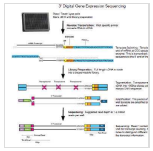

Version 2

May 17, 2021

3'-DGE High Throughput RNA Library Preparation V.2

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dx.doi.org/10.17504/protocols.io.bumynu7w

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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 asses 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

DOI

dx.doi.org/10.17504/protocols.io.bumynu7w

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.bumynu7w
Version created by Sarah Boswell

WHAT'S NEW

Walk through with new user found a few mistakes that were corrected in V2.

KEYWORDS

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

CREATED

Apr 29, 2021

LAST MODIFIED

May 17, 2021

PROTOCOL INTEGER ID

49560

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[BC6][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

oligodT.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 TEXT

MATERIALS

Laboratory equipment is not listed here.
See Guidelines for the primers needed with these materials.

ß -mercaptoethanol **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**

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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 Catalog #FC-131-1096**

Nextera Flex Sample preparation kit **illumina Catalog #20018705**

Agilent High Sensitivity DNA Kit **Agilent Technologies Catalog #5067-4626**

Perkin Elmer CellCarrier ULTRA **Perkin Elmer**

BEFORE STARTING

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, µCLEAR **now sold as Perkin Elmer CellCarrier ULTRA**

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

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.)

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

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

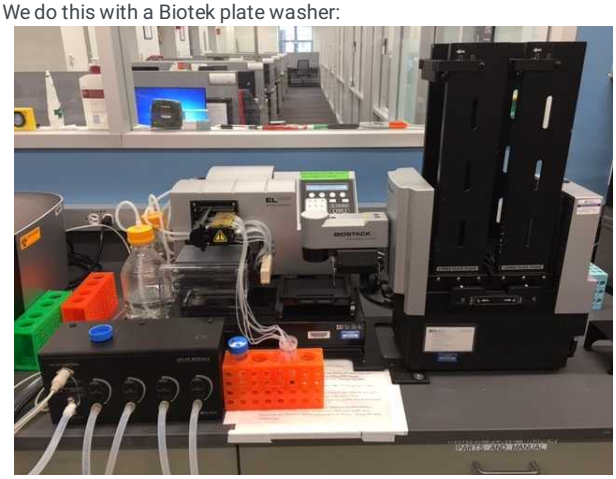
3. Lysate from 4 x 96 well plates can also be used. 96 well plates are re-arrayed to 384 well format in the first stage of RNA purification.

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

A	B	C
Reagent	**Well**	**Plate**
TCL Buffer	9.9 µL	4.22 mL
ß-mercaptoethanol	0.1 µL	42.2 µl
Total	**10 µL**	**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 µl of PBS.

- *In a 96 well plate about 15-20 µl of PBS is left behind*

6 Add **10 µl** of lysis buffer to each well of your 384-well plate.

- *In a 96 well plate use 30 µl of lysis buffer.*

7 Incubate for **00:15:00** **Room temperature** on belly dancer to aid in lysis. Freeze **-80 °C** until ready to proceed with RNA extraction.

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.

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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 (**15 mL**)
- Fresh 80% Ethanol (**50 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.

*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** .

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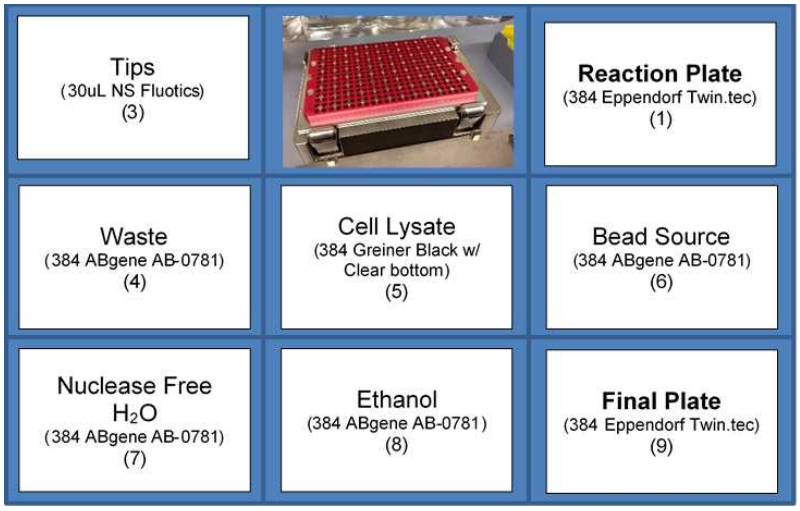
Spin down lysate plate and water plate **00:01:00** 1000 xg before opening seal.

