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Smart-seq3xpress V.3

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Protocol status: Working

We use this protocol and it's working

Created: January 08, 2025

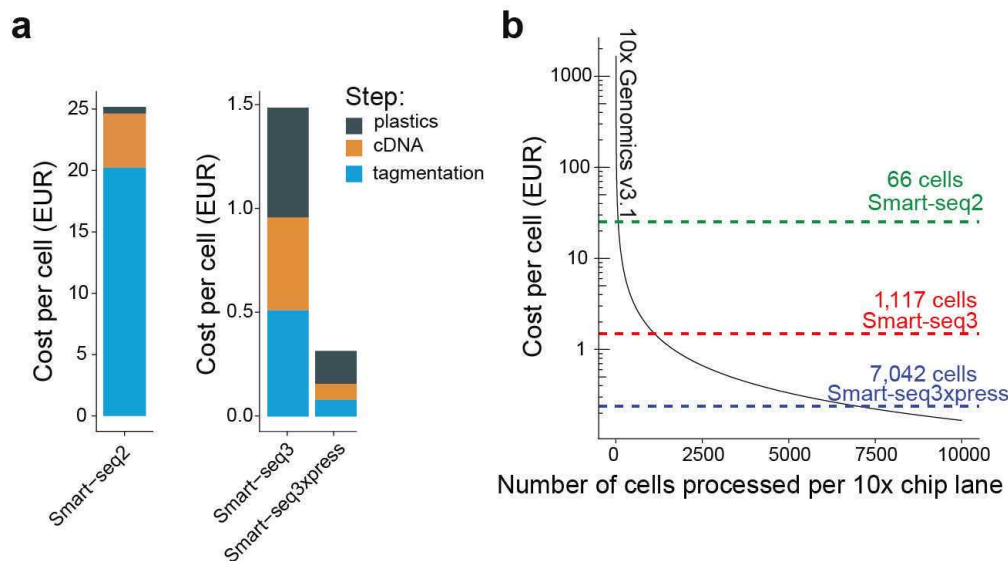
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Abstract

Plate-based single-cell RNA-sequencing methods with full-transcript coverage typically excel at sensitivity but are more resource and time-consuming. Here, we miniaturized and streamlined the Smart-seq3 protocol for drastically reduced cost and increased throughput.



(a) Cost of Smart-seq library preparations by summing up list prices of reagents and consumables. Shown is the cost per cell divided by costs relating to cDNA synthesis (Lysis, RT, PCR), tagmentation (including indexing PCR) and plastics. **(b)** Approximate cost per cell of Smart-seq library preparations and a single lane of 10x Genomics v3.1 relative to the number of cells analyzed.

Guidelines

List of Oligos:

	A	B	C	D	E
	Oligo Name	Vend or	Recommend ed Purification	Working Concentrati on	Sequence
	OligodTVN30	IDT	RNase-Free HPLC	100/10uM	/5BiosG/ACGAGCATCAGCAGCATAC GATTTTTTTTTTTTTTTTTTTTTTTT TTTTTTVN
	Smart-seq3xpress TSO	IDT	RNase-Free HPLC	100uM	/5BiosG/AGAGACAGATTGCGCAATG NNNNNNNNWwGrGrG
	Forward Primer	IDT	HPLC	100uM	TCGTCGGCAGCGTCAGATGTGTATA AGAGACAGATTGCGCAA*T*G
	Reverse Primer	IDT	HPLC	100uM	ACGAGCATCAGCAGCATAC*G*A

*phosphorothioate bonds

Index primers:

We use custom Nextera Indexes primers (standard 25 nmol oligo preps from IDT, delivered at 200 uM concentration in IDTE buffer) and we typically get performance that is indistinguishable from Illumina's primers.

For making your own primers, we recommend using the "DNABarcodes" R package. using the following settings:
Barcode length: 10 bp (or 8bp like Illumina primers, depending on the amount of cells you need indexed and sequenced at the same time)

Minimal levenshtein distance: 3

Filter out homopolymers >= 3

Filter for uneven GC content

Additionally, there seems to be an artifact on the NovaSeq v1 reagent kits for i5 index primers starting with the bases "AC", so we recommend to avoid those too!

(see supplementary information in this paper: <https://bmcbgenomics.biomedcentral.com/articles/10.1186/s12864-018-4703-0#Sec13>)

Attached here are dual unique Nextera i5 and i7 10bp barcodes and full sequences. Remember these are used in combination. So you do not need to order full 384 well plate of both i5 and i7 indexes. Mixing columns of i5 with rows of i7 creates quickly a large number of combinatorial indexes possible.



384set_Nextera_BC_order.xls

Materials

This is a comprehensive list of the reagents and consumables we use for the method.

