CEL-Seq Protocol

Hashimshony, Wagner, Sher & Yanai. CEL-Seq: Single cell RNA-Seq by multiplexed linear amplification. 2012 (Cell Reports).

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

<u>For RNA amplification</u>:
ERCC RNA spike-in mix – Ambion 4456740
MessageAmpII kit – Ambion AM1751
Optional: extra columns for cDNA/aRNA purification – Ambion10066G
Fragmentation buffer: 200mM Tris-acetate, pH 8.1, 500 mM KOAc, 150 mM MgOAc
Fragmentation stop buffer: 0.5 M EDTA pH8

<u>For Library preparation</u>:
RNase OUT – invitrogen 100000840
Superscript II – invitrogen 18064-014
T4 RNA ligase 2, truncated – NEB M0242
AMPure XP beads – Beckman Coulter A63880
TruSeq small RNA sample prep kit – Illumina RS-200-0012 (or -0024, -0036, -0048)
Optional (to supplement Illumina's Small RNA kit):
Phusion® High-Fidelity PCR Master Mix with HF Buffer – NEB M0531
RNA RT primer, RNA PCR primers (sequences available from Illumina)

Equipment:
Thermocycler with lid with adjustable temperature (one that can also fit 0.5 ml PCR tubes is convenient)
Speed vac
Oven
Heat block
Magnetic stand (for 0.5 ml tubes)
Qubit® Fluorometer - invitrogen
Bioanalyzer – Agilent

Primers:

CEL-Seq primer design: The RT primer was designed with an anchored polyT, a 6 bp unique barcode, a 5 bp UMI (unique molecular identifier), the 5' Illumina adapter (as used in the Illumina small RNA kit) and a T7 promoter. The barcodes were designed such that each pair is different by at least two nucleotides, so that a single sequencing error will not produce the wrong barcode. Primers are desalted at the lowest scale (0.025), stock solution 1 μg/μl (34 μM), working concentration 25ng/μl (0.85 μM).

