CEL-Seq2 protocol using 384 well plate

Modified by Akiko Noma at BTL 1/11/2017

Reagents:

LoBind tubes – 0.5 ml – Eppendorf 022431005
Ultra pure RNase free water
Ethanol
Bioanalyzer kits - Agilent RNA pico kit (5067-1513), high sensitivity DNA kit (5067-4626)
Qubit reagents: dsDNA HS Assay – invitrogen Q32851 or Q32854

For RNA amplification:
ERCC RNA spike-in mix – Ambion 4456740
dNTPs mix 10mM
SuperScript II – Invitrogen 18064-014
RNaseOUT – Invitrogen 10777-019
Second strand buffer – Invitrogen 10812-014
DNA Plymerase I (E. coli) – Invitrogen 18010-025
E. coli DNA ligase – Invitrogen 18052-019
RnaseH (E. coli) – Invitrogen 18021-071
MEGAscript T7 Transcription Kit – Ambion AM1334
ExoSAP-IT For PCR Product Clean-Up – Affymetrix 78200
Fragmentation buffer: 200mM Tris-acetate, pH 8.1, 500 mM KOAc, 150 mM MgOAc
Fragmentation stop buffer: 0.5 M EDTA pH8
AMPure XP beads – Beckman Coulter A63880
RNAClean XP beads - Beckman coulter A63987
Bead binding buffer - 20% PEG8000, 2.5M Nacl

For Library preparation:
SuperScript II – Invitrogen 18064-014
RNaseOUT – Invitrogen 10777-019
AMPure XP beads – Beckman Coulter A63880
Phusion® High-Fidelity PCR Master Mix with HF Buffer – NEB M0531
randomhexRT primer - GCCTTGGCACCCGAGAATTCCANNNNNN
RNA PCR primers (sequences available from Illumina)
Primers re-suspended at 100μM, PCR primers used at 10μM.

Equipment:

Thermocycler with lid with adjustable temperature (one that can also fit 0.5 ml PCR tubes is convenient)
Mantis
Echo550
BRAVO
Magnetic stand (for 0.5 ml tubes)

Qubit® Fluorometer - invitrogen
Bioanalyzer – Agilent

Cell plate preparation

- Cells are sorted in 0.6 µl of 1%NP40, immediately frozen on dry ice and kept at -80 °C.

Breaking cell open and annealing with primer:

- For sorted cells in a plate – remove from -80°C, put it on ice.
- Dispense 0.6ul of dNTP mix by Mantis:

Reagent	**Volume**
10mM dNTPs	0.1 µl
Water	0.5 µl
total	**0.6 µl**

- Dispense 20nl of diluted ERCC spikeins with Echo(Dilution depends on cell types: e.g. for T cells, 1:800,000)
- Dispense 5nl of 1 µg/µl barcode RT primers to each well with Echo
- Go to a 5 minute incubation at 65°C. Move immediately to ice. Spin at maximal speed for a few seconds to collect as many droplets as possible before next step, and then return to ice.

RT reaction:

- Add 0.8µl of the following mix to each reaction by Mantis:

Reagent	**1 sample**
5x First strand buffer	0.4 µl
0.1M DTT	0.2 µl
RNaseOUT	0.1 µl
SuperScriptII	0.1 µl
total	**0.8 µl**

- Incubate 1hr at 42°C (on thermal cycler with lid at 85 °C)
- Heat inactivate 10min at 70 °C

Second strand reaction:

- Move previous step to ice so it cools below 16°C.
- Add 10µL of the following mix to each well with BRAVO:

Reagent	**1 sample**
Second strand buffer	2.31 µl
10mM dNTPs	0.23 µl
E. coli DNA ligase	0.08 µl

E. coli DNA polymerase I	0.3 µl
RNaseH	0.08 µl
Water	7 µl
total	**10 µl**

Mix and spin samples (at maximal speed for a few seconds).