Overlays:

 Vapor-Lock **Qiagen Catalog #981611**

Alternative Overlays:

 Silicone Oil 5 cSt **Merck MilliporeSigma (Sigma-Aldrich) Catalog #317667-250ML**

 Silicone Oil 20 cSt **Merck MilliporeSigma (Sigma-Aldrich) Catalog #378348-250ML**

 Silicone Oil 100 cSt **Merck MilliporeSigma (Sigma-Aldrich) Catalog #378364-250ML**

Reagents:

 Triton X-100 Solution **Merck MilliporeSigma (Sigma-Aldrich) Catalog #93443-100ML**

 Recombinant RNase Inhibitor (RRI) **Takara Bio Inc. Catalog #2313A**

 Poly Ethylene Glycol (PEG) 8000 (40% Solution) **Merck MilliporeSigma (Sigma-Aldrich) Catalog #P1458-25ML**

 dNTPs (10mM / each) **Thermo Fisher Scientific Catalog #R0192**

 UltraPure DNase/RNase-Free Distilled Water **Thermo Fisher Scientific Catalog #10977035**

 Trizma® base **Merck MilliporeSigma (Sigma-Aldrich) Catalog #T4661**

 NaCl (5M) **Thermo Fisher Scientific Catalog #AM9760G**

 MgCl₂ (1M) **Thermo Fisher Scientific Catalog #AM9530G**

 GTP (100mM Tris buffered solution) **Thermo Fisher Scientific Catalog #R1461**

 DTT (100mM Solution) **Thermo Fisher Scientific Catalog #707265ML**

 Maxima H-minus Reverse Transcriptase **Thermo Fisher Scientific Catalog #EP0753**

 SeqAmp DNA polymerase **Takara Bio Inc. Catalog #638509**

 N.N-DimethylFormamide **Merck MilliporeSigma (Sigma-Aldrich) Catalog #900638-4X2ML**

 Tagment DNA Enzyme 1 (TDE1) **Illumina, Inc. Catalog #20034198**

 SDS Solution 10% **Thermo Fisher Scientific Catalog #15553027**

 Tween-20 **Merck MilliporeSigma (Sigma-Aldrich) Catalog #P9416-50ML**

 Phusion HF DNA Polymerase **Thermo Fisher Scientific Catalog #F530L**

Reagents for making 22% Clean-up beads



⊗ Sera-Mag Speed Beads **GE Healthcare Catalog #65152105050250**

⊗ Poly Ethylene Glycol (PEG) 8000 **Merck MilliporeSigma (Sigma-Aldrich) Catalog #89510**

⊗ Sodium Azide **Merck MilliporeSigma (Sigma-Aldrich) Catalog #S2002-100G**

⊗ NaCl (5M) **Thermo Fisher Scientific Catalog #AM9760G**

⊗ IGEPAL CA-630 **Merck MilliporeSigma (Sigma-Aldrich) Catalog #I8896-50ML**

⊗ Tris-HCl (1M pH 8) **Thermo Fisher Scientific Catalog #AM9856**

⊗ EDTA (0.5M solution) **Thermo Fisher Scientific Catalog #AM9260G**

Plastics & other consumables:

⊗ Armadillo 384 well PCR plates **Thermo Fisher Scientific Catalog #AB2384**

⊗ Nalgene Disposable Polypropylene Robotic Reservoirs **Thermo Fisher Scientific Catalog #1200-1300**

⊗ Adhesive PCR Plate Seals **Thermo Fisher Scientific Catalog #AB0558**

⊗ Axygen Aluminium Sealing Film **Corning Catalog #PCR-AS-200** For  -80 °C Storage

Protocol materials

⊗ Protector RNase Inhibitor **Merck MilliporeSigma (Sigma-Aldrich) Catalog #3335399001**

⊗ Recombinant RNase Inhibitor **Takara Bio Inc. Catalog #2313A**

⊗ NXGen RNase Inhibitor **Lucigen Catalog #30281-2**

⊗ RNaseOUT™ Recombinant Ribonuclease **Thermo Fisher Scientific Catalog #10777019**

⊗ Agencourt AMPure XP **Beckman Coulter Catalog #A63880**

⊗ Nuclease-free Water

⊗ Nalgene Disposable Polypropylene Robotic Reservoirs **Thermo Fisher Scientific Catalog #1200-1300**

Considerations (PLEASE READ BEFORE START)

- 1
 - This protocol requires a some type of liquid handler capable of doing nanoliter dispenses. We have tested and used (**Formulatrix Mantis, Dispendix I.Dot & Dispendix I.Dot Mini**). Other (non contact) liquid dispensers should work as well, as long as they can dispense the required volumes accurately.
 - All volumes have been scaled to nanoliter volumes, as such the volume your cell is dispensed in matters. We have tested this protocol with an array of FACS machines and cell printers including BD FACSMelody, BD Fusion, BD Influx, Sony SH800S, Cellenion CellenOne, Cytana F.SIGHT Omics, which all typically dispenses the cell in ~5-10nl or less. If your instrument dispenses in higher volumes (> 50nl) , the protocol may either not work or not be as efficient.
 - Consider what buffer you use to dispense / sort your single cells in. Since the relative difference between sorted cell volume and lysis volume has overall decreased, common additives like FBS, BSA, EDTA can potentially interfere and affect downstream molecular reaction if present in high enough amounts. As such we recommend if possible to sort in PBS alone, or as recommended by 10x Genomics a solution of PBS + 0.04% BSA at most. Refrain also from using buffers with Mg²⁺ and Ca²⁺ or other metal ions for sorting. If EDTA is an absolute must, try and keep the amounts low. Avoid other additives like DNaseI, and Sodium Azide.
 - Not all RNase Inhibitors are compatible and some can have severe negative impact on the downstream reaction and ultimately library quality. It is highly recommended to use the RNase Inhibitor used in the protocol (

 Recombinant RNase Inhibitor **Takara Bio Inc. Catalog #2313A**). Alternative RNase Inhibitors, that have been tested and seemed compatible are,

 NXGen RNase Inhibitor **Lucigen Catalog #30281-2** ,

 RNaseOUT™ Recombinant Ribonuclease **Thermo Fisher Scientific Catalog #10777019**

 Protector RNase Inhibitor **Merck MilliporeSigma (Sigma-Aldrich) Catalog #3335399001**
- This protocol in it's current form is **not compatible** with cell-picking and mouth pipetting for capturing and dispensing single cells.

