VERSION 1

FEB 03, 2021

OPEN ACCESS

DOI:
dx.doi.org/10.17504/protocols.io.veie3ce

Protocol Citation: Sarah Boswell, Caitlin Mills, Feodor Price, Stewart Rudnicki 2021. 3'-DGE High Throughput RNA Library Preparation. **protocols.io** <https://dx.doi.org/10.17504/protocols.io.veie3ce>

License: This is an open access protocol distributed under the terms of the [Creative Commons Attribution License](#), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited

Protocol status: Working
 We use this protocol and it works for us.

Created: Nov 07, 2018

Last Modified: Feb 05, 2021

PROTOCOL integer ID:
 17578

3'-DGE High Throughput RNA Library Preparation V.1

Sarah Boswell¹, Caitlin Mills¹, Feodor Price², Stewart Rudnicki³

¹Laboratory of Systems Pharmacology, Harvard Medical School, Boston MA;

²Stem Cell and Regenerative Biology, Harvard University, Cambridge MA;

³CCB-Longwood Screening Facility, Harvard Medical School, Boston MA

Sarah Boswell

Harvard Medical School, Laboratory of Systems Pharmacology

ABSTRACT

This protocol is for the high-throughput transcriptome screening method called, 3' Digital Gene Expression (3'-DGE). In this method RNA is purified from cell lysate and cDNA is generated by oligo dT priming, during which unique molecular identifiers (UMIs) are incorporated and strand-specificity is preserved. The 3'-DGE protocol has been optimized for 384 well plate experiments with a few thousand cells per well. The 3'-DGE method and a similar method known as DRUG-seq have been shown to be able to recapitulate 85% of the transcriptome you can obtain with standard mRNA-seq. In our experience using 3'-DGE we sequence each well at a low depth, aiming for ~1 million reads per sample which allows us to assess 40-80% of the transcriptome depending on the cell type. It is possible to sequence these samples to a greater depth if warranted. It is important to keep in mind that in 3'-DGE, and most low-input methods, only the 3' poly-A end of the transcript is preserved. Therefore, this technique cannot be used if it is important to capture splice-isoforms or transcripts that do not have a poly-A tail.

This is a modification of the SCRB-seq protocol originally published here:

<https://www.biorxiv.org/content/10.1101/003236v1>;

Recognition also goes to the Enard group and their modifications to SCRB-seq found here:

<https://www.protocols.io/view/mcscrb-seq-protocol-p9kdr4w>

The home-brew SPRI beads used in this study are made with this protocol:

dx.doi.org/10.17504/protocols.io.bkppkvmn

Keywords: bulk RNA sequencing, RNA-seq, transcriptomics, SCRB-seq

GUIDELINES

- The complete list of reagents and plasticware with catalog numbers can be found in the 'Materials' section.
- Make sure all steps involving cell lysate and RNA before reverse transcription are carried out swiftly.
- All primer sequences are listed below:

Oligo	Vendor	Purification	Concentration	Sequence
barcoded oligo-dT (E3V6NEXT)	IDT	TruGrade	2 µM	Biotin-ACACTCTTTCCCTACACGACGCTCTTCCGATCT[B C6][UMI10][T30]VN
TSO unblocked (E5V6NEXT)	IDT	HPLC	100 µM	ACACTCTTTCCCTACACGACGCrGrGrG
PreAmp (SINGV6)	IDT	Desalted	10 µM	Biotin-ACACTCTTTCCCTACACGACGC
3' enrichment primer (P5NEXTPT5)	IDT	HPLC	5 µM	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCG*A*T*C*T
i7 Index Primer (N7XX)	IDT	TruGrade	5 µM	CAAGCAGAAGACGGCATACGAGAT[i7]GTCTCGTGGGCTCGG

oligoD.T.txt List of RT primers, updated with p11 primer that works Aug 2020

Primers can be ordered resuspended in TE and should be stored as 10 µl aliquots upon arrival at -80°C. Primers are good indefinitely when stored properly.

MATERIALS

MATERIALS

Laboratory equipment is not listed here.

See Guidelines for the primers needed with these materials.