- Incubate at 16°C for 2hr (in thermal cycler with unheated lid).
- Stopping point: Samples can be kept at -20°C

cDNA cleanup:

- Prewarm AMPure XP beads to room temperature.
- Pool all cells that are to go to same IVT with BRAVO. Should have ~12µl from each cell.
- Add 0.8X volume of AMPure XP beads+beads binding buffer mixture(Sample:beads:beads binding buffer=1:0.13:0.67 example: for 576 µl sample, add 386 µl beads binding buffer and 75 µl of beads.) to sample. Pipette mix, try to minimize air bubble
- Incubate at room temperature for 15 min.
- Place on magnetic stand for at least 5 min, until liquid appears clear.
- Remove and discard supernatant.
- Add 800µl freshly prepared 80% EtOH.
- Incubate at least 30 seconds, then remove and discard supernatant without disturbing beads.
- Add 800µl freshly prepared 80% EtOH
- Incubate at least 30 seconds, then remove and discard supernatant without disturbing beads.
- Air dry beads for 15 min, or until completely dry.
- Resuspend with 6.4µl water. Pipette entire volume up and down ten times to mix thoroughly.
- Incubate at room temperature for 2 min.
- Go straight to IVT in 96 well plate (transfer beafds+elution mixture to plate)

IVT (Ambion kit):

- Prepare the following mix and add 9.6µl per well

Reagent	**1 well (96samples pooled)**
ATP	1.6 µl
GTP	1.6 µl
CTP	1.6 µl
UTP	1.6 µl
10x T7 buffer	1.6 µl
T7 enzyme	1.6 µl

total	**9.6 µl**

- Incubate in a thermal cycler at 37°C for 13 hrs, with lid at 70°C. Set cycler to go to 4°C at end of incubation. aRNA (amplified RNA) is stable for at least several hours.

EXO-SAP treatment (to remove primers):

- Add 6 µl enzyme
- Incubate at 15 minutes at 37 °C

RNA fragmentation:

- Mix the following on ice:

aRNA	22µl
Fragmentation buffer	5.5µl

- Incubate for 3 min. at 80°C.
- Immediately move to ice and add 2.75µl fragmentation stop buffer.

Remove beads:

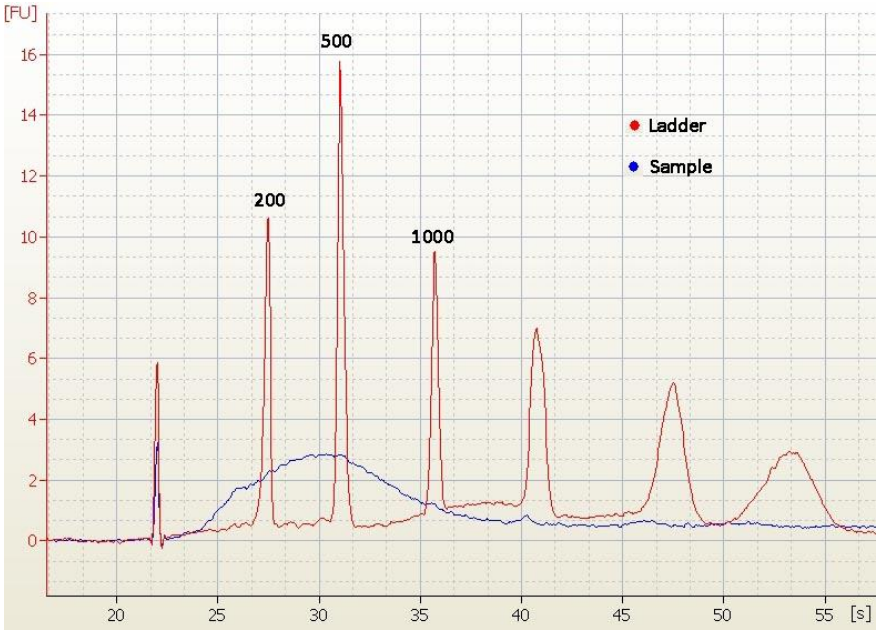
- Place on magnetic stand for at least 5 min, until liquid appears clear.
- Transfer the supernatant to new tube.

aRNA cleanup:

- Prewarm RNAClean XP beads to room temperature.
- Vortex RNAClean XP beads until well dispersed, add to sample 55µl beads. (1.8 volumes)
- Incubate at room temperature for 10 min.
- Place on magnetic stand for at least 5 min, until liquid appears clear.
- Remove and discard ~80µl of the supernatant.
- Add 200µl freshly prepared 70% EtOH.
- Incubate at least 30 seconds, then remove and discard supernatant without disturbing beads.
- Repeat wash two more times.
- Air dry beads for 15 min, or until completely dry.
- Resuspend with 7µl water. Pipette entire volume up and down ten times to mix thoroughly.
- Incubate at room temperature for 2 min.
- Place on magnetic stand for 5 min, until liquid appears clear.
- Transfer supernatant to new tube.

Check aRNA amount and quality:

- Load 1µl onto Bioanalyzer RNA pico chip after heating an aliquot of the sample to 70° for 2 min.
- When starting the IVT with ~0.1ng total RNA, the expected yield is 500-1000 pg/µl. Size distribution should peak at ~500 nt (See Bioanalyzer plot for example).



Library preparation:

RT reaction:

- Make 5 µl of RNA+water mix in new 96 well plate, on ice. You can use all 5 µl RNA without adding water, but it depends on RNA amount you got.
- Add 1 µl randomhexRT primer and 0.5 µl dNTPs .
- Incubate 5min at 65 °C, quick chill on ice.
- Add 4µl of the following mix at room temperature to each reaction:

Reagent	**1 well (96samples pooled)**
5X First strand buffer	2 µl
0.1M DTT	1 µl
RNaseOUT	0.5 µl
SuperSctiptII	0.5 µl
total	**4 µl**

- Incubate 10 min at 25 °C.
- Incubate 1hr at 42°C (in hybridization oven, or pre heated thermal cycler with lid at 50 °C)

PCR amplification:

Use a half RT product for PCR (5 µl)

- To each 5 µl of reverse transcription reaction add 38µl of the following mix:

Reagent	**1 well (96samples pooled)**
Ultra-pure water	16 µl
Phusion HiFi mix	25 µl
10µM RNA PCR Primer (RP1)	2 µl
total	43 µl

- To each reaction add 2µl of a uniquely indexed RNA PCR Primer (RPIX, sequences from Illumina kit, choose balanced primers according to Illumina's pooling guide)
- Amplify the tube in the thermal cycler using the following PCR cycling conditions:

#1. 98 °C 00:30
#2. 98 °C 00:10
#3. 60 °C 00:30
#4. 72 °C 00:30
#5. Go to #2, 10-14 cycles (total 11-15 cycles), depends on cell type
#6. 72 °C 10:00
#7. 4 °C ∞

Can go up to 15 cycles if necessary, if aRNA concentration was low.
Stopping point: samples can be kept at -20°C.

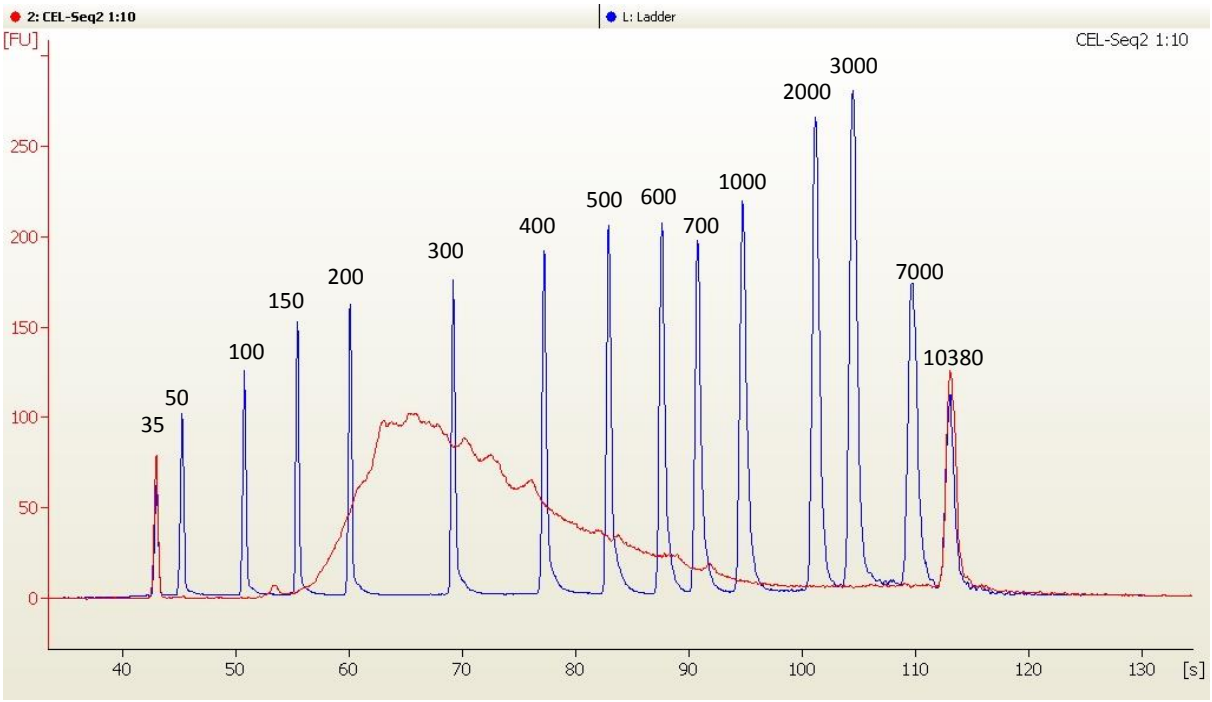
Bead Cleanup of PCR products :

- Prewarm Ampure XP beads at room temperature.
- Add 40 µl beads to 50 µl PCR reaction (0.8X), mix by pipetting
- Incubate at room temperature for 15min
- Place on magnet stand, wait for at least 5min or until liquid appears clear.
- Remove sup, add 200 µl of fresh 80% ethanol, incubate for 30sec then remove supernatant.
- Remove sup, add 200 µl of fresh 80% ethanol, incubate for 30sec then remove supernatant.
- Air dry beads for 15min or until completely dry.
- Resuspend with 25 µl of water. Pipette mix well.
- Incubate at room temperature for 2min.
- Place on magnet stand, wait for 5min or until liquid appears clear.
- **Transfer supernatant** (**keep** supernatant) to **new well** in the same plate.
- Add 20 µl beads to supernatant, then incubate at room temperature for 15min.
- Place on magnet stand, wait for at least 5min or until liquid appears clear.
- Remove sup, add 200 µl of fresh 80% ethanol, incubate for 30sec then remove supernatant.
- Remove sup, add 200 µl of fresh 80% ethanol, incubate for 30sec then remove supernatant.
- Air dry beads for 15min or until completely dry.
- Resuspend with 10 µl of water. Pipette mix well.

- Incubate at room temperature for 2min.
- Place on magnet stand, wait for 5min or until liquid appears clear.
- Transfer supernatant to new 1.5ml Eppendorf tube /96 pooled sample.

Check library amount and quality:

- Check concentration of DNA by Qubit, 1µl should be enough to measure using the high sensitivity reagent; expected concentration is at least ~1ng/µl.
- Run 1µl of each sample on Bioanalyzer using a high sensitivity DNA chip to see size distribution. Expected peak at 200-400bp (See Bioanalyzer plot for example).



Sequencing:

- Concentration to be loaded for sequencing should be calibrated by the sequencing facility.
- Pool libraries up to 4x96 samples for sequencing.
- For Miseq run, use 8pM load with 10% PhiX.
- For Hiseq run, ask to mix 5% PhiX.
- Paired end sequencing is performed, 15 bases for read 1, 6 for the illumine index and 36 bases for read 2. Throughout the Illumina sequencing the libraries should be considered Small-RNA libraries.
- Currently, CEL-Seq libraries are not compatible with Illumina HiSeq high throughput v.4 reagents.