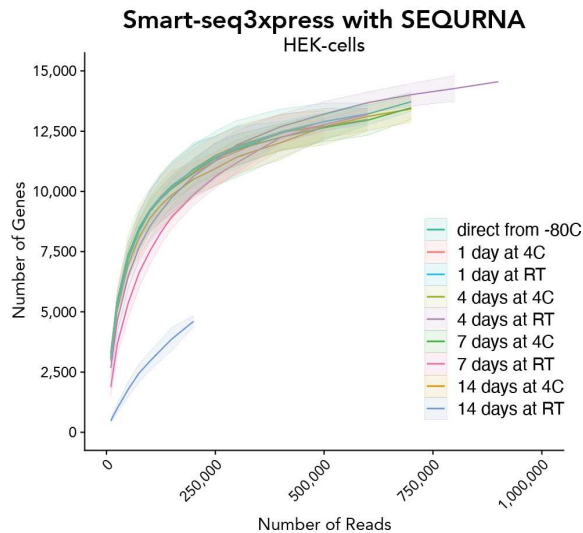
- Due to the viscosity of the overlays they can compromise the adhesive abilities of PCR seals; especially after storage in -80C. This can be unusual to work with in the beginning. However loose seals have no practical implications, as the method works perfectly well even without seals, or even without heated thermocycler lids during reverse transcription and preamplification PCR. However, heated lid thermocyclers are necessary for the tagmentation reaction, that run independent of overlays. The inert overlay should fully encapsulate the reaction. Furthermore the seal will adhere again better after the initial 72C denaturation step. Even not strictly required, we do recommended to run the method with seals to protect from contamination.
- Take a look at the Guidelines section for more info about Oligos etc. used in this protocol. **Recommended storage for oligos are**  -20 °C **for OligodT30VN and PCR primers and**  -80 °C **for TSO (The TSO contains RNA bases, so please store it at -80C)**
- Can I change reagent A to reagent B, and oligo X to oligo Y?
 - Usually yes it is possible, but user optimization might be required, and results may vary. It is no longer Smart-seq3xpress, and our support will therefore be limited.
- **For further details and considerations on method design and decisions please read the supplementary note in the manuscript**

Protocol updates!

- 2 **This section will highlight and address some updates, improvements and changes to the protocol that I/we/the team behind Smart-seq3xpress have started implementing.**

SEQURNA:

- SEQURNA Thermo stable RNase Inhibitor ("SEQURNA", <https://www.genovis.com/genomics/sequrna/>) has replaced Takara's Recombinant RNase Inhibitor. SEQURNA only needs to be added to the lysis buffer and is not added again during reverse transcription (RT). SEQURNA a synthetic and heat-tolerant inhibitor that protects RNA integrity.
- We primarily made the switch to SEQURNA due its improved capabilities to stabilize and protect RNA in lysis. This can buffer out some of the common logistical hurdles with storing and shipping plates. To illustrate that we performed an experiment where 384 well plates containing HEK cells in lysis buffer with SEQURNA were left either at room temperature (drawer on the lab-bench) or at 4C (fridge) for 1, 4, 7 and 14 days before being processed with Smart-seq3xpress library preparation.



Plot showcases library complexity as genes detected from a comparison of the different storage conditions and timepoints. Each condition is a 384 well plate of single HEK cells prepared with Smart-seq3xpress containing SEQRNA

- **Follow sub-section 6.1 & 9.1 for the Smart-seq3xpress with SEQRNA protocol.** These subsections contain a modified lysis plate generation and RT mix respectively with SEQRNA included

- **Please use the SEQRNA at the specified amount/range:** For Smart-Seq3xpress: 0.2 mass units/ μ l in lysis buffer (optimal range 0.1-0.3), resulting in ~0.15 mass units/ μ l in the RT reaction. Using more of the inhibitor will eventually cause downstream inhibition and lead to decline in library quality and complexity.

- For more information please check out the recent publication that showcases SEQRNA in Smart-seq library preparations:

Citation

Noble JC, Lentini A, Hagemann-Jensen M, Sandberg R, Reinius B (2024)
. Introducing synthetic thermostable RNase inhibitors to single-cell RNA-seq.

<https://doi.org/10.1038/s41467-024-52717-4>

LINK

Before Starting

- 3 This protocol should be carried out in a clean environment. Use ethanol, RNaseZAP, DNA-OFF, or similar to prepare work bench before start.

Work quickly and preferably on ice.

Try and prepare master-mixes right before use, while the plate(s) are finishing the previous incubation step.

Prepare 22% PEG beads for final library clean-up (Optional, but recommended)

- 4 This step is optional and can be disregarded if you choose to use  Agencourt AMPure XP **Beckman Coulter Catalog #A63880** or similar beads for cleaning up your final libraries.