12 Set up reagents and plates on BRAVO as follows:



BRAVO DGE program for RNA purification (step 1 of DGE program)

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".

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 (**25 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)

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

- Load tips #2 [Fluotics AGI-30.NS]. 1m

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16
- Leaving the reaction plate on the magnet, remove liquid and dispense into the waste plate.
- Wash the beads with **25 µl** 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 µl** 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** .

18
- Load tips #3 [Fluotics AGI-30.NS].
- While the reaction plate is still on the magnet, add **20 µl** 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 µl** 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.

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.

If samples this hard to dissolve / look clumpy it may be worth aborting the experiment unless samples are precious

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
- Use 2µl of sample to check concentration
- If starting from 96 well plates concentrations are often much higher 5-10 ng/µl

Can also take some sample to run RNA pico BioAnalyzer to check RNA quality.
This is usually done in the pilot 3'-DGE run. On the actual sample plate BioA is not usually run.

Safe Stop Point

Citation: Sarah Boswell, Caitlin Mills, Feodor Price, Stewart Rudnicki (05/17/2021). 3'-DGE High Throughput RNA Library Preparation. https://dx.doi.org/10.17504/protocols.io.bumynu7w

22

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

BEST to proceed directly to next step in reaction.

Seal and spin RNA plate before using for RT step.

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 RNA and adapter plates **00:01:00** 1000 xg.

26 Set up reagents and plates on BRAVO as follows:

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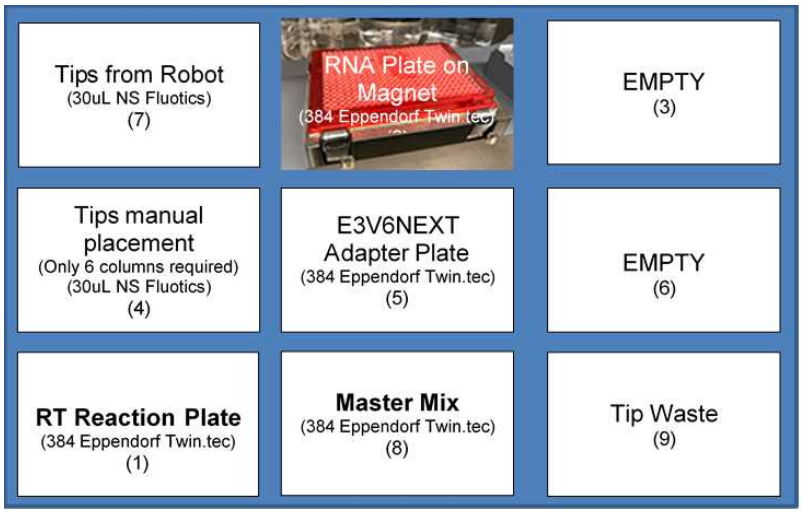

If using RNA as input, make sure to normalize concentrations (minimum: 0.5 ng/µl, optimum 20 ng/µl) across samples.

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.

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 ([M] **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 µl** (assume ~5 ng/µl) of the RNA from the RNA plate to the PCR Plate.
(Be sure the RNA plate is taped down to the magnet.)

Mix **5 µl** 3x

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 °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.

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

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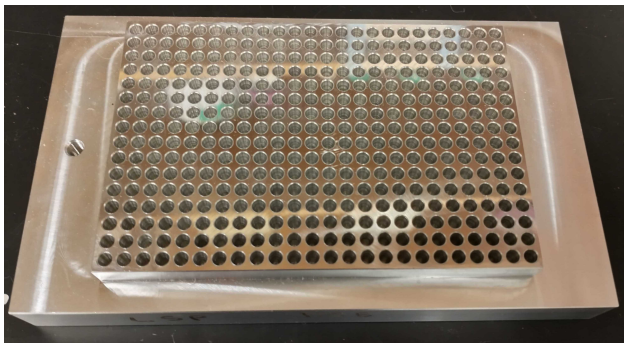

31 Incubate for **01:30:00** at **42 °C** .

32 After incubation give plate a quick spin 1000xg **00:01:00** 1m
Then carefully remove the seal

cDNA Pooling & Purification

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

34 Spin 1,000 xg for **00:02:00** .

35 Collect cDNA into 50 mL Falcon tube. Expected volume ~3.2-3.6 mL.

36 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.