1	CGATTGAGGCCGGTAATACGACTCACTATAGGGGTTCAGAGTTCTACAGTCCGACGATCNNNNNAGACTCTTTTTTTTTTTTTTTTTTTTTTV
2	CGATTGAGGCCGGTAATACGACTCACTATAGGGGTTCAGAGTTCTACAGTCCGACGATCNNNNNAGCTAGTTTTTTTTTTTTTTTTTTTTTTV
3	CGATTGAGGCCGGTAATACGACTCACTATAGGGGTTCAGAGTTCTACAGTCCGACGATCNNNNNAGCTCATTTTTTTTTTTTTTTTTTTTTTV
4	CGATTGAGGCCGGTAATACGACTCACTATAGGGGTTCAGAGTTCTACAGTCCGACGATCNNNNNAGCTTCTTTTTTTTTTTTTTTTTTTTTTV
5	CGATTGAGGCCGGTAATACGACTCACTATAGGGGTTCAGAGTTCTACAGTCCGACGATCNNNNNCATGAGTTTTTTTTTTTTTTTTTTTTTTV
6	CGATTGAGGCCGGTAATACGACTCACTATAGGGGTTCAGAGTTCTACAGTCCGACGATCNNNNNCATGCATTTTTTTTTTTTTTTTTTTTTTV
7	CGATTGAGGCCGGTAATACGACTCACTATAGGGGTTCAGAGTTCTACAGTCCGACGATCNNNNNCATGTCTTTTTTTTTTTTTTTTTTTTTTV
8	CGATTGAGGCCGGTAATACGACTCACTATAGGGGTTCAGAGTTCTACAGTCCGACGATCNNNNNCACTAGTTTTTTTTTTTTTTTTTTTTTTV
9	CGATTGAGGCCGGTAATACGACTCACTATAGGGGTTCAGAGTTCTACAGTCCGACGATCNNNNNCAGATCTTTTTTTTTTTTTTTTTTTTTTV
10	CGATTGAGGCCGGTAATACGACTCACTATAGGGGTTCAGAGTTCTACAGTCCGACGATCNNNNNTCACAGTTTTTTTTTTTTTTTTTTTTTTV
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14	CGATTGAGGCCGGTAATACGACTCACTATAGGGGTTCAGAGTTCTACAGTCCGACGATCNNNNNTCCTAGTTTTTTTTTTTTTTTTTTTTTTV
15	CGATTGAGGCCGGTAATACGACTCACTATAGGGGTTCAGAGTTCTACAGTCCGACGATCNNNNNTCTGAGTTTTTTTTTTTTTTTTTTTTTTV
16	CGATTGAGGCCGGTAATACGACTCACTATAGGGGTTCAGAGTTCTACAGTCCGACGATCNNNNNTCTGCATTTTTTTTTTTTTTTTTTTTTTV
17	CGATTGAGGCCGGTAATACGACTCACTATAGGGGTTCAGAGTTCTACAGTCCGACGATCNNNNNTCGAAGTTTTTTTTTTTTTTTTTTTTTTV
18	CGATTGAGGCCGGTAATACGACTCACTATAGGGGTTCAGAGTTCTACAGTCCGACGATCNNNNNTCGACATTTTTTTTTTTTTTTTTTTTTTV
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20	CGATTGAGGCCGGTAATACGACTCACTATAGGGGTTCAGAGTTCTACAGTCCGACGATCNNNNNGTACAGTTTTTTTTTTTTTTTTTTTTTTV
21	CGATTGAGGCCGGTAATACGACTCACTATAGGGGTTCAGAGTTCTACAGTCCGACGATCNNNNNGTACCATTTTTTTTTTTTTTTTTTTTTTV
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36	CGATTGAGGCCGGTAATACGACTCACTATAGGGGTTCAGAGTTCTACAGTCCGACGATCNNNNNCTAGTGTTTTTTTTTTTTTTTTTTTTTTV
37	CGATTGAGGCCGGTAATACGACTCACTATAGGGGTTCAGAGTTCTACAGTCCGACGATCNNNNNCTAGGATTTTTTTTTTTTTTTTTTTTTTV
38	CGATTGAGGCCGGTAATACGACTCACTATAGGGGTTCAGAGTTCTACAGTCCGACGATCNNNNNCTCATGTTTTTTTTTTTTTTTTTTTTTTV
39	CGATTGAGGCCGGTAATACGACTCACTATAGGGGTTCAGAGTTCTACAGTCCGACGATCNNNNNCTCAGATTTTTTTTTTTTTTTTTTTTTTV
40	CGATTGAGGCCGGTAATACGACTCACTATAGGGGTTCAGAGTTCTACAGTCCGACGATCNNNNNCTTCGATTTTTTTTTTTTTTTTTTTTTTV
41	CGATTGAGGCCGGTAATACGACTCACTATAGGGGTTCAGAGTTCTACAGTCCGACGATCNNNNNCTGTACTTTTTTTTTTTTTTTTTTTTTTV
42	CGATTGAGGCCGGTAATACGACTCACTATAGGGGTTCAGAGTTCTACAGTCCGACGATCNNNNNCTGTGATTTTTTTTTTTTTTTTTTTTTTV
43	CGATTGAGGCCGGTAATACGACTCACTATAGGGGTTCAGAGTTCTACAGTCCGACGATCNNNNNTGAGACTTTTTTTTTTTTTTTTTTTTTTV
44	CGATTGAGGCCGGTAATACGACTCACTATAGGGGTTCAGAGTTCTACAGTCCGACGATCNNNNNTGCAACTTTTTTTTTTTTTTTTTTTTTTV
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46	CGATTGAGGCCGGTAATACGACTCACTATAGGGGTTCAGAGTTCTACAGTCCGACGATCNNNNNTGCAGATTTTTTTTTTTTTTTTTTTTTTV
47	CGATTGAGGCCGGTAATACGACTCACTATAGGGGTTCAGAGTTCTACAGTCCGACGATCNNNNNTGTCACTTTTTTTTTTTTTTTTTTTTTTV
48	CGATTGAGGCCGGTAATACGACTCACTATAGGGGTTCAGAGTTCTACAGTCCGACGATCNNNNNTGTCGATTTTTTTTTTTTTTTTTTTTTTV

Single cell isolation:

Individual cells (so far we've worked with *C. elegans* blastomeres or trypsinised tissue culture cells) are transferred with a micro-pipette into a 0.5µl drop of appropriate buffer (egg salts or PBS) placed on the cap of a 0.5 ml LoBind Eppendorf tube. Location of cell should be marked. Excess liquid is aspirated off leaving only the cell behind, and tube is frozen in liquid nitrogen. Samples are stored at -80°C.

RNA Amplification:

Prepare primer mix (for each different primer used):

Primer (25ng/µl)	1µl
ERCC Spike-in	Xµl
water	Yµl
	6µl

Spike-in dilution should be appropriate for sample size – see protocol of ERCC RNA spike in mix. For single cells we add 1ul of spike-in at 1:500,000 dilution.

Breaking cell open and annealing with primer:

- Add 1.2µl primer mix to marked location of single cell on cap of tube (Keep cell frozen until adding the primer mix, handle up to 12 cells in parallel).
- Incubate 5 min. at 70^{o}C (with lid of thermal cycler set to 70^{o}C).
- Brief spin down.
- Incubate for an additional 5 min. at 70^{o}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.

Alternative protocol – using clean RNA:

- Prepare an RNA/primer/spike-in. mix. Amount of primer as above, appropriate amount of spike-in can be added to the clean RNA or directly to the sample before RNA prep.
- Transfer 1.2µl of mix to new tube (left over mix can be kept at -70^{o}C).
- Incubate for 10 min. at 70^{o}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 (Ambion kit)

- Add 0.8µl of the following mix to each reaction (final conc of primer is 85 nM):

First Strand buffer	0.2µl
dNTP	0.4µl
RNase Inhibitor	0.1µl
ArrayScript	0.1µl

- Incubate 2hr at 42°C (in hybridization oven, or thermal cycler with lid at 50 °C)

Second strand reaction (Ambion kit):

- Move previous step to ice so it cools below 16°C.
- Add 8uL of the following mix to each reaction tube:

DDW	6.3µl
Second strand buf.	1µl
dNTP	0.4µl
DNA Pol	0.2µl
RNaseH	0.1µl

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

(At this point samples are already barcoded, so if all samples are going to the same IVT, a single tip can be used.)

- Incubate at 16°C for 2hr (in thermal cycler with unheated or open lid).

cDNA cleanup and speedvac:

- Pool all cells that are to go to same IVT. Should have ~10µl from each cell.
- Adjust volume to 100µl with nuclease free water. If more than 10 cells in a pool, just add all together.
- Add 250µl cDNA binding buffer to each 100ul sample (if total sample volume exceeds 100uL, adjust cDNA binding buffer volume according to sample volume). Load onto Ambion cDNA cleanup column. Volume of up to 24 pooled samples can be loaded. If more than 24 samples are pooled, after spin load remaining volume and spin again.
- Spin 1 min at 10,000g to bind cDNA, discard flow through (repeat if more than 24 samples are pooled).
- Add 500µl wash buffer, spin as above, discard flow through.
- Spin for an additional minute to dry.
- Transfer column to clean round bottom 2 ml tube (important to reduce drying time, see below).
- Elute by adding 9µl warm water (55°C), incubating for 2 minutes at room temperature and spinning 1.5 min at 10,000g.
- Repeat elution.
- Adjust volume to 6.4µl by drying in a speedvac (~8 min).

Stopping point: Samples can be kept at -20°C

IVT (Ambion kit):

- Prepare the following mix and add 9.6µl per tube.

A	1.6µl
G	1.6µl
C	1.6µl
U	1.6µl
10xT7 buffer	1.6µl
T7 enzyme	1.6µl

- 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.

RNA fragmentation and cleanup:

- Mix the following on ice:

aRNA	16µl
Fragmentation buffer	4µl

- Incubate for 3 min. at 94°C.
- Immediately move to ice and add 2µl fragmentation stop buffer.

Depending on the samples, follow one of the following clean-up protocols:

If total RNA in pooled samples (either cells or clean RNA) is 400-2000pg:

- Adjust volume to 30ul by adding 8µl water.
- Add 105µl aRNA binding buffer, followed by 75µl EtOH, immediately mix by pipetting 3-4 times and load onto **small** spin-column (the ones labeled as cDNA clean-up). Bind sample by immediately spinning for 1 min at 10,000g, discard flow through. (If cleaning more than one reaction, this step should be done for each sample separately)
- Add 500µl wash buffer (samples can wait at this step until binding of all samples is complete).
- Spin as above, discard flow through.
- Spin for an additional minute to dry.
- Transfer column to clean tube.
- Elute by adding 10µl warm water (55°C), incubating for 2 minutes at room temperature and spinning 1.5 min at 10,000 g.
- Repeat elution.

If total RNA in pooled samples (either cells or clean RNA) is more than 2 ng:

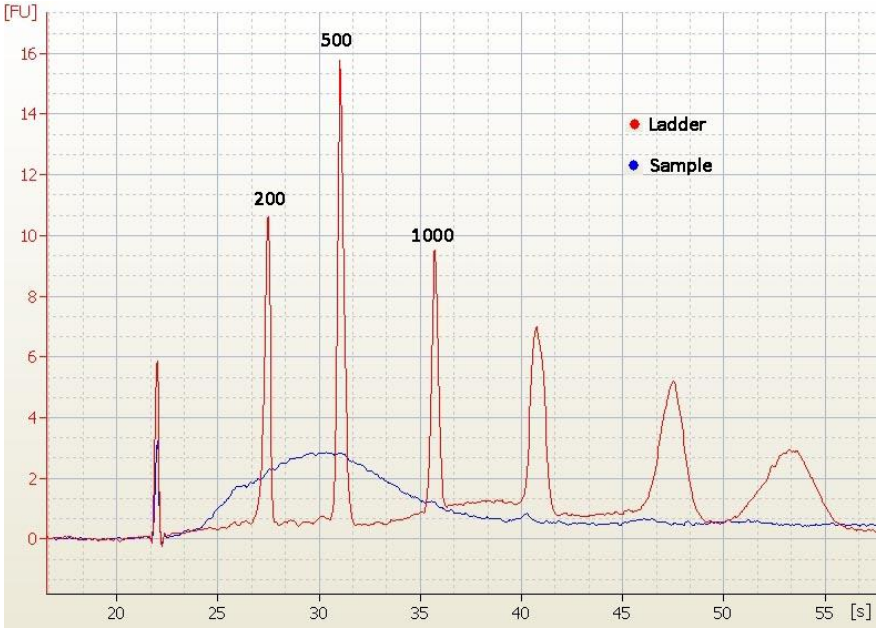
- Adjust volume to 100ul by adding 78µl water (make sure samples are in tubes with minimal volume of 0.7ml).

- Add 350µl aRNA binding buffer, followed by 250µl EtOH, immediately mix by pipetting 3-4 times and load onto spin-column (aRNA purification tubes supplied with the kit). Bind sample by immediately spinning for 1 min at 10,000g, discard flow through. (If cleaning more than one reaction, this step should be done for each sample separately)
- Add 650µl wash buffer (samples can wait at this step until binding of all samples is complete).
- Spin as above, discard flow through.
- Spin for an additional minute to dry.
- Transfer column to clean tube.
- Elute by adding 100µl warm water (55^{o}C), incubating for 2 minutes at room temperature and spinning 1.5 min at 10,000 g.

Stopping point: Samples can be kept at -80^{o}C

<u>Check aRNA amount and quality:</u>

- Load 1µl onto Bioanalyzer RNA pico chip after heating an aliquot of the sample to 70^{o} for 2 min.
- When starting the IVT with ~0.5ng total RNA, the expected yield is 500-1000 pg/µl. Size distribution should peak at ~500 bp (See Bioanalyzer plot for example).
- If starting the amplification with more than 5ng total RNA, concentration can be checked by qubit prior to running the bioanalyser.



Library preparation:

Protocol designed for 5-10ng amplified RNA, although as little as 1-2ng can be used, but then additional PCR cycles are required. Sample volume should be adjusted to 5µl, either by adding water or drying down in a speedvac, depending on RNA concentration. IVTs can be pooled at this point if there is no overlap in barcodes used.

Ligate 3' adapter:

Dilute 3' adapter (RA3, from Illumina kit) 5 fold.

To 5µl RNA add 1µl of the diluted 3' adaptor.

Incubate at 70°C for 2 minutes and then immediately place the tube on ice to prevent secondary structure formation.

Add 4µl of the following mix:

- 5X Ligation Buffer (HML, Illumina kit) 2µL
- RNase Inhibitor (Illumina kit) 1µL
- T4 RNA Ligase 2, truncated 1µL

Incubate the tube on the pre-heated thermal cycler at 28°C for 1 hour (with unheated or open lid).

Dilute the Stop Solution (STP, Illumina kit) 5 fold.

With the reaction tube remaining on the thermal cycler, add 1µl diluted Stop Solution and gently pipette the entire volume up and down 6–8 times to mix thoroughly. Continue to incubate the reaction tube on the thermal cycler at 28°C for 15 minutes, and then place the tube on ice.

Add 3µL nuclease free water.

Reverse transcription reaction:

Dilute dNTPs (from Illumina kit) two fold with nuclease free water (prepare at least 1µl per sample)

Combine the following in a PCR tube (the remaining 3' adapter-ligated RNA may be stored at -80°C):

- Adapter-ligated RNA 6µL
- RNA RT Primer (RTP, from Illumina kit) 1µL

Incubate the tube at 70°C for 2 minutes and then immediately place the tube on ice.