These 22% PEG beads for clean-up is prepared similar to the mcSCRB-seq protocol (**mcSCRB-seq**)

A	B
Reagent	Amount
PEG 8000	11g
NaCl (5M)	10mL
Tris-HCl (1M, pH 8)	500uL
EDTA (0.5M)	100uL
IGEPAL CA-630 (10% Solution)	50uL
Sodium Azide (10% Solution)	250uL
H2O	Up to 49mL
Total	50mL

Weight out the PEG8000 in a 50mL Falcon tube, and add all ingredients except IGEPAL CA-630 together. DO NOT add the total amount of water but wait until the PEG is completely dissolved until filling the Falcon tube up to 50mL.

Incubate at  37-40 °C and vortex regularly to help dissolve the PEG8000.

Meanwhile the PEG8000 solution is dissolving prepare the Sera-Mag Speed Beads.



Resuspend the bead stock, and pipette  1000 μL of bead stock into a 1.5mL tube.

Place on magnet stand and let beads collect. Remove supernatant

Add  1000 μL of a 10mM Tris-HCl pH 8, 1mM EDTA (TE) solution and resuspend beads before retuning the tube on the magnet stand.

Remove supernatant and repeat the wash above once more.

Afterwards remove supernatant and add and re suspend the beads in  900 μL 10mM Tris-HCl pH 8, 1mM EDTA (TE).

Add the bead solution to the PEG8000 solution above, after it is dissolved.

Add IGEPAL-CA630, and fill up with the remaining H₂O to 50mL. Mix it well.

Prepare "overlay" plates

- 5 We have tested multiple types of hydrophobic inert overlays such as Silicone Oils, Hydrocarbons, and the commercial Vapor-Lock (Qiagen). They can have different properties, such as viscosities and solidifying temperature. We commonly use Vapor-Lock, Silicone oil 25 cSt, Silicone Oil 100 cSt (The higher viscosity is better suited for shipping plates).

CAUTION!!! Do not dispense these silicone oils / overlays with your non contact liquid handler. The solutions can "creep" everywhere. Use either manual multichannel pipettes or semi-manual (e.g. Integra ViaFlow) / automatic dispensing (e.g. Agilent Bravo, Tecan Fluent) with tips, and prepare and store in bulk.

Add  3 μL of overlay to each well of a 384 well plate. The amount of overlay can be increased if desired.

Quick pulse centrifugation to  1000 x g to ensure all is collected in the bottom.

Put on seal and store at  Room temperature until use.

Prepare lysis plates

- 6 Prepare **lysis buffer reaction mix**. The mix is prepared for 500 samples. Adjust this to suit your liquid handlers dead volume and pipetting comfort level.

	A	B	C	D
	Reagent	Reaction conc.	uL per reaction	384 well plate (uL)
	Triton X100 (10% solution)	0.1%	0.003	1.5
	Poly-ethylene Glycol 8000 (40% solution)	5%	0.05	25
	Spike-ins (optional)	-	-	-
	RNase Inhibitor (40u/uL)	0.4u	0.003	1.5
	OligodTVN30 (10uM)	0.125uM	0.005	2.5
	dNTPs (10mM / each)	0.5mM/each	0.02	10
	H2O		0.22	109.5
	Total		0.3uL	150uL

Reaction concentrations for PEG8000, OligodT30VN and dNTPs, are adjusted to and reflect their final concentration in the reverse transcription reaction (0.4uL).

- Ensure that PEG is fully mixed into solution, by either pipetting up and down until the liquid is clear, or start with vortexing the required master-mix volume of water and PEG together before adding the remaining reagents.

Add  0.3 µL of lysis mix to each well of a 384 well plate containing overlay.

Quick pulse centrifugation to  1000 x g to ensure lysis mix is collected in the bottom underneath the overlay before storage until use. For short term storage  On ice . For longer term storage, store the lysis plates in a freezer at  -20 °C or at  -80 °C if the lysis plates contain Spike-in RNAs.

6.1 Prepare lysis plates with SEQRNA RNase Inhibitor

Prepare **lysis buffer reaction mix**. The mix is prepared for 500 samples. Adjust this to suit your liquid handlers dead volume and pipetting comfort level.

	A	B	C	D
	Reagent	Reaction conc.	uL per reaction	384 well plate (uL)
	Triton X100 (10% solution)	0.1%	0.003	1.5
	Poly-ethylene Glycol 8000 (40% solution)	5%	0.05	25
	Spike-ins (optional)	-	-	-
	SEQURNA Inhibitor (50 Mass U/ μ L)	0.2 Mass U/uL	0.0012	0.6
	OligodTVN30 (10uM)	0.125uM	0.005	2.5
	dNTPs (10mM / each)	0.5mM/each	0.02	10
	H2O		0.22	110.5
	Total		0.3uL	150uL

Reaction concentrations for PEG8000, OligodT30VN and dNTPs, are adjusted to and reflect their final concentration in the reverse transcription reaction (0.4uL).

Ensure that PEG is fully mixed into solution, by either pipetting up and down until the liquid is clear, or start with vortexing the required master-mix volume of water and PEG together before adding the remaining reagents.

Add  0.3 μ L of lysis mix to each well of a 384 well plate containing overlay.