37 After Inverting the Falcon tube to mix, give it a quick spin 1000xg **00:01:00** 1m

Load the sample onto QIAquick PCR purification column using vacuum filter system.

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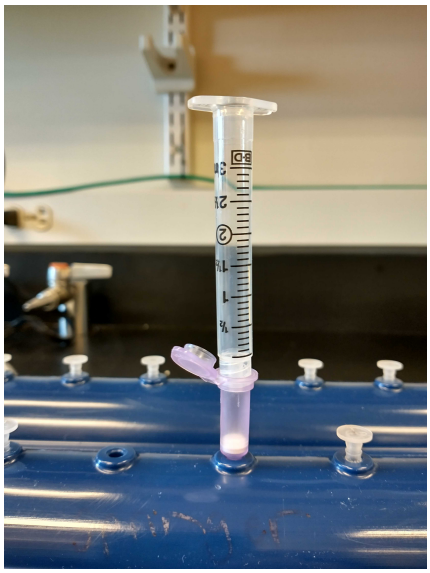
38 The loading will slow down as more sample is loaded.

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.



39 Once all the sample has been loaded, wash the syringe barrel column with **750 µl** of PE buffer.

40 Remove the syringe barrel column and wash the column once more with **750 µl** of PE buffer.

41 Dry the column by centrifuging at >10,000 xg for **00:02:00** .

42 Elute cDNA with **21 µl** of nuclease free water (usually with water warmed to ~ **60 °C** .
Let water sit on column for **00:01:00** , then spin at >10,000 xg for **00:01:00** .

43 ⚠

Check concentration of sample using dsDNA HS Qubit Assay.
Expected yield 30-80 ng/µl.

Do NOT pause protocol here.
Proceed through Exo I treatment and amplification before pausing protocol.

Exonuclease I Treatment

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44 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

NOTE: if you have >17ul of material take ALL of it to the next step. You can increase the amount of 10x buffer and still use 1ul of Exonuclease 1.

Full length cDNA Amplification

45

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
Advantage 2 Polymerase Mix	1 µl
H20	22 µl
Total	**50 µl**

NOTE: If you have >20ul of material from step 45 you can decrease the amount of water in the recipe above.

You can also use KAPA HiFi 2x ReadyMix.
If using KAPA mix, change the **95 °C** steps below to **98 °C** .

46 Incubate the Pre Amplification PCR as follows:

A	B	C	D
Step	**Temperature**	**Time**	**Cycles**
Initial Denaturation	95˚C	1 min	1 cycle
Denaturation	95˚C	15 sec	**5 cycles***
Annealing	65˚C	30 sec	
Extension	68˚C	6 min	
Final Extension	72˚C	10 min	
Storage	4˚C	forever	

*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** .

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Safe Stop Point

47 ⏸

Can leave reaction at 4 °C overnight.

cDNA Bead Purification (0.7x)

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

49 Mix sample with 70 µl Ampure XP beads at a ratio of 1:0.7
Be sure Ampure beads are at Room Temperature and fully resuspended before use.

> 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.

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

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

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

53 Repeat wash step once more.
Use aspirator to remove residual ethanol to speed up drying time.

54 Air dry beads for 00:05:00 .

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

55 Take sample off the magnet and elute cDNA in 16 µl H_2O.

56 Incubate sample for 00:02:00 at RT to elute the cDNA 2m

57 Then place the sample back on the magnet and remove 14 µl . Use residual sample on beads to quantitate sample with Qubit.

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Safe Stop Point

58 ⏸

Can leave sample at -20 °C until ready to continue.

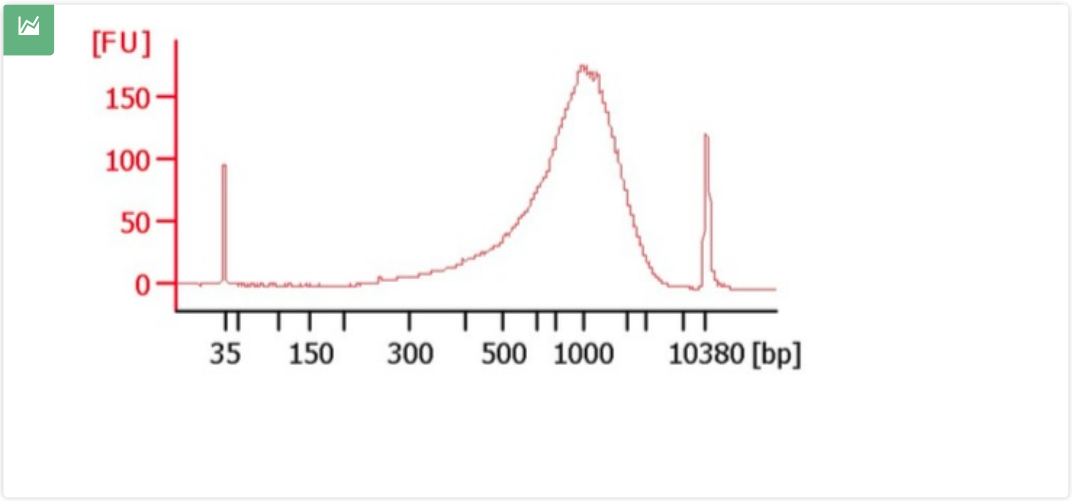
cDNA Quantification and Quality Check

59 Quantify the cDNA using the Quant-iT PicoGreen dsDNA assay kit or dsDNA HS Qubit following the manufacturer's protocol. Note will QC again with Qubit after diluting sample for Nextera XT protocol.

Use 1 µl of clean cDNA for quantification.

Usually ~15-60 ng/µl

60 Optional: Quality check the cDNA using the Agilent 2100 Bioanalyzer with High Sensitivity DNA Analysis Kits. (Usually do not bother with this step, but can be informative when you are new to the protocol.)



Tagmentation (Nextera XT)

61 Prepare 20ul of a 1 ng/µL solution of cDNA for Nextera XT Tagmentation.
Use 5ul to check concentration by Qubit.

Further dilute sample to 0.8ng/ul for Nextera XT protocol.

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 x5 cycles of amplification. For the final purification, follow the protocol which includes a double sided SPRI selection (0.5x followed by additional 1x).

62 Prepare Tagmentation Mix (enough for 5 replicate reactions).
NOTE - add Amplicon Tagment Mix last (ATM)

A	B	C
Reagent	**1x**	**5x**
cDNA (0.8 ng/µL)	1 µL	5 µl
Tagment DNA Buffer	10 µL	50 µl
H2O	4 µL	20 µl
Amplicon Tagment Mix	5 µL	25 µl
Total	**20 µL**	**100 µl**

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Do tagmentation reaction in replicates of 5 in a PCR strip.

63 Distribute **20 µl** of Tagmentation Mix in 5 reactions in PCR strip and incubate the reaction for **00:10:00** at **55 °C** .

64 Add **5 µl** NT Buffer to each reaction in PCR strip, mix, and incubate for **00:05:00** at **Room temperature** .

Nextera Index PCR

65

Nextera Index PCR Mix

A	B	C
Reagent	**1x**	**5x**
Indexing primer N7XX (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**

66 Add **25 µl** Nextera Index PCR mix to each replicate and incubate as follows:

A	B	C	D
Step	**Temperature**	**Time**	**Cycles**
Gap Fill	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	1 min	1 cycle
Hold	4˚C	∞	

Library Bead Purification

67 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.

68 Mix Index PCR with **240 µl** Ampure XP beads at a a ratio of 1:0.8

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.

