 30 sec, then remove all the remaining ethanol with a pipette.

19. Let the sample tube rest open at room temperature for 3-5 min until the pellet appears dry. You may see a tiny crack in the pellet.

20. Once the beads are dry, add 16 µl of Tris Buffer to cover the beads. Close the tube, remove the tube from the magnetic separation device and mix thoroughly by vortexing until all the beads have been washed off the sides of the tube.

21. Incubate at room temperature for 5 min to rehydrate.

22. Briefly spin the sample tube. Place the sample tube on the magnetic separation device for 2 min or longer, until the solution is completely clear.

23. Transfer the supernatants (15 µl) to non-sticky low-bind tube.

24. Proceed immediately to validation or store at –20°C.