Quick pulse centrifugation to  1000 x g to ensure lysis mix is collected in the bottom underneath the overlay before storage until use. For short term storage  On ice . For longer term storage, store the lysis plates in a freezer at  -20 $^{\circ}$ C or at  -80 $^{\circ}$ C if the lysis plates contain Spike-in RNAs.

Sample collection / Cell sorting

- Sort single cells into each well containing  0.3 μ L lysis reaction mix overlayed by  3 μ L of preferred overlay.

As mentioned in the **Considerations section**. Be mindful of the solution cells are sorted in, as additives such as BSA, FBS and EDTA, can have negative impact on the downstream molecular reactions.

Seal plates with a cold storage foil seal, do a quick pulse centrifugation to 1000 x g and immediately store at -80 °C or on dry ice.

Cell lysis

10m

- 8 Remove the plate of sorted cells from the -80 freezer and incubate in a thermocycler with heated lid at 72 °C for 00:10:00 followed by a 4 °C hold. Keep the -80C storage seal on for this step, even if it seems loose (read above).

10m

Reverse transcription

2h 15m

- 9 While the plate is incubating in step 7, prepare **RT reaction mix**. The mix is prepared for 500 samples. Adjust this to suit your liquid handlers dead volume and pipetting comfort level.

2h 15m

	A	B	C	D
	Reagents	Reaction conc.	uL per reaction	384 well plate (uL)
	Tris-HCl pH 8.0-8.4 (1M)	25mM	0.01	5
	NaCl (2.5M)	30mM	0.0048	2.4
	MgCl ₂ (100mM)	2.5mM	0.01	5
	GTP (100mM)	1mM	0.004	2
	DTT (100mM)	8mM	0.032	16
	RNase Inhibitor (40u/uL)	0.25u	0.0025	1.25
	Smart-seq3xpress TSO (100uM)	0.75uM	0.003	1.5
	Maxima H-minus RT enzyme (200u/uL)	2u	0.004	2
	H ₂ O		0.03	14.9
	Total		0.1uL	50uL

Add  0.1 µL of **RT reaction mix** into each well, seal the plate with a PCR seal and pulse centrifuge the plate quickly to  1000 x g to ensure RT mix merges properly with the lysed cell mix.

Incubate the plate in a thermocycler as following.

A	B	C
Temperature	Time	Cycles
42C	90min	1x
50C	2min	10x
42C	2min	
85C	5min	1x
4C	Hold	-

9.1 Reverse transcription with SEQRNA RNase inhibitor in lysis

While the plate is incubating in step 7, prepare **RT reaction mix**. The mix is prepared for 500 samples. Adjust this to suit your liquid handlers dead volume and pipetting comfort level.

A	B	C	D
Reagents	Reaction conc.	uL per reaction	384 well plate (uL)
Tris-HCl pH 8.0-8.4 (1M)	25mM	0.01	5
NaCl (2.5M)	30mM	0.0048	2.4
MgCl ₂ (100mM)	2.5mM	0.01	5
GTP (100mM)	1mM	0.004	2
DTT (100mM)	8mM	0.032	16
Smart-seq3xpress TSO (100uM)	0.75uM	0.003	1.5
Maxima H-minus RT enzyme (200u/uL)	2u	0.004	2

	A	B	C	D
	H2O		0.03	16.25
	Total		0.1uL	50uL

Add  0.1 µL of **RT reaction mix** into each well, seal the plate with a PCR seal and pulse centrifuge the plate quickly to  1000 x g to ensure RT mix merges properly with the lysed cell mix.

Incubate the plate in a thermocycler as following.

	A	B	C
	Temperatur e	Time	Cycles
	42C	90min	1x
	50C	2min	10x
	42C	2min	
	85C	5min	1x
	4C	Hold	-

Preamplification PCR

10

While the plate is incubating in step 8, prepare **PCR reaction mix**. The mix is prepared for 500 samples. Adjust this to suit your liquid handlers dead volume and pipetting comfort level.

	A	B	C	D
	Reagent	Reaction conc.	uL per reaction	384 well plate (uL)
	SeqAmp PCR buffer (2x)	1x	0.5	250

	A	B	C	D
	Forward Primer (100uM)	0.5uM	0.005	2.5
	Reverse Primer (100uM)	0.5uM	0.005	2.5
	SeqAmp DNA polymerase (1.25u/uL)	0.025u	0.02	10
	H2O		0.07	35
	Total		0.6uL	300uL

Add  0.6 µL of **PCR reaction mix** to each well seal the plate with PCR seal and pulse centrifuge the plate quickly to  1000 x g to ensure RT mix merges properly with the lysed cell mix.

Run the following PCR program in a thermocycler.

	A	B	C	D
	Step	Temperature	Time	Cycles
	Initial denaturation	95C	1min	1x
	Denaturation	98C	10sec	
	Annealing	65C	30sec	12-16x*
	Elongation	68C	4min	
	Final Elongation	72C	10min	1x
	Hold	4C	Hold	-

*To determine the number of PCR cycles it mainly depends on your cells and RNA content. We use as a standard 12 cycles for HEK cells, and 16 cycles for PBMCs.