Citation: Sarah Boswell, Caitlin Mills, Feodor Price, Stewart Rudnicki (05/17/2021). 3'-DGE High Throughput RNA Library Preparation. https://dx.doi.org/10.17504/protocols.io.bumynu7w

69 Incubate for **00:15:00** at **Room temperature** .

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

71 Wash with **500 µl** of 80% EtOH while the tube is on the magnet. Discard the supernatant.

72 Repeat wash step once more.

73 Air dry beads for **00:04:00** in **37 °C** air incubator then finish drying at RT. You can dry at RT only, but will take much longer to dry. 4m

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

74 Remove tube from the magnet and elute library in **15 µl** H_2O.

75 Incubate beads **00:02:00** at RT 2m

76 Put tube with eluted beads back on magnet for 2min

Remove **12 µl** for a final library

Use remining volume on beads for QC steps below

Final Sample QC

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

If yields are much higher could cycle for fewer cycles during final amplification.

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

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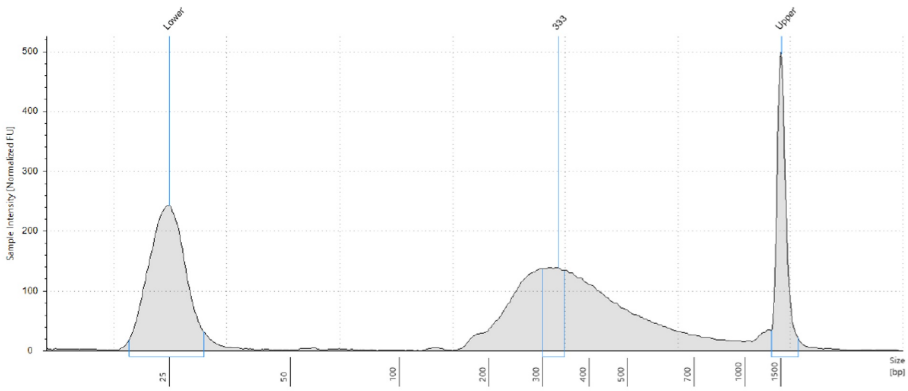
B1: IDG

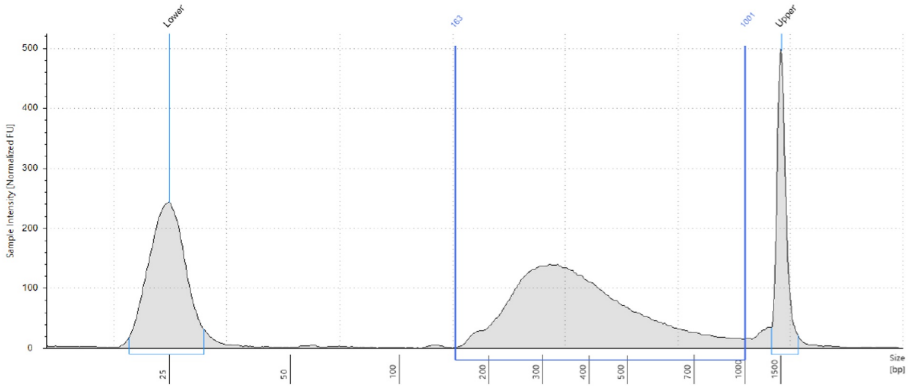


Sample Table

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

Peak Table

Size [bp]	Calibrated Conc. [pg/µl]	Assigned Conc. [pg/µl]	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/µl]	Region Molarity [pmol/l]	%of Total	Region Comment	Color
163	1001	399	873	4040	94.82		

Safe Stop Point

79 Store sample at **-20 °C** until sequenced.

Sequencing

80 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!)

81 Libraries are run in a paired-end manner:

17 cycles Read 1	Well index and UMI
8 cycles Index 1	Nextera Plate Index

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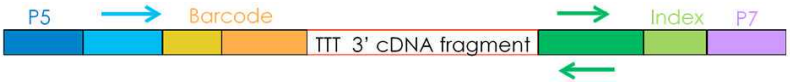
60 (or more) cycles Read 2 cDNA Fragment



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

82 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-adapter-reads 0 --use-bases-mask y17,i8,y46

Citation: Sarah Boswell, Caitlin Mills, Feodor Price, Stewart Rudnicki (05/17/2021). 3'-DGE High Throughput RNA Library Preparation. https://dx.doi.org/10.17504/protocols.io.bumynu7w