*Quartz-seq2 protocol\_v6*

*This protocol mostly follows the detailed protocol sent by Dr. Yohei Sasagawa. All steps from priming and reverse transcription are as per the new protocol. The preparation of lysis plates and sorting is still as per our original GIH protocol, as these steps have already been performed. This new protocol incorporates many changes, including reduced RT concentration and different first strand cDNA purification. Strong emphasis is also placed on controlling TdT concentration and reaction time during A-tailing step. This protocol differs from v5 only with decreased cDNA amplification cycles and a final 0.6X Ampure cleanup.*

# Preparation of plates

## ◆Step 1<a> Preparation of oligo master stock plates (time)

Purpose: Resuspend supplied lyophilized barcoded RT oligos at high concentration for long-term storage.

1. Spin down 96 well plates to collect lyophilized oligo in the bottom of the well.
2. Place 250 µl TE (low E; 10mM Tris, 0.1mM EDTA) buffer in wells of 8-strip tubes for multichannel pipette use. Will need to be topped up with more buffer every 3 columns.
3. Add 80 µl TE to each well of 96 well plate to give a 50µM concentration. These are the ‘master stock’ plates.
4. Place Microseal F plate seal securely on plate and vortex to thoroughly resuspend oligo.
5. Quick spin and leave at RT for 30min to ensure oligo is completely resuspended.
6. Store at -80C.

## ◆Step 1<b> Preparation of oligo working stock plate (time)

Purpose: Dilute oligos to a concentration useful for preparing lysis plates.

1. Place 250 µl UltraPure H2O in wells of 8-strip tubes for multichannel pipette use. Will need to be topped up with more water every 2 columns.
2. Add 98 µl water to each well of new 96 well plates.
3. Transfer 2 µl of oligo master stock from 96 well plates to new plate to give 1 µM concentration. These are the ‘working stock’ plates.

## ◆Step 1<c> Preparation of lysis buffer master mix (time)

Purpose: Combine all components of lysis buffer together to allow aliquoting into single use plates.

1. Thaw oligo working stock plate (1 µM) on ice.
2. Prepare lysis buffer for appropriate number of plates in LoBind 50mL tubes [Table 1]. Mix well by pipetting slowly 15 times. Place the tube on ice until just before use.

Table 1 : Lysis buffer master mix (factoring in 1 in 1.125 dilution with addition of oligos)

|  |  |  |
| --- | --- | --- |
| Reagent | 1 plate | 6 plates (7.5x) |
| H2O | 355.05 µl | 2662.87 µl |
| 10mM dNTPs | 5.19 µl | 38.93 µl |
| 10% NP-40 | 12.96 µl | 97.2 µl |
| RNasin Plus | 10.8 µl | 81 µl |
| Total volume | 384 µl | 2880 µl |

## ◆Step 1<d> Aliquoting of lysis buffer into 384 well plates (time)

Purpose: Prepare final lysis plates for sample collection. Volumes are for 10 plates (scale up or down as necessary).

1. Dispense 170 μl of lysis buffer master mix into each tube of 2x 8-tube PCR strip. Spin down the tubes in a centrifuge to collect the solution to the bottom. Place the tubes on ice until just before use.
2. Add 6 μl lysis buffer to each well of 384 well plate.
3. Add 0.75 μl/well of oligo working stock into appropriate wells of 384 well plate containing 6 μl lysis buffer. Mix by pipetting gently 15x. This is the ‘prep’ plate.
4. Pipette 1uL from prep plate into each well of 6x 384 well LoBind twin.tec plates. These are the ‘lysis’ plates ready for sample collection. Seal securely with foil seal for short-term storage at -80C, or PCR seal for transport to flow cytometry facility. Keep on ice until loaded onto flow cytometer.

# Sample collection

## ◆Step 2<a> Sorting cells into 384 well plates (time)

Purpose: Collect cells into wells of lysis plates.

1. Ensure flow cytometer is set up for single cell sorting (highest purity option) into twin.tec 384 well plates (empty plate may be required to set up instrument).
2. Randomly assign ‘negative’ control wells (10/plate) to receive no cells, and set up on sorting template.
3. Sort desired cell population into wells. Immediately after completing a plate, seal well with Microseal F and spin 10,000g 1 min at 4C (if possible).
4. Vortex mix for 1 min, and repeat centrifugation 10,000g 1 min.
5. Store at -80C until ready to perform next step. Transport from FACS facility on dry ice.
6. Repeat for as many plates as desired (use different random negative control wells for each plate).