Add 5.5µl of the following mix:

- 5X First Strand Buffer 2µL

- 12.5 mM dNTP mix (diluted dNTP) 0.5µL
- 100 mM DTT 1µL
- RNase Inhibitor (Illumina kit) 1µL
- SuperScript II Reverse Transcriptase 1µL

Incubate the tube in the pre-heated thermal cycler at 50°C for 1 hour and then place the tube on ice.

PCR amplification:

To each reverse transcription reaction add 35.5µl of the following mix:

- Ultra Pure Water 8.5µL
- PCR mix (PML, from Illumina kit)) 25µL
- RNA PCR Primer (RP1, from Illumina kit) 2µL

To each reaction add 2µl of a uniquely indexed RNA PCR Primer (RPIX, from Illumina kit)

(Half a PCR reaction is usually enough)

Amplify the tube in the thermal cycler using the following PCR cycling conditions:

- 30 seconds at 98°C
- 12 cycles of:
  - 10 seconds at 98°C
  - 30 seconds at 60°C
  - 30 seconds at 72°C
- 10 minutes at 72°C
- Hold at 4°C

Can go up to 15 cycles if necessary, or down to 11 if starting with the full 10ng.

Stopping point: samples can be kept at -20^{o}C.

Bead Cleanup of PCR products – Repeat 1:

- Prewarm beads to room temperature.
- Vortex AMPure XP Beads until well dispersed, then add 50µl to the 50µl PCR reaction. Mix entire volume up ten times to mix thoroughly.
- Incubate at room temperature for 15 min.
- Place on magnetic stand for at least 5 min, until liquid appears clear.
- Remove and discard 95µl of the supernatant.
- Add 200µl freshly prepared 80% EtOH.
- Incubate at least 30 seconds, then remove and discard supernatant without disturbing beads.

- Add 200µl 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 32.5µl Resuspension Buffer (from Illumina kit). 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 30µl of supernatant to new tube.

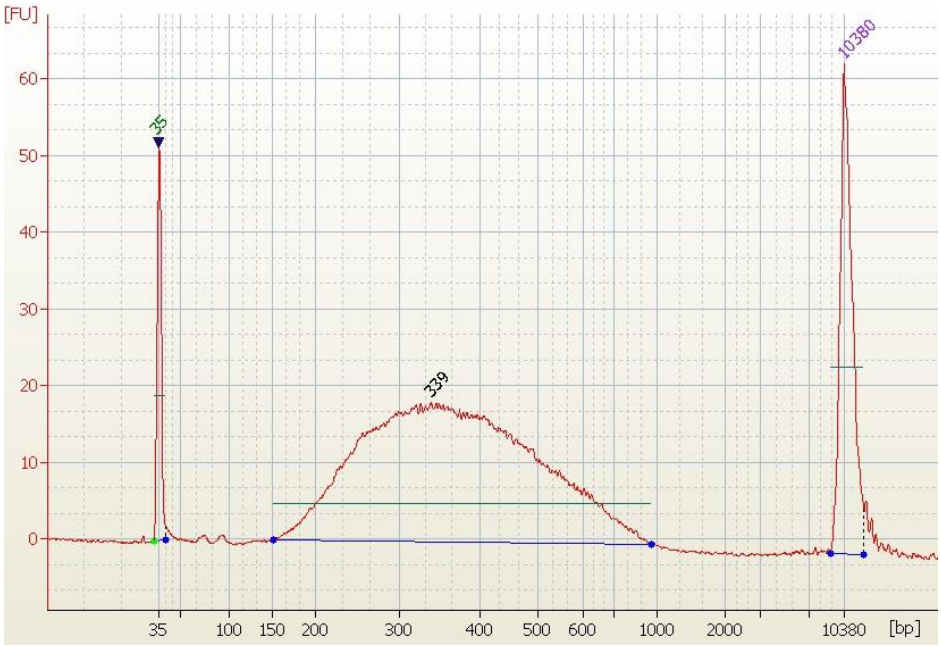
Bead Cleanup of PCR products – Repeat 2:

Repeat as above, but adding 39µl beads and eluting in 12.5µl resuspension buffer at the end, transferring 10µl to a new tube.

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 300-400bp (See Bioanalyzer plot for example).



Concentration to be loaded for sequencing should be calibrated by the sequencing facility. For us, 5pM on Hi-Seq v.1 reagents gave good cluster density. Currently we are using 8pM with v.3 reagents. Paired end sequencing is performed, 15 bases for read 1, 7 for the illumine index (when needed) and 36 bases for read 2. Throughout the Illumina sequencing the libraries should be considered Small-RNA libraries. For example, in Illumina Rehyb kits, some Illumina kits are not for Small-RNA.