However, for most cell types a wide range of PCR cycles can be used, yielding similar quality data. Below is a schematic of selected sequencing output metrics from a PCR

cycle evaluation run on PBMCs. Each 384 well plate of sorted PBMCs from the same donor, same batch, underwent a different number of PCR cyclers in preamplification. (Take into consideration heterogeneity of PBMCs as each column is a unique 384 well plate of cells)

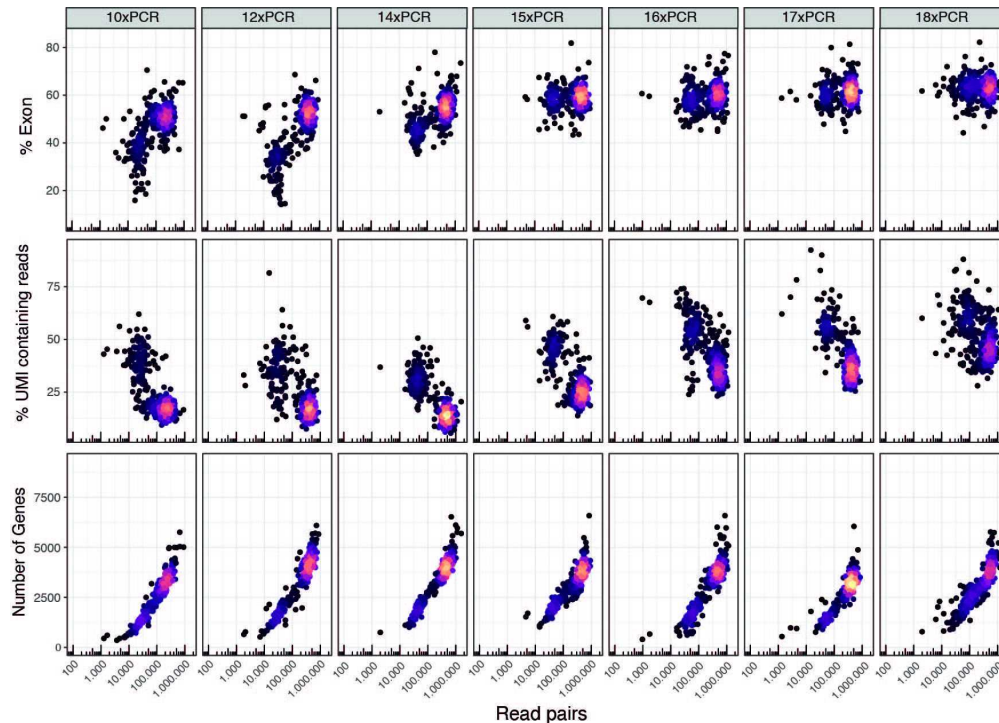


Figure shows (top) Percent reads mapping to Exon, (middle) percent UMI containing reads (bottom) number of genes detected for PBMCs undergoing different PCR cycles in Preamplification. All plates were from same donor, sorted in same batch and were processed similarly, apart from receiving varying cycles of Preamplification PCR. Amount of TDE1 Tn5 used 0.002uL per cell.

The primary concerns when choosing PCR cycles is

1. To amplify cDNA enough to be well above the genomic DNA levels.
2. Using more cycles means also needing to use more TDE1 Tn5 enzyme per cell to be able to get a good ratio of UMI containing reads to internal reads (~50%). This is not such a massive concern since we use minuscule amounts of TDE1 per cell, If you need to adjust the amount of TDE1 Tn5, we recommend adjustments in the order of +/- 0.0005uL per cell increments.
3. Choosing lower PCR cycles might mean you will need to scale up the amounts of PCR cycles for the tagmentation PCR (see below)

Above image in higher quality format.

 PBMC_PCRcycles_statsv2.pdf

Dilution

- 11 After the preamplification PCR dilute the  1 μL cDNA by adding  9 μL of  Nuclease-free Water

Again, seal the plate and pulse centrifuge to  1000 x g to ensure that everything is collected beneath the overlay.

Note

It is not the intention of this method to check your preamplified cDNA. Using low preamplification cycles leaves your cDNA below the limit of detection for Bioanalyzer and Tapestation!

Tagmentation (with index plates)

- 12 1. Transfer  1 μL of diluted cDNA to a new 384 well plate, and put  On ice for until tagmentation.
2. Prepare 4x Tagmentation buffer as following. Aliquots of 4xTD buffer can be stored at  -20 $^{\circ}\text{C}$ for later use.

Safety information

Dimethylformamide (DMF) should be handled in a fume hood and according to local safety regulations.

	A	B	C
	Reagent	Amount (μL)	Concentration at 4x
	Tris-HCl pH 7.5 (1M)	40	40mM
	MgCl ₂ (100mM)	200	20mM
	DMF	200	20%



	A	B	C
	H2O	560	
	Total	1000uL	

3. Prepare **Tagmentation Mix**. Mix is for 500 samples.

	A	B	C	D
	Reagent	Reaction concentration	uL per reaction	384 well plate (uL)
	4x Tagmentation buffer	1x	0.5	250
	TDE1 Tn5		0.002*	1
	H2O		0.498	249
	Total		1	500

NB !!! TDE1 Tn5 enzyme comes as a glycerol viscous solution. When pipetting these small uL amounts to your tagmentation mix, be careful to not transfer extra drops that are stuck to the pipette tip. This can cause overtagmentation, which is not bad, but can cause less than expected UMI reads to be captured in sequencing.