# Generation of full-length amplified cDNA

## ◆Step 3<a> Reverse transcription (time)

Purpose: Generate cDNA from each single cell in separate wells.

1. Remove plate (1 at a time) from -80C and thaw on ice
2. Spin down to collect lysis buffer in bottom of wells.
3. Denature and hybridise primers to RNA according to Table 2.

Table 2 : Primer hybridisation

|  |  |
| --- | --- |
| Temperature | Time |
| 70℃ | 90 seconds |
| 35℃ | 15 seconds |
| 4℃ | hold |

1. Spin down to collect volume in the bottom of wells and place on ice.
2. Prepare RT premix according to Table 3.

Table 3 : RT premix (RT6.25 condition)

|  |  |  |
| --- | --- | --- |
| Reagent | 1 plate | 1.3 plates |
| H2O | 303.46 µl | 394.49 µl |
| ThermoPol Buffer | 76.8 µl | 99.85 µl |
| SuperScript III | 2.41 µl | 3.13 µl |
| RNAsin Plus | 1.33 µl | 1.73 µl |
| Total volume | 384 µl | 499.2 µl |

1. Remove seal from plate and add 1uL/well RT pre-mix.
2. Seal well with PCR adhesive seal and spin down to collect volume in the bottom of wells.
3. Vortex mix on low speed for 1min, and return to ice for 1 min.
4. Spin down to collect volume in the bottom of wells.
5. Perform RT reaction as per Table 4.

Table 4 : Reverse transcription reaction

|  |  |
| --- | --- |
| Temperature | Time |
| 35C | 5 min |
| 50C | 50 min |
| 70C | 15 min |
| 4C | hold |

1. Spin down to collect volume in the bottom of wells.
2. Proceed directly to next step.

## ◆Step 3<b> First strand cDNA purification (time)

Purpose: Combine first-strand cDNA from all wells and column purify.

1. Spin down plates to ensure liquid is in bottom of wells.
2. Remove seal and place VBLOK reservoir over top of plate.
3. Invert and centrifuge 500g 3 min to collect sample in reservoir (typical volume recovered ~700uL).
4. Add 3.5mL Binding Buffer from Zymo DCC-5 kit to reservoir (total volume 4.2mL).
5. Transfer total volume to 5mL Lo-Bind Eppendorf tube.
6. Quick-spin to 1000g to collect volume in the bottom of the tube.
7. Add 700uL/column to 3x DNA Clean and Concentrator-5 Zymo Spin columns, spin down 11 000g for 1 min and discard flow-through.
8. Apply an additional 700uL/column to all three columns and spin down 11 000g for 1 min (2 spins x 700uL x 3 columns should be sufficient. If volume still remains in 5mL tube, repeat spins until all has been added to columns).
9. Replace column into new collection tube.
10. Add 200uL DNA wash buffer to column (make sure ethanol has been added) and invert column carefully 5x.
11. Incubate at RT for 1 min.
12. Spin 11 000g for 1 min.
13. Replace column into new collection tube.
14. Add 200uL DNA wash buffer to column, and invert column carefully 5x.
15. Incubate at RT for 1 min.
16. Spin 11 000g for 1 min.
17. Transfer each column to a 1.5mL LoBind tube, add 10.5uL nuclease-free water, and incubate at room temperature for 1 minute.
18. Spin down 16 000g for 2 min to elute sscDNA.
19. Add a further 10.5uL nuclease-free water to each column, and incubate at room temperature for 1 min.
20. Spin down 16 000g for 2 min.
21. Recovery should be ~20uL/tube.

## ◆Step 3<c> polyA tagging and cDNA amplification (time)

1. Transfer eluted cDNA from each 1.5mL tube to PCR strip tube and place on ice (3 tubes/384well plate).
2. Prepare TdT solution on ice according to Table 5.

\*IMPORTANT NOTE – to avoid by-product synthesis, volume of TdT needs to be measured very accurately. Perform reverse pipetting or dispense to the first stop only!\*

Table 5. TdT solution

|  |  |  |
| --- | --- | --- |
| Reagent | 1 tube | 3.3 tubes |
| H2O | 19.74 µl | 65.15 µl |
| Thermopol buffer | 2.5 µl | 8.25 µl |
| dATP | 0.6 µl | 1.98 µl |
| RNaseH | 0.48 µl | 1.58 µl |
| TdT | 1.68 µl | 5.54 µl |
| Total volume | 25 µl | 82.5 µl |

1. Keeping tubes on ice, add 25 µl TdT solution into each tube and pipette 15x to mix. Spin down briefly.
2. Place tubes into thermocycler and perform A-tailing reaction according to Table 6.

Table 6 : A-tailing reaction

|  |  |
| --- | --- |
| Temperature | Time |
| 0C | Hold (pre-chill) |
| 37C | 50 seconds |
| 65C | 10 min |
| 4C | hold |

1. Quick spin tubes and place on ice.
2. For each tube, divide across 4 tubes containing 11uL/tube (will be a total of 12 tubes from one 384 well plate).
3. Prepare PCR I premix according to Table 7.

Table 7: PCR I premix

|  |  |  |
| --- | --- | --- |
| Reagent | 1 tube | 12.5 tubes |
| H2O | 18.8 µl | 235 µl |
| Terra Direct PCR Buffer | 25.04 µl | 313 µl |
| Tagging primer (10uM) | 0.32 µl | 4 µl |
| Terra Direct DNA polymerase | 2 µl | 25 µl |
| Total volume | 46.16 µl | 577 µl |

1. Add 46.16 µl PCR I premix to each tube and mix carefully by pipetting up and down 15 times, followed by a brief spin-down.
2. Place tubes in thermocycler and perform second strand synthesis according to Table 8.

Table 8 : Second strand synthesis

|  |  |
| --- | --- |
| Temperature | Time |
| 98C | 130 seconds |
| 40C | 1 min |
| 68C | 5 min – ramp at 0.2C/s |

1. Quick spin tubes and place on ice.
2. Prepare PCR II premix according to Table 9.

Table 9: PCR II premix

|  |  |  |
| --- | --- | --- |
| Reagent | 1 tube | 12.5 tubes |
| H2O | 24.24 µl | 303 µl |
| Terra Direct PCR Buffer | 25.04 µl | 313 µl |
| gM primer (100uM) | 0.95 µl | 11.88 µl |
| Total volume | 50.23 µl | 627.88 µl |

1. Add 50.23 µl PCR II premix to each tube and mix carefully by pipetting up and down 15 times, followed by a brief spin-down.
2. Place tubes in thermocycler and perform cDNA amplification according to Table 10.

Table 10 : cDNA amplification

|  |  |
| --- | --- |
| Temperature | Time |
| 68C | Hold (pre-heat) |
| 98C | 10 seconds |
| 65C | 15 seconds |
| 68C | 5 min |
| Return to Step 2 | 11X (12 cycles total) |
| 68C | 5 min |
| 4C | hold |

## ◆Step 3<d> cDNA clean-up (time)

<Column purification>

1. Spin tubes briefly, and transfer volume from all tubes originating from 12 wells/plate to 50mL LoBind tube (total volume ~ 1284uL).
2. Add 32.1 uL 3M NaOAc (pH 5.2) and 6.42 mL Buffer PB (from Qiagen MinElute kit) to tube and mix well.
3. Centrifuge the tube at 2000g for 1 min.
4. Apply 700 uL to a MinElute spin column within a 2mL collection tube, spin down 16 000g for 1 min, and discard flow-through.
5. Repeat with additional 700uL/column until all sample has been loaded onto column (11-12 spins total).
6. Add 750 uL Buffer PE to column, spin 16 000g 1 min, and discard flow-through (ensure ethanol has been added to wash buffer).
7. Centrifuge for an additional 1 min at 16 000g to dry the membrane.
8. Place column into clean 1.5mL LoBind tube and add 21 uL nuclease-free water.
9. Incubate at room temperature for 1 min and spin 16 000g for 2 min.
10. Add a further 21uL nuclease-free water to column.
11. Incubate at room temperature for 1 min and spin 16 000g for 2 min.
12. Recovery should be ~40uL of amplified cDNA.

<Ampure purification>

1. Add 24uL Ampure beads to 40uL eluted cDNA (0.6X), pipette 15x to mix, and incubate at room temperature 10 min.
2. Place tube on magnetic separator for 5 min or until solution is clear.
3. Remove supernatant, add 200uL 80% ethanol, and incubate 30 seconds.
4. Remove ethanol, and repeat ethanol wash with additional 200uL
5. Quick spin, remove residual ethanol, and air dry for 2 minutes. Do not overdry the beads.
6. Remove tube from magnet and add 25uL Buffer EB. Pipette mix 15x and incubate at room temperature for 2 min.
7. Place tube on magnetic separator for 5 min or until solution is clear.
8. Collect up to 25uL volume and transfer to new 1.5mL LoBind tube. Store at -20C.

**<QC 1>**

Check concentration and length distribution by running 1uL of amplified cDNA on a High Sensitivity BioAnalyzer chip. Expected average size is ~1400bp, expected concentration is ~1ng/uL.

# Generation of libraries

## ◆Step 4<a> Preparation of truncated adaptors (time)

1. Resuspend adaptor oligos (rYshapeP5, rYshapeP7LTxx) in adaptor buffer (10mM Tris-HCl pH 8, 0.1mM EDTA pH 8, 50mM NaCl) to 100uM.
2. For each indexed adaptor, add 5uL 100uM rYshapeP5 and 5uL 100uM rYshapeP7LTxx into a single PCR tube, mix by pipetting and spin down.
3. Place tubes in thermocycler and perform adaptor annealing according to Table 11.

Table 11: Adaptor annealing

|  |  |  |  |
| --- | --- | --- | --- |
| Step | Temperature | Time | Number cycles |
| 1 | 23C | hold | 1 |
| 2 | 90C | 1 min | 1 |
| 3 | 90C | 30 seconds | 1 |
| 4 | Return to step 3, increment -0.5C/cycle | 30 seconds | 160 |
| 5 | 4C | Hold | 1 |

1. Place tubes on ice and add 90uL ice-cold adaptor buffer to each tube to give 5uM adaptors.
2. Aliquot 2.5uL into tubes on ice and store at -80C.

## ◆Step 4<b> Fragmentation (time)

1. Prepare Covaris S2 sonicator and equilibrate to ~7C, water level 12 (can take ~1-2 hours).
2. According to QC 1, dilute 5-10ng of amplified cDNA with nuclease-free water to a total volume of 130uL, and transfer to a snap-cap microTUBE.
3. Fragment using a Covaris sonicator with the following conditions: duty factor 10%; intensity 4; cycles per burst 200; treatment time 110s (2x55s).
4. Transfer fragmented cDNA to 1.5mL tube and add 650 uL DNA Binding Buffer (Zymo DCC kit). Vortex to mix and spin down.
5. Add to DNA Clean and Concentrator-5 Zymo Spin column and spin down 11 000g for 1 min. Place column in a new collection tube.
6. Add 200uL DNA wash buffer to column (make sure ethanol has been added) and spin 11 000g for 1 min. Place column in a new collection tube.
7. Repeat wash with further 200uL DNA wash buffer.
8. Transfer column to a 1.5mL LoBind tube, add 10.5uL nuclease-free water, and incubate at room temperature for 1 minute.
9. Spin down 16 000g for 2 min to elute DNA.
10. **Optional:** Run 1uL on High Sensitivity DNA BioAnalyzer chip to check cDNA size post-fragmentation. If performing this test, add 1uL nuclease-free water to make up volume.

## ◆Step 4<c> Adaptor ligation (time)

1. Transfer volume to PCR tube.
2. Prepare End-Repair and A-Tailing mix according to Table 12.

Table 12: End-Repair and A-Tailing Mix

|  |  |  |  |
| --- | --- | --- | --- |
| Reagent | 1 tube | 7 tubes | 7.7 tubes |
| End Repair and A-Tailing Buffer | 1.4 µl | 9.8 µl | 10.78 µl |
| End Repair and A-Tailing Enzyme | 0.6 µl | 4.2 µl | 4.62 µl |
| Total volume | 2 µl | 14 µl | 15.4 µl |

1. Add 2 uL End-Repair and A-tailing mix to each tube of purified fragmented DNA, and mix well by pipetting 15x on ice.
2. Place tubes in thermocycler and perform End repair and A-tailing according to Table 13.

Table 13: End Repair and A-Tailing

|  |  |
| --- | --- |
| Temperature | Time |
| 20C | Hold |
| 37C | 60 min |
| 65C | 30 min |
| 4C | Hold |

1. Add 2uL 5uM annealed truncated adaptor to end-repaired solution on ice. \***IMPORTANT –** use different adaptor for each tube.\* Mix well by pipetting 15x on ice.
2. Prepare ligation mix according to Table 14.

Table 14: Ligation mix

|  |  |  |  |
| --- | --- | --- | --- |
| Reagent | 1 tube | 7 tubes | 7.7 tubes |
| Ligation Buffer | 6 µl | 42 µl | 46.2 µl |
| DNA ligase | 2 µl | 14 µl | 15.4 µl |
| Total volume | 8 µl | 56 µl | 61.6 µl |

1. Add 8 µl ligation mix to each tube, and mix well by pipetting 15x on ice.
2. Place tubes in thermocycler and incubate 20C for 15 minutes.
3. Remove tubes from thermocycler and add 18uL Ampure beads/tube (0.82x cleanup).
4. Pipette 15x to mix, and incubate at room temperature 10 min.
5. Place tube on magnetic separator for 5 min or until solution is clear.
6. Remove supernatant, add 200uL 80% ethanol, and incubate 30 seconds.
7. Remove ethanol, and repeat ethanol wash with additional 200uL.
8. Quick spin, remove residual ethanol, and air dry for 1 minute, or until pellet is dry. Do not overdry the beads.
9. Remove tube from magnet and add 20uL nuclease free water. Pipette mix 15x and incubate at room temperature for 2 min.
10. Place tube on magnetic separator for 5 min or until solution is clear.
11. Collect up to 20uL volume and transfer to new PCR tube.

## ◆Step 4<d> Library PCR (time)

1. Prepare PCR premix according to Table 15.

Table 15: PCR premix

|  |  |  |  |
| --- | --- | --- | --- |
| Reagent | 1 tube | 7 tubes | 7.7 tubes |
| 2x KAPA HiFi ReadyMix | 25 µl | 175 µl | 192.5 µl |
| TPC2 primer (10uM) | 1.75 µl | 12.25 µl | 13.48 µl |
| P5-gMac hybrid primer (10uM) | 1.75 µl | 12.25 µl | 13.48 µl |
| Nuclease-free water | 1.5 µl | 10.5 µl | 11.54 µl |
| Total volume | 30 µl | 210 µl | 231 µl |

1. Add 30 uL PCR premix to 20 uL purified, adaptor-ligated cDNA in PCR tube and pipette mix 15x.
2. Perform PCR according to Table 16.

Table 16: Library PCR

|  |  |
| --- | --- |
| Temperature | Time |
| 37C | hold |
| 95C | 3 min |
| 98C | 15 seconds |
| 60C | 30 seconds |
| 72C | 1 min |
| Return to Step 3 | 7X (8 cycles total) |
| 72C | 5 min |
| 4C | Hold |

1. Add 40 uL Ampure beads to each tube (0.8x cleanup), pipette mix 15x, and incubate at room temperature 10 min.
2. Place tube on magnetic separator for 5 min or until solution is clear.
3. Remove supernatant, add 200uL 80% ethanol, and incubate 30 seconds.
4. Remove ethanol, and repeat ethanol wash with additional 200uL.
5. Quick spin, remove residual ethanol, and air dry for 2 minutes. Do not overdry the beads.
6. Remove tube from magnet and add 25uL Buffer EB. Pipette mix 15x and incubate at room temperature for 2 min.
7. Place tube on magnetic separator for 5 min or until solution is clear.
8. Collect up to 25uL volume and transfer to new 1.5mL LoBind tube. Store at -20C.

**<QC 2>**

Check concentration and length distribution by running 1uL of a 1/2 dilution of final library on a High Sensitivity BioAnalyzer chip.