⊗	β -mercaptoethanol	Merck MilliporeSigma (Sigma-Aldrich) Catalog #M3148
⊗	Buffer TCL	Qiagen Catalog #1031576
⊗	Reagent Reservoirs (DNase/RNase Free)	Thomas Scientific Catalog #1228K15
⊗	Marsh Plates 384 Well Polypropylene Storage Plates	Thermo Fisher Scientific Catalog #AB0781
⊗	Eppendorf twin.tec® PCR Plates	Eppendorf Catalog # 951020745
⊗	384 well magnet	Contributed by users Catalog # 771G-7AM-1
⊗	Fluotics 30 uL NS tips	Agilent Technologies Catalog #AGI-30.NS
⊗	80% Ethanol	Contributed by users
⊗	nuclease free water	Contributed by users
⊗	SPRI beads (homemade) or Ampure XP beads	Contributed by users
⊗	Qubit™ RNA HS Assay Kit	Thermo Fisher Catalog #Q32855
⊗	Maxima H Minus Reverse Transcriptase (200 U/uL)	Thermo Fisher Scientific Catalog #EP0752
⊗	Deoxynucleotide Solution Mix - 40 umol of each	New England Biolabs Catalog #N0447L
⊗	VBlok reservoir from Clickbio	Thomas Scientific Catalog # VBLOK200
⊗	Exonuclease I Reaction Buffer - 6.0 ml	New England Biolabs Catalog #B0293S
⊗	Exonuclease I (E.coli) - 3,000 units	New England Biolabs Catalog #M0293S
⊗	QIAquick PCR purification kit	Qiagen
⊗	Advantage® 2 PCR Kit, 100 reactions	Clontech Catalog #639206
⊗	Agencourt AmPure XP beads	Contributed by users Catalog #A63880
⊗	Qubit dsDNA HS (High sensitivity) Assays	Thermo Fisher Scientific Catalog #Q32851
⊗	Nextera XT DNA Sample Preparation Kit, 96 samples	Illumina, Inc. Catalog #FC-131-1096
⊗	Nextera Flex Sample preparation kit	Illumina, Inc. Catalog #20018705
⊗	Agilent High Sensitivity DNA Kit	Agilent Technologies Catalog #5067-4626

BEFORE START INSTRUCTIONS

Wipe bench surfaces with RNase Away/Zap and keep working environment clean.

Cell Plating

1h

- 1 Cell plating conditions will vary depending on your experiment and cell type. Usually 2,000-10,000 cells per well.

For best compatibility with ICCB-L robotics please use **GREINER MICROPLATE, 384 WELL, PS, F-BOTTOM, μ CLEAR**

Note

This protocol has only been tested with adherent mammalian cells.

Note that you should **ONLY** plate cells in wells you want data from. Any unused wells need to be empty of cells. PBS or Media is fine, but no cells.

If you are setting this up on your own robotic system you can use a different plate. Just be consistent with the plates used for proper robotic head height.

Cell Lysis

1h

- 2 Purified RNA or intact adherent cells in 384 well format can be used for 3'-DGE. (Note, we have had better experience with starting from intact cells.)

1. If starting with purified RNA, skip to Step 22.

2. If starting with intact cells, proceed to Step 3.

3. Lysate from 4 x 96 well plates can also be used. 96 well plates are re-arrayed to 384 well format in the first stage of RNA purification.

- 3 Prepare enough **Lysis Buffer** for the number of samples. (Protocol is for 384 well format)
TCL buffer + 1 % β -mercaptoethanol

A	B	C
Reagent	Well	Plate
TCL Buffer	9.9 μ L	4.22 mL
β -mercaptoethanol	0.1 μ L	42.2 μ L

A	B	C
Total	10 μ L	4.22 mL

- 4 Aspirate media and wash wells once with PBS.

We do this with a Biotek plate washer:

- 5 Remove most of the PBS. Our robotic system usually leaves behind about 10 μ L of PBS.
 - *In a 96 well plate about 15-20 μ L of PBS is left behind*
- 6 Add 10 μ L of lysis buffer to each well of your 384-well plate.
 - *In a 96 well plate use 30 μ L of lysis buffer.*
- 7 Incubate for 00:15:00 Room temperature on belly dancer to aid in lysis. Freeze -80 $^{\circ}$ C until ready to proceed with RNA extraction.

Note

Incubation can be 0-30 min. Be consistent with your samples.

Safe Stop Point

- 8 Lysate plate can be stored at -80 °C until RNA extraction. Use within a few weeks of lysis is best, but we have stored samples for months with successful extraction.

Set up for RNA purification on BRAVO

- 9 Bring 384 well lysate plate to ICCB-L with the following reagents:

- Home Brew SPRI Beads [dx.doi.org/10.17504/protocols.io.bkppkvmn](https://doi.org/10.17504/protocols.io.bkppkvmn) (15 mL)
- Fresh 80% Ethanol (30 mL)
- Water (10 mL)
- Reagent Reservoirs
- 384 well multichannel pipette & Tips
- Set of standard pipettes & Filter tips
- Qubit
 - instrument
 - reagents & standards
 - tubes
- Repeater & 2x 1 mL tips
- 1x Marsh waste plate
- 3x Marsh plates for reagents
- 2x Eppendorf Twintec PCR plates (*need 2x more for RT step*)
- 3x BRAVO tip boxes for reaction (*need 2.25x more for RT step*)

10 Fill 3 separate clean MARSH plates with:

- 1) 40 μL SPRI beads/well
- 2) 75 μL 80% Ethanol
- 3) 35 μL RNase free water

We use a 384 well multichannel (16 channel) pipette to make this easier.

Note

*Important - seal water plate and spin down 00:01:00 1000 xg.

Removes any bubbles that form under the water in the plate.

If purifying multiple plates, can double the volume of water. Other plates need to be fresh for each reaction.

11 Thaw lysate plate in the hood for around 00:05:00 .

Spin down plate 00:01:00 1000 xg before opening seal.

12 Set up reagents and plates on BRAVO as follows:

Tips (30uL NS Fluotics) (3)		Reaction Plate (384 Eppendorf Twin.tec) (1)
Waste (384 ABgene AB-0781) (4)	Cell Lysate (384 Greiner Black w/ Clear bottom) (5)	Bead Source (384 ABgene AB-0781) (6)
Nuclease Free H₂O (384 ABgene AB-0781) (7)	Ethanol (384 ABgene AB-0781) (8)	Final Plate (384 Eppendorf Twin.tec) (9)

BRAVO DGE program for RNA purification (step 1 of DGE pr...

- 13
- Load tips #1 [Fluotics AGI-30.NS].
 - Mix cell lysate (**WATCH ROBOT – be sure can see efficient mixing**).
 - Transfer 10 µL of cell lysate to a 384-well Eppendorf Twintec PCR plate.
This is the "Reaction Plate".

Note

Need to watch during mixing. If lysate mixing is not achieved reaction will not work.
If a different plate was used for cell plating you may need to adjust the height of tips on the BRAVO.

- 14
- Mix SPRI beads in bead source plate (30 µL 2x)
 - Add 28 µL of beads to the reaction plate and mix (15 µL 5x).
This is a 1.5x SPRI clean. Ratio of sample to beads is 1:1.5.

- Incubate Room temperature for 00:05:00 .

15

- Transfer the reaction plate to the magnet (V&P Scientific 771G-7AM-1).

- Incubate Room temperature for 00:05:00 .

(TAPE DOWN PLATE ON MAGNET)

Note

Need to tape plate onto magnet or it gets pulled up by Bravo head.

16

- Load tips #2 [Fluotics AGI-30.NS].
- Leaving the reaction plate on the magnet, remove liquid and dispense into the waste plate.
- Wash the beads with 25 μ L of 80% EtOH (00:00:30 delay before removing the liquid).
- Leaving the reaction plate on the magnet, remove liquid and dispense into the waste plate.
- Wash the beads a second time with 25 μ L of 80% EtOH (00:00:30 delay before removing the liquid).
- Leaving the reaction plate on the magnet, remove liquid and dispense into the waste plate.

1m

17

Perform one last aspirate to remove all of the EtOH from the reaction plate.

Dry beads for 00:01:00 .

1m

18

- Load tips #3 [Fluotics AGI-30.NS].
- While the reaction plate is still on the magnet, add 20 μ L of nuclease free water.

(Remove tape from magnet and manually separate plate from magnet if stuck.)

19

Remove the reaction plate from the magnet and resuspend the beads.
(15 μ L 15x at a height of 3 mm from the bottom of the plate; 3x).

Look at plate after mixing and be sure it looks well mixed.

Note

If mixing is problematic you can take plate off the Bravo, seal it, smack it down then vortex it. Then quick spin followed by another mix step on the Bravo.

If the SPRI beads look clumpy/sandy when mixing it is possible the extraction failed due to an issue with the lysis buffer, beads or ethanol. Heating the plate up to 50°C can help with the elution. This should be a last resort option.

- 20
- Incubate Room temperature for 00:02:00 .
 - Transfer the reaction plate to the magnet.
 - Incubate 00:05:00 at Room temperature .
 - Transfer all of the supernatant to a fresh 384-well Twintec PCR plate.
This is the "Final Plate".

- 21
- Pick random wells to check RNA concentration by Qubit.
- 2-5 ng/μl is the goal
 - If starting from 96 well plates concentrations are often much higher 5-10 ng/μl

Note

Can also take some sample to run RNApico BioAnalyzer to check RNA quality.

Safe Stop Point

- 22
- RNA can be frozen here at -80 °C before moving on to RT/Library prep.

Note

Usually proceed directly to next step in reaction.

Set up for reverse transcription on BRAVO

- 23
- Bring reagents for RT:

- Repeater & 2x 1 mL tips
- 2x Eppendorf Twintec PCR plates
- 2.25x BRAVO tip boxes for reaction
- Set of standard pipettes & Filter tips

5x RT Buffer 850 µl
 dNTPs (NEB - #N0447L) 425 µl
 E5V6NEXT (10uM) 425 µl
 Maxima H- RT Enzyme (Thermo Scientific #EP0753) 50 µl

24 Make master mix

A	B	C
Reagent	Well	Plate
Maxima H Minus RT	0.125 µl	50 µl
Maxima RT Buffer (5x)	2 µl	850 µl
dNTPs (25 mM)	1 µl	425 µl
E5V6NEXT (10 uM)	1 µl	425 µl
Total	4.125 µl	1750 µl

Dispense 36 µL master mix into the first 3 columns of a 384-well eppendorf twintec plate. This is your "Master Mix Plate".

25 Thaw the RNA plate and the E3V6Next adapter plate (10 µL) 00:05:00 in hood.

Spin down plates 00:01:00 1000 xg.

26 Set up reagents and plates on BRAVO as follows:

PCR Plate (384 Eppendorf Twin.tec) (1)		Tips (30uL NS Fluotics) (3)
Tips (Only 6 columns required) (30uL NS Fluotics) (4)	Primers (384 Eppendorf Twin.tec) (5)	EMPTY (6)
Empty Tip Box (30uL NS Fluotics) (7)	Master Mix (384 Eppendorf Twin.tec) (8)	EMPTY (9)

Note

If using RNA as input, make sure to normalize concentrations (minimum: 0.5 ng/μl, optimum 20 ng/μl) across samples.

BRAVO DGE program for RT (step 2 of DGE program)

- 27 Using the BRAVO Add 4.125 μL of the master mix to each well of the "PCR plate".

Change tips after every 4 dispense steps.

Note

There is JUST barely enough master mix for a whole plate. In the last dispense watch to make sure enough master mix went into all wells. It is sometimes necessary to dispense into a few wells by hand.

- 28 Add 1 μL of the unique barcoded E3V6NEXT adapters (10 micromolar (μM)) per well from the Primer plate to the PCR Plate.
(Be sure the adapter plate is taped down.)

- 29 ■ Using the BRAVO, transfer $4.875\ \mu\text{L}$ (assume $\sim 5\ \text{ng}/\mu\text{L}$) of the RNA from the RNA plate to the PCR Plate.

(Be sure the RNA plate is taped down to the magnet.)

Mix $5\ \mu\text{L}$ 3x

Note

It is important the RNA plate is on the magnet for 1-2 minutes before transferring. There are often residual SPRI beads in the RNA plate that you do not want carried over into the PCR plate for the RT reaction.

- 30 Seal the plate and bring to $42\ ^\circ\text{C}$ incubator. This could be done in qPCR machine. We use an air incubator with a metal block pre-equilibrated in the incubator for even heating.

Note

Block: Labnet D12384 Aluminum Dry Bath Dual Block, Holds 384 Well PCR Plate

- 31 Incubate for 01:30:00 at $42\ ^\circ\text{C}$.

cDNA Pooling & Purification

- 32** Fit 384 well plate upside down onto collection plate (*VBlok reservoir from Clickbio VBLOK200*).

- 33** Spin 1,000 xg for 00:02:00 .

- 34** Collect cDNA into 50 mL Falcon tube. Expected volume ~3.4-3.6 mL.

- 35** Add 5x volume Qiagen PB buffer (3.4 mL = 17 mL, 3.6 mL = 18 mL)

Add 10 mL directly to a 50 mL Falcon tube.

Use the rest to rinse out collection plate then pipette into the 50 mL Falcon tube with the rest of the sample.

- 36** Load the sample onto QIAquick PCR purification column using vacuum filter system.
The loading will slow down as more sample is loaded.

Note

Jam the luer-lock end of a 3 or 5 mL syringe barrel onto the column to make a "tube extender" to allow for easier loading of the column.

- 37 Once all the sample has been loaded, wash the syringe barrel column with 750 μL of PE buffer.
- 38 Remove the syringe barrel column and wash the column once more with 750 μL of PE buffer.
- 39 Dry the column by centrifuging at $>10,000 \text{ xg}$ for 00:02:00 .

40 Elute cDNA with 21 µL of nuclease free water.

Let water sit on column for 00:01:00 , then spin at >10,000 xg for 00:01:00 .

41 Check concentration of sample using dsDNA HS Qubit Assay.

Expected yield 30-80 ng/µl.

Note

Do NOT pause protocol here.

Proceed through Exo I treatment and amplification before pausing protocol.

Exonuclease I Treatment

42 Mix 17 µL of purified cDNA with 2 µL of Exonuclease I Buffer (10x) and 1 µL of Exonuclease I.

Incubate as follows:

A	B	C
Step	Temperature	Time
Incubation	37°C	30 min
Heat Inactivation	80°C	20 min

Full length cDNA Amplification

43 Prepare Amplification Mix

A	B
Reagent	1x
cDNA from previous step	20 µl
10x Advantage 2 PCR buffer	5 µl
dNTPs	1 µl
SINGV6 primer (10 µM)	1 µl

A	B
Advantage 2 Polymerase Mix	1 µl
H2O	22 µl
Total	50 µl

Note

You can also use KAPA HiFi 2x ReadyMix.

If using KAPA mix, change the 95 °C steps below to 98 °C .

44 Incubate the Pre Amplification PCR as follows:

A	B	C	D
Step	Temperature	Time	Cycles
Elongation	72°C	3 min	1 cycle
Initial Denaturation	95°C	30 sec	1 cycle
Denaturation	95°C	10 sec	5 cycles*
Annealing	55°C	30 sec	
Elongation	72°C	1 min	
Final Elongation	72°C	5 min	1 cycle
Storage	4°C	∞	

Note

*For a 384 well plate 5 cycles is enough.

For 96 well plate, run 10 cycles.

If using KAPA HiFi mix, change 95 °C steps to 98 °C .

Safe Stop Point

45 Can leave reaction at 4 °C overnight.

cDNA Bead Purification (0.7x)

46 Dilute PCR mix with 50 µL Water for 100 µl total reaction volume.

47 Mix sample with 70 µL Ampure XP beads at a ratio of 1:0.7

Note

Ampure XP is used instead of homebrew SPRI beads in order to ensure best size selection at this critical stage where we are removing any primer dimers.

48 Incubate for 00:15:00 at Room temperature .

49 Place the tube on the magnet stand until clear and discard supernatant.

50 Wash with 500 µL of 80% EtOH while the tube is on the magnet. Discard the supernatant.

51 Repeat wash step once more.

52 Air dry beads for 00:05:00 .

Note

Depending on temperature and humidity, the beads may dry faster. Therefore it is important to regularly check the beads to avoid over-drying.

53 Elute cDNA in 16 μL H_2O .

Safe Stop Point

54 Can leave sample at $-20\text{ }^{\circ}\text{C}$ until ready to continue.

cDNA Quantification and Quality Check

55 Quantify the cDNA using the Quant-iT PicoGreen dsDNA assay kit or dsDNA HS Qubit following the manufacturer's protocol.

Use 1 μL of clean cDNA for quantification.

Usually $\sim 15\text{-}60\text{ ng}/\mu\text{L}$

56 Optional: Quality check the cDNA using the Agilent 2100 Bioanalyzer with High Sensitivity DNA Analysis Kits.

(Usually do not bother with this step.)

Expected result

Tagmentation (Nextera XT)

- 57** Prepare a 0.8 ng/μL solution of cDNA for Nextera XT Tagmentation using ~1 ng/μl diluted sample from QC step.

Note

Can also use **Nextera FLEX kit** instead of XT kit. Use mid range amount of sample input ~250ng and use 1/2 volume reactions. Follow manual instructions performing x6 cycles of amplification. For the final purification, follow the protocol which includes a double sided SPRI selection (0.5x followed by additional 1x).

- 58** Prepare Tagmentation Mix (enough for 5 replicate reactions)

A	B	C
Reagent	1x	5x
cDNA (0.8 ng/μL)	1 μL	5 μl
Tagment DNA Buffer	10 μL	50 μl
Amplicon Tagment Mix	5 μL	25 μl

A	B	C
H2O	4 µL	20 µl
Total	20 µL	100 µl

Note

Do tagmentation reaction in replicates of 5.

59 Distribute 20 µL of Tagmentation Mix in 5 wells and incubate the reaction for 00:10:00 at 55 °C .

60 Add 5 µL NT Buffer to each well, mix, and incubate for 00:05:00 at Room temperature .

Nextera Index PCR

61 Nextera Index PCR Mix

A	B	C
Reagent	1x	5x
P7NXX (5 µM)	0.5 µL	2.5 µl
P5NextPT5 (5 µM)	0.5 µL	2.5 µl
NPM PCR Mix	15 µL	75 µl
H2O	9 µL	45 µl
Total	25 µL	125 µl

62 Add 25 µL Nextera Index PCR mix to each replicate and incubate as follows:

A	B	C	D

A	B	C	D
Step	Temperature	Time	Cycles
Elongation	72°C	3 min	1 cycle
Initial Denaturation	95°C	30 sec	1 cycle
Denaturation	95°C	10 sec	12 cycles
Annealing	55°C	30 sec	
Elongation	72°C	1 min	
Final Elongation	72°C	5 min	1 cycle
Hold	4°C	∞	

Library Bead Purification

63 Pool all 5 PCR Replicates; rinse PCR wells with 50 µL of water (total). Combine with pooled sample for a total of 300 µL of sample.

64 Mix Index PCR with 240 µL Ampure XP beads at a ratio of 1:0.8

Note

If doing a gel cleanup use a 1:1.2 ratio to capture as much cDNA since the primers will be cut out from the gel.

65 Incubate for 00:15:00 at Room temperature .

66 Place the plate on the magnet stand until clear and discard supernatant.

67 Wash with 500 µL of 80% EtOH while the plate is on the magnet. Discard the supernatant.

68 Repeat wash step once more.

69 Air dry beads for 00:05:00 .

Note

Depending on temperature and humidity, the beads may dry faster. Therefore it is important to regularly check the beads and avoid over-drying.

70 Elute library in 16 µL H₂O.

Final Sample QC

71 Quantify sample using Qubit dsDNA HS. Expected yield is low 1-4 ng/µl (which is ~4 nM).

72 Run sample on Tapestation or Bioanalyzer to assess size. Below is an example from the tapestation.

B1: IDG

Sample Table

Well	Conc. [pg/ul]	Sample Description	Alert	Observations
B1	150	IDG		

Peak Table

Size [bp]	Calibrated Conc. [pg/ul]	Assigned Conc. [pg/ul]	Peak Molarity [pmol/l]	% Integrated Area	Peak Comment	Observations
25	489	-	30100	-		Lower Marker
333	150	-	691	100.00		
1500	250	250	256	-		Upper Marker

Region Table

From [bp]	To [bp]	Average Size [bp]	Conc. [pg/ul]	Region Molarity [pmol/l]	% of Total	Region Comment	Color
163	1001	399	873	4040	94.82		

Safe Stop Point

73 Store sample at -20 °C until sequenced.

Sequencing

- 74** We have found that running a single 384 well DGE plate at a time is best for clustering on a NextSeq. For a NovaSeq run, multiple plates can be pooled together.

These libraries are difficult to cluster appropriately: usually you need to load almost 2x the concentration you would normally run to get good clustering. (Test this in your own hands!)

- 75** Libraries are run in a paired-end manner:

17 cycles Read 1	Well index and UMI
8 cycles Index 1	Nextera Plate Index
60 (or more) cycles Read 2	cDNA Fragment

Barcode: Well barcode (N6) + Unique Molecular Identifier (N10)
 Index: Plate indexing

- 76** BCL file to FASTQ file conversion is a bit tricky due to the short length of Read 1. Speak to your sequencing core to ensure you get your data correctly.

The code below has worked for our team:

```
bcl2fastq --adapter-stringency 0.9 --barcode-mismatches 0 --fastq-compression-level 4 --ignore-missing-bcls --ignore-missing-filter --ignore-missing-positions --min-log-level INFO --minimum-trimmed-read-length 0 --sample-sheet /n/boslfs/INSTRUMENTS/illumina/190107_NB501677_0376_AHTLM5BGX7/SampleSheet.csv --runfolder-dir /n/boslfs/INSTRUMENTS/illumina/190107_NB501677_0376_AHTLM5BGX7 --output-dir /n/boslfs/ANALYSIS/190107_NB501677_0376_AHTLM5BGX7 --processing-threads 8 --no-lane-splitting --mask-short-adaptor-reads 0 --use-bases-mask y17,i8,y46
```