*This is a suggestive amount of Tn5, but overall a good starting point that works well with HEK cells (12xPCR preamplification) and PBMCs (16xPCR preamplification). The amount can be changed to meet user specific criteria in terms of ratio of UMI containing reads vs Internal reads, and can be affected by cell-type or amount of preamplification PCR cycles given.

4. Dispense  1 μ L of Tagmentation Mix to each well of the 384 well plate containing  1 μ L of diluted and pre-dispensed cDNA. Seal and pulse centrifuge the plate down at  1000 x g .

5. Incubate the plate at  55 °C for  00:10:00 in a thermocycler.

6. To stop the tagmentation reaction and strip off the Tn5 enzyme, add  0.5 μ L of 0.2% SDS to each well. Seal the plate and quickly centrifuge the plate down before incubation at  Room temperature for  00:05:00

7. Proceed to either substep 11.1 or substep 11.2 depending on your index primer concentration and whether you want to PCR your tagmented libraries in higher or lower volumes. For adding Nextera index primers we recommend preparing index plates with already combined Nextera i5 and i7 indexes at a certain working dilution.

12.1 Lower volume indexing PCR, final volume 5uL per well. This can save a bit of cost on Phusion Polymerase

8.1 Add  1 µL of premixed index primers.

A	B	C
Reagent	Reaction conc.	uL per reaction
Premixed custom S50X / N70X index primers (1uM /each)	0.2uM /each	1uL

9.1 Prepare **Index PCR mix**. Again the calculated amount here for ease is for 500 sample. Please scale this to suit your specific dead volume.

A	B	C	D
Reagent	Reaction conc.	uL per reaction	384 well plate (uL)
Phusion HF buffer (5x)	1x	1	500
dNTPs (10mM/each)	0.2mM/each	0.1	50
Tween-20 (10%) (*necessary)	0.025%	0.0125	6.25
Phusion HF Polymerase	0.01u/uL	0.025	12.5
H2O		0.3625	181.25
Total		1.5uL	750

*Addition of Tween-20 to the PCR reaction is necessary to avoid having the remaining SDS affect Phusion DNA polymerase, at the given concentration and amount used in the stop reaction. Tested working amounts of Tween-20 used with Phusion polymerase is



0.005-0.05%. Above 0.05% the reaction starts to get negatively affected by it in our hands. If you change these parameters try and match or be in slight excess with the amount of Tween-20 to the concentration of leftover SDS from the stop solution.

10.1 Add  1.5 μL of **index PCR mix** to each well, seal the plates, apply quick centrifugation to settle everything in the bottom, and incubate in a thermocycler as following:

	A	B	C	D
	Step	Temperature	Time	Cycles
	Gap filling	72C	3min	1x
	Initial denaturation	98C	30sec	1x
	Denaturation	98C	10sec	
	Annealing	55C	30sec	10-14x*
	Elongation	72C	1min	
	Final elongation	72C	5min	1x
	Hold	4C	Hold	

*The amount of PCR cycles needed depends on the starting material, preamplification cycles given, and amount of tagmentation performed (amount TDE1 Tn5 used per cell). Therefore some user specific optimization may be necessary to suit the needs and wants and final material required.

However as a point of reference:

- HEK cells (12xPCR cycles in preamplification PCR) we use a standard 12xPCR cycles in tagmentation PCR
- PBMCs (10-18xPCR cycles in preamplification PCR) we use 14xPCR cycles in tagmentation PCR.

12.2 **Standard volume index PCR, final volume 8uL. If you have index plates in the working concentration used for Smartseq3.** (0.5 micromolar (μM))

8.2 Add  3.5 μL of premixed index primers to each well.



A	B	C	D
Reagent	Reaction conc.	uL per reaction	
Premixed custom S50X / N70X index primers (0.5uM/each)	~0.22uM/each	3.5uL	

9.2 Prepare **index PCR mix**. Again the calculated amount here for ease is for 500 sample. Please scale this to suit your specific dead volume.

A	B	C	D
Reagent	Reaction conc.	uL per reaction	384 well plate (uL)
Phusion HF buffer (5x)	1x	1.6	800
dNTPs (10mM/each)	0.2mM/each	0.16	80
Tween-20 (10%) (optional*)	0.01%	0.008	4
Phusion HF Polymerase	0.01u/uL	0.04	20
H2O		0.192	96
Total		2uL	1000uL

*Adding a bit of Tween-20 to the PCR reaction can help protect the DNA polymerase a bit against SDS from previous stop reaction. This trick can also help other polymerases to work better like KAPA, Vent etc.

10.2 Add  2 μ L of **index PCR mix** to each well, seal the plates, apply quick centrifugation to settle everything in the bottom, and incubate in a thermocycler as following:

A	B	C	D
Step	Temperature	Time	Cycles
Gap filling	72C	3min	1x

	A	B	C	D
	Initial denaturation	98C	30sec	1x
	Denaturation	98C	10sec	
	Annealing	55C	30sec	10-14x*
	Elongation	72C	1min	
	Final Elongation	72C	5min	1x
	Hold	4C	Hold	-

*The amount of PCR cycles needed depends on the starting material, preamplification cycles given, and amount of tagmentation performed (TDE1 Tn5 amount added per cell). As such it might require a bit of user optimization, depending on wants, needs, and final material needed.

However as a reference point:

- HEK cells (12xPCR cycles in preamplification PCR) we use as standard 12xPCR cycles in tagmentation PCR. (10xPCR is confirmed working as well)
- PBMCs (10x-18xPCR cycles preamplification PCR) we use 14xPCR cycles in tagmentation PCR.

Tagmentation (Dessicated index primer startegy)

- 13 **To save even further on general plastic consumption and tip use, we designed a way of tagmenton & indexing in which one prepares predisposed "tagmentation" plates containing premixed dessicated indexes. Hence you can with the same set of tips prepare multiple tagmentation plates containing the same indexes for storage. Of course it is very important to note that you need to change tips when/if you change premixed source indexes so as to not mix/contaminate your premixed stock indexes with different set of indexes.**

This method is the most cost-effective implementation of Smart-seq3xpress and especially suited for large-scale projects or preparing large amounts of plates in advance.

Depending on your premixed index plate concentrations, dispense $\text{[Volume]} \times \mu\text{L}$ of indexes aiming for a concentration of between $\text{[Conc]} 0.2 \text{ micromolar } (\mu\text{M})$ -

[M] 0.5 micromolar (μM) /each for a 5uL final volume reaction. It can also be performed in a 7.5uL final volume reaction, simply adjust the concentration accordingly to fit the higher volume

Examples for a 5uL final reaction volume

For [M] 0.5 micromolar (μM) /each premixed indices add $\text{2.5 } \mu\text{L}$ to each well of a 384 well PCR plate (results in [M] 0.25 micromolar (μM)).

For [M] 1.0 micromolar (μM) /each premixed indices add $\text{1.25 } \mu\text{L}$ to each well of a 384 well PCR plate. ([M] 0.25 micromolar (μM)).

Put the PCR plates onto an open thermocycler and incubate at 95°C without seal for $00:01:00$ - $00:05:00$ depending on the amount if index primers added to each well.

In the process or after visually inspect that all the liquid has evaporated from the wells.

Seal and store the plates at -20°C until use.

13.1 1. Transfer $\text{1 } \mu\text{L}$ of prediluted cDNA from step 10 into a 384 well PCR plate containing dessicated index primers.

2. Prepare tagmentation mix (see Step 11 for the recipe for 4xTagmentation buffer). Shown mix is for 500 samples.

	A	B	C	D
	Reagent	Reaction concentration	uL per reaction	384 well plate (uL)
	4x tagmentation buffer	1x	0.5	250
	TDE1 Tn5		0.002*	1
	H2O		0.498	249
	Total		1uL	500uL

NB !!! TDE1 Tn5 enzyme comes as a glycerol viscous solution. When pipetting these small uL amounts to your tagmentation mix, be careful to not transfer extra drops that are stuck to the

pipette tip. This can cause overtagmentation, which is not bad, but can cause less than expected UMI reads to be captured in sequencing.

*This is a suggestive amount of Tn5, but overall a good starting point that works well with HEK cells (12xPCR preamplification) and PBMCs (16xPCR preamplification). The amount can be changed to meet user specific criteria in terms of ratio of UMI containing reads vs Internal reads, and can be affected by cell-type, amount of preamplification PCR cycles given.

3. Dispense  1 μ L of Tagmentation Mix to each well of the 384 well plate containing  1 μ L of cDNA. Seal and pulse centrifuge the plate down at  1000 x g .

4. Incubate the plate at  55 $^{\circ}$ C for  00:10:00 in a thermocycler.

5. To stop the tagmentation reaction and strip off the Tn5 enzyme, add  0.5 μ L of 0.2% SDS to each well. Seal the plate and quickly centrifuge the plate down before incubation at  Room temperature for  00:05:00

6. Prepare **Index PCR mix**. Again the calculated amount here for ease is for 500 sample. Please scale this to suit your specific dead volume.

	A	B	C	D	E	F
	Reagent	Reaction concentration	μ L per reaction (5 μ L final)	384 well plate (μ L/5 μ L final)	μ L per reaction (7.5 μ L final)	384 well plate (μ L/7.5 μ L final)
	Phusion HF buffer (5x)	1x	1	500	1.5	750
	dNTPs (10mM/each)	0.2mM/each	0.1	50	0.15	75
	Tween-20 (10%)	0.025% / 0.01%	0.0125	6.25	0.0075	3.75
	Phusion DNA polymerase (2u/ μ L)	0.01u/ μ L	0.025	12.5	0.0375	18.75
	H2O		1.3625	681.25	3.305	1652.5

	A	B	C	D	E	F
	Total		2.5uL	1250uL	5uL	2500uL

7 Add either  2.5 µL or  5 µL of **index PCR mix** to each well depending on chosen final volume, seal the plates, apply quick centrifugation to settle everything in the bottom, and incubate in a thermocycler as following:

	A	B	C	D
	Step	Temperature	Time	Cycles
	Gap filling	72C	3min	1x
	Initial denaturation	98C	30sec	1x
	Denaturation	98C	10sec	
	Annealing	55C	30sec	10-14x*
	Elongation	72C	1min	
	Final elongation	72C	5min	1x
	Hold	4C	Hold	-

*The amount of PCR cycles needed depends on the starting material, preamplification cycles given, and amount of tagmentation performed (amount TDE1 Tn5 used per cell). Therefore some user specