CEL-Seq2 Protocol in the C1™

This document is a merging of Supplementary file 1 and Supplementary file 2 from the publication T. Hashimshony, T. N. Senderovich, G. Avital, A. Klochendler, Y. de Leeuw, L. Anavy, D. Gennery, S. Li, K. J. Livak, O. Rozenblatt-Rosen, Y Dor, A. Regev, and I. Yanai. CEL-Seq2: sensitive highly-multiplexed single-cell RNA-Seq. *Genome Biol* **17**:77 (2016).

Reagents:
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 – Thermo Fisher Scientific 4456740
C1 Open App IFC – Fluidigm 100-8133 (5–10 µm), 100-8134 (10–17 µm), 100-8135 (17–25 µm)
C1 Open App Reagent Kit – Fluidigm 100-8920
NP-40, 10% – Thermo Fisher Scientific 28324
5× Lysis buffer: 2.5% NP-40, 250 mM Tris-HCl, pH 8.4, 5 mM EDTA
DTT (dithiothreitol) – Teknova D9750
dNTP Mix, 25 mM each – Thermo Fisher Scientific R1121
rNTP Mix, 25 mM each – New England BioLabs N0466S
SuperScript II – Thermo Fisher Scientific 18064014 (includes 5× First Strand Buffer)
RNaseOUT – Thermo Fisher Scientific 10777019
Second Strand Buffer – Thermo Fisher Scientific 10812014
DNA Polymerase I (E. coli) – Thermo Fisher Scientific 18010025
E. coli DNA ligase – Thermo Fisher Scientific 18052019
RNaseH (E. coli) – Thermo Fisher Scientific 18021071
MEGAscript T7 Transcription Kit – Thermo Fisher Scientific AM1334
ExoSAP-IT for PCR Product Clean-Up – Affymetrix 78200
Fragmentation buffer: 200 mM Tris-acetate, pH 8.1, 500 mM KOAc, 150 mM MgOAc
Fragmentation stop buffer: 0.5 M EDTA pH 8
AMPure XP beads – Beckman Coulter A63880
RNAClean XP beads - Beckman Coulter A63987
Bead binding buffer - 20% PEG8000, 2.5 M NaCl

For Library preparation:
SuperScript II – Thermo Fisher Scientific 18064014
RNaseOUT – Thermo Fisher Scientific 10777019
AMPure XP beads – Beckman Coulter A63880
Phusion® High-Fidelity PCR Master Mix with HF Buffer – New England BioLabs M0531

randomhexRT primer – GCCTTGGCACCCGAGAATTCCANNNNNN
RNA PCR primers (sequences available from Illumina)
RP1 – AATGATACGGCGACCACCGAGATCTACACGTTCAGAGTTCTACAGTCCG*A, *=phosphorothioate
RPi1 – CAAGCAGAAGACGGCATACGAGATCGTGATGTGACTGGAGTTCCTTGGCACCCGAGAATTCC*A
Primers re-suspended at 100 μM, PCR primers used at 10 μM.

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

Primers:
CEL-Seq2 primer design: The RT primer was designed with an anchored polyT, a 6 bp unique barcode, a 6 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 possible scale, stock solution 1 μg/μl, working concentration 25 ng/μL.

1s	GCCGGTAATACGACTCACTATAGGGAGTTCTACAGTCCGACGATCNNNNNNAGACTCTTTTTTTTTTTTTTTTTTTTTV
2s	GCCGGTAATACGACTCACTATAGGGAGTTCTACAGTCCGACGATCNNNNNNAGCTAGTTTTTTTTTTTTTTTTTTTTTV
3s	GCCGGTAATACGACTCACTATAGGGAGTTCTACAGTCCGACGATCNNNNNNAGCTCATTTTTTTTTTTTTTTTTTTTTV
4s	GCCGGTAATACGACTCACTATAGGGAGTTCTACAGTCCGACGATCNNNNNNAGCTTCTTTTTTTTTTTTTTTTTTTTTV
5s	GCCGGTAATACGACTCACTATAGGGAGTTCTACAGTCCGACGATCNNNNNNCATGAGTTTTTTTTTTTTTTTTTTTTTV
6s	GCCGGTAATACGACTCACTATAGGGAGTTCTACAGTCCGACGATCNNNNNNCATGCATTTTTTTTTTTTTTTTTTTTTV
7s	GCCGGTAATACGACTCACTATAGGGAGTTCTACAGTCCGACGATCNNNNNNCATGTCTTTTTTTTTTTTTTTTTTTTTV
8s	GCCGGTAATACGACTCACTATAGGGAGTTCTACAGTCCGACGATCNNNNNNCACTAGTTTTTTTTTTTTTTTTTTTTTV
9s	GCCGGTAATACGACTCACTATAGGGAGTTCTACAGTCCGACGATCNNNNNNCAGATCTTTTTTTTTTTTTTTTTTTTTV
10s	GCCGGTAATACGACTCACTATAGGGAGTTCTACAGTCCGACGATCNNNNNNTCACAGTTTTTTTTTTTTTTTTTTTTTV
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13s	GCCGGTAATACGACTCACTATAGGGAGTTCTACAGTCCGACGATCNNNNNNAGTGTCTTTTTTTTTTTTTTTTTTTTTV
14s	GCCGGTAATACGACTCACTATAGGGAGTTCTACAGTCCGACGATCNNNNNNTCCTAGTTTTTTTTTTTTTTTTTTTTTV
15s	GCCGGTAATACGACTCACTATAGGGAGTTCTACAGTCCGACGATCNNNNNNTCTGAGTTTTTTTTTTTTTTTTTTTTTV
16s	GCCGGTAATACGACTCACTATAGGGAGTTCTACAGTCCGACGATCNNNNNNTCTGCATTTTTTTTTTTTTTTTTTTTTV
17s	GCCGGTAATACGACTCACTATAGGGAGTTCTACAGTCCGACGATCNNNNNNTCGAAGTTTTTTTTTTTTTTTTTTTTTV
18s	GCCGGTAATACGACTCACTATAGGGAGTTCTACAGTCCGACGATCNNNNNNTCGACATTTTTTTTTTTTTTTTTTTTTV
19s	GCCGGTAATACGACTCACTATAGGGAGTTCTACAGTCCGACGATCNNNNNNTCGATCTTTTTTTTTTTTTTTTTTTTTV
20s	GCCGGTAATACGACTCACTATAGGGAGTTCTACAGTCCGACGATCNNNNNNGTACAGTTTTTTTTTTTTTTTTTTTTTV
21s	GCCGGTAATACGACTCACTATAGGGAGTTCTACAGTCCGACGATCNNNNNNGTACCATTTTTTTTTTTTTTTTTTTTTV

22s GCCGGTAATACGACTCACTATAGGGAGTTCTACAGTCCGACGATCNNNNNNGTACTCTTTTTTTTTTTTTTTTTTTTTTV
23s GCCGGTAATACGACTCACTATAGGGAGTTCTACAGTCCGACGATCNNNNNNGTCTAGTTTTTTTTTTTTTTTTTTTTTTTV
24s GCCGGTAATACGACTCACTATAGGGAGTTCTACAGTCCGACGATCNNNNNNGTCTCATTTTTTTTTTTTTTTTTTTTTTTV
25s GCCGGTAATACGACTCACTATAGGGAGTTCTACAGTCCGACGATCNNNNNNGTTGCATTTTTTTTTTTTTTTTTTTTTTTV
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27s GCCGGTAATACGACTCACTATAGGGAGTTCTACAGTCCGACGATCNNNNNNGTGATCTTTTTTTTTTTTTTTTTTTTTTTV
28s GCCGGTAATACGACTCACTATAGGGAGTTCTACAGTCCGACGATCNNNNNNACAGTGTTTTTTTTTTTTTTTTTTTTTTTV
29s GCCGGTAATACGACTCACTATAGGGAGTTCTACAGTCCGACGATCNNNNNNACCATGTTTTTTTTTTTTTTTTTTTTTTTV
30s GCCGGTAATACGACTCACTATAGGGAGTTCTACAGTCCGACGATCNNNNNNACTCTGTTTTTTTTTTTTTTTTTTTTTTTV
31s GCCGGTAATACGACTCACTATAGGGAGTTCTACAGTCCGACGATCNNNNNNACTCGATTTTTTTTTTTTTTTTTTTTTTTV
32s GCCGGTAATACGACTCACTATAGGGAGTTCTACAGTCCGACGATCNNNNNNACGTACTTTTTTTTTTTTTTTTTTTTTTTV
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34s GCCGGTAATACGACTCACTATAGGGAGTTCTACAGTCCGACGATCNNNNNNACGTGATTTTTTTTTTTTTTTTTTTTTTTV
35s GCCGGTAATACGACTCACTATAGGGAGTTCTACAGTCCGACGATCNNNNNNCTAGACTTTTTTTTTTTTTTTTTTTTTTTV
36s GCCGGTAATACGACTCACTATAGGGAGTTCTACAGTCCGACGATCNNNNNNCTAGTGTTTTTTTTTTTTTTTTTTTTTTTV
37s GCCGGTAATACGACTCACTATAGGGAGTTCTACAGTCCGACGATCNNNNNNCTAGGATTTTTTTTTTTTTTTTTTTTTTTV
38s GCCGGTAATACGACTCACTATAGGGAGTTCTACAGTCCGACGATCNNNNNNCTCATGTTTTTTTTTTTTTTTTTTTTTTTV
39s GCCGGTAATACGACTCACTATAGGGAGTTCTACAGTCCGACGATCNNNNNNCTCAGATTTTTTTTTTTTTTTTTTTTTTTV
40s GCCGGTAATACGACTCACTATAGGGAGTTCTACAGTCCGACGATCNNNNNNCTTCGATTTTTTTTTTTTTTTTTTTTTTTV
41s GCCGGTAATACGACTCACTATAGGGAGTTCTACAGTCCGACGATCNNNNNNCTGTACTTTTTTTTTTTTTTTTTTTTTTTV
42s GCCGGTAATACGACTCACTATAGGGAGTTCTACAGTCCGACGATCNNNNNNCTGTGATTTTTTTTTTTTTTTTTTTTTTTV
43s GCCGGTAATACGACTCACTATAGGGAGTTCTACAGTCCGACGATCNNNNNNTGAGACTTTTTTTTTTTTTTTTTTTTTTTV
44s GCCGGTAATACGACTCACTATAGGGAGTTCTACAGTCCGACGATCNNNNNNTGCAACTTTTTTTTTTTTTTTTTTTTTTTV
45s GCCGGTAATACGACTCACTATAGGGAGTTCTACAGTCCGACGATCNNNNNNTGCATGTTTTTTTTTTTTTTTTTTTTTTTV
46s GCCGGTAATACGACTCACTATAGGGAGTTCTACAGTCCGACGATCNNNNNNTGCAGATTTTTTTTTTTTTTTTTTTTTTTV
47s GCCGGTAATACGACTCACTATAGGGAGTTCTACAGTCCGACGATCNNNNNNTGTCACTTTTTTTTTTTTTTTTTTTTTTTV
48s GCCGGTAATACGACTCACTATAGGGAGTTCTACAGTCCGACGATCNNNNNNTGTCGATTTTTTTTTTTTTTTTTTTTTTTV
49s GCCGGTAATACGACTCACTATAGGGAGTTCTACAGTCCGACGATCNNNNNNTGGTACTTTTTTTTTTTTTTTTTTTTTTTV
50s GCCGGTAATACGACTCACTATAGGGAGTTCTACAGTCCGACGATCNNNNNNGACATGTTTTTTTTTTTTTTTTTTTTTTTV
51s GCCGGTAATACGACTCACTATAGGGAGTTCTACAGTCCGACGATCNNNNNNGATCACTTTTTTTTTTTTTTTTTTTTTTTV
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68s GCCGGTAATACGACTCACTATAGGGAGTTCTACAGTCCGACGATCNNNNNNTCACTCTTTTTTTTTTTTTTTTTTTTTTV

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93s GCCGGTAATACGACTCACTATAGGGAGTTCTACAGTCCGACGATCNNNNNNGACAACTTTTTTTTTTTTTTTTTTTTTTV

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95s GCCGGTAATACGACTCACTATAGGGAGTTCTACAGTCCGACGATCNNNNNNGAGTTGTTTTTTTTTTTTTTTTTTTTTTV

96s GCCGGTAATACGACTCACTATAGGGAGTTCTACAGTCCGACGATCNNNNNNGAGTGATTTTTTTTTTTTTTTTTTTTTTV

C1 Mixes

Lysis Mix (1000 µL)

280	µL	5× Lysis Buffer
28	µL	1 M DTT
4	µL	40 units/µL RNaseOUT
8	µL	1:10,000 dilution of ERCC RNA Spike-In Mix
70	µL	C1 Loading Reagent
610	µL	H_2O

7 µL Lysis Mix to Inlet #3

Diluted CEL-Seq2 Primers

Dispense 8 µL Lysis Mix to each of 96 wells

Add 2 µL 25 ng/µL CEL-Seq2 Primer to each well

5 µL Diluted CEL-Seq2 Primer to each of 96 Harvest Outlets

RT Mix (28.6 µL)

20	µL	5× First Strand Buffer
2.6	µL	40 units/µL RNaseOUT
2	µL	25 mM each dNTP
2.6	µL	200 U/µL SuperScript II Reverse Transcriptase
1.4	µL	C1 Loading Reagent

7 µL RT Mix to Inlet #4

2^{nd} Strand Mix (100 µL)

24.7	µL	5× Second Strand Buffer
1	µL	25 mM each dNTP
3.3	µL	10 units/µL E.coli DNA polymerase
1	µL	10 units/µL E. coli DNA ligase
1	µL	2 units/µL RNase H
5	µL	C1 Loading Reagent
64	µL	H_2O

24 µL 2^{nd} Strand Mix to Inlet #7

IVT Mix (29.6 µL)

6.6	µL	T7 10× Reaction Buffer (MEGAscript kit)
14.9	µL	25 mM each rNTP
6.6	µL	T7 Enzyme Mix (MEGAscript kit)
1.5	µL	C1 Loading Reagent

24 µL IVT Mix to Inlet #8

C1 Steps

1. 96 Diluted CEL-Seq2 Primers to chamber 1 from the 96 Outlet wells

2. Lysis Mix to chambers 0+1+2 from Inlet #3
   65°C, 5 min (300 sec)
   10°C, 1 min (60 sec)

3. RT Mix to chambers 0+1+2+3 from Inlet #4
   42°C, 120 min (7200 sec)
   10°C, 1 min (60 sec)

4. 2nd Strand Mix to chambers 0+1+2+3+4 from Inlet #7
   16°C, 120 min (7200 sec)
   65°C, 20 min (1200 sec)
   10°C, 1 min (60 sec)

5. IVT Mix to chambers 0+1+2+3+4+5 from Inlet #8
   37°C, 12 hr (12× 3600 sec)
   10°C, 1 min (60 sec)

6. Harvest

aRNA Clean up

1) Pool 2.5 µL of each of the harvested aRNA libraries (~240 µL total)
2) Add 1.8× volumes (430 µL) of RNAClean XP beads to the harvested aRNA. Mix well until the liquid appears homogeneous. Incubate for 10 min at room temperature.
3) Bind beads on a magnetic stand for 5 min.
4) Discard the supernatant.
5) Wash 3 times with 70% ethanol.
6) Air dry for 10 min or until completely dry.
7) Resuspend the beads with 16 µl RNase-free water. Incubate in room temperature for 2 min.
8) Bind beads on a magnetic stand for 5 min.
9) Transfer the eluted aRNA to a new Eppendorf, continue with ExoSAP treatment, as in regular CEL-Seq2 protocol.

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	22 µL
Fragmentation buffer	5.5 µL

- Incubate for 3 min. at 94°C.
- Immediately move to ice and add 2.75 µL Fragmentation stop buffer.

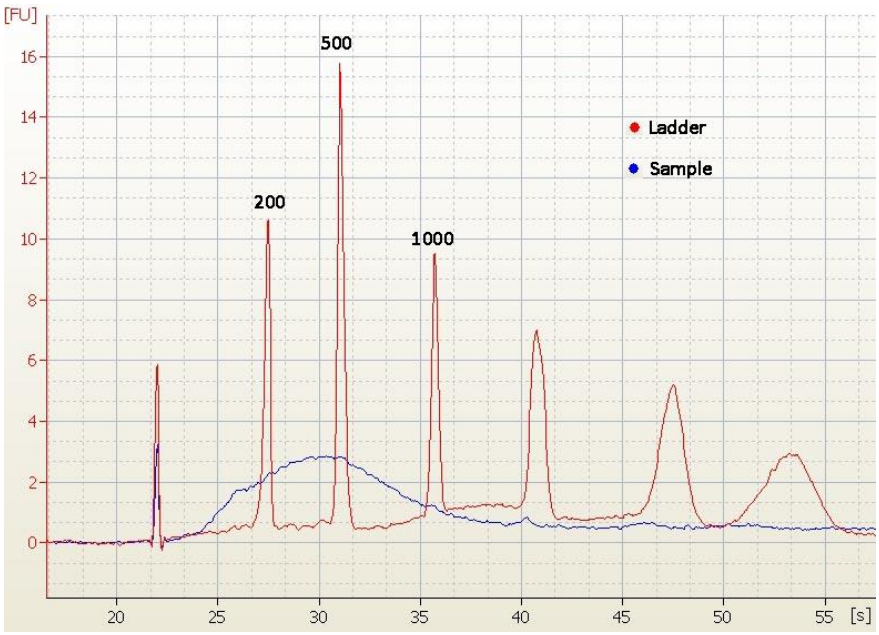
aRNA cleanup:

- Prewarm RNAClean XP beads to room temperature.
- Vortex RNAClean XP beads until well dispersed, add to sample 55 µL 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 50 µL of the supernatant.
- Add 200 µL 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 7 µL 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.

Stopping point: Samples can be kept at -80°C

Check aRNA amount and quality:

- Load 1 µL onto Bioanalyzer RNA pico chip after heating an aliquot of the sample to 70° for 2 min.
- When starting the IVT with ~0.1 ng total RNA, the expected yield is 500-1000 pg/µL. Size distribution should peak at ~500 bp (See Bioanalyzer plot for example). If yield is higher, dilute a portion of aRNA to 1 ng/µL for the RT step below.



Library preparation:

RT reaction:

To 5 µL aRNA, add 1 µL randomhexRT primer and 0.5 µL 10 mM each dNTP.

Incubate 5 min at 65 °C, quick chill on ice.

- Add 4µl of the following mix at room temperature to each reaction:

First Strand buffer	2 µL
DTT 0.1M	1 µL
RNaseOUT	0.5 µL
SuperscriptII	0.5 µL

Incubate 10 min at 25 °C.

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

PCR amplification:

To each reverse transcription reaction add 38 µL of the following mix:

- Ultra Pure Water 11 µL
- PCR Master Mix 25 µL
- RNA PCR Primer (RP1, from Illumina kit) 2 µL

To each reaction add 2 µL of a uniquely indexed RNA PCR Primer (e.g. RPi1; if mixing multiple libraries for sequencing, choose balanced primers according to Illumina's pooling guide)

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

- 30 seconds at 98°C
- 11 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, if aRNA concentration was low.

Stopping point: samples can be kept at -20°C.

Bead Cleanup of PCR products – Repeat 1:

- Prewarm beads to room temperature.
- Vortex AMPure XP Beads until well dispersed, then add 50 µL to the 50 µL 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 95 µL of the supernatant.
- Add 200 µL freshly prepared 80% EtOH.
- Incubate at least 30 seconds, then remove and discard supernatant without disturbing beads.
- Add 200 µL 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 25 µL 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 25 µL of supernatant to new tube.

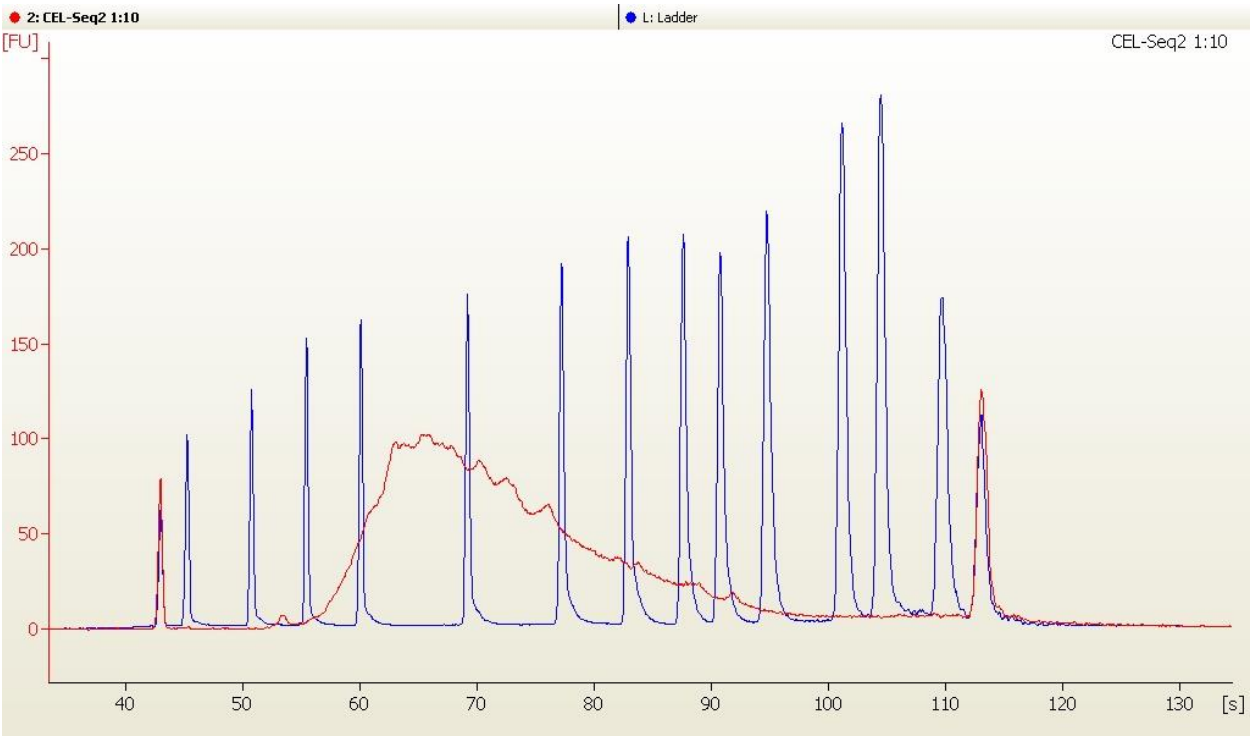
Bead Cleanup of PCR products – Repeat 2:

Repeat as above, adding 25 µL beads and eluting in 10 µL water at the end, transferring 10 µL to a new tube.

Check library amount and quality:

Check concentration of DNA by Qubit, 1 µL should be enough to measure using the high sensitivity reagent; expected concentration is at least ~1ng/µl.

Run 1 µL of each sample on Bioanalyzer using a high sensitivity DNA chip to see size distribution. Expected peak at 200-400 bp (See Bioanalyzer plot for example).



Concentration to be loaded for sequencing should be calibrated by the sequencing facility. We are using 8 pM with HiSeq high throughput v.3 reagents, or 12 pM on MiSeq and HiSeq rapid mode. 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.

Currently, CEL-Seq2 libraries are not compatible with Illumina HiSeq high throughput v.4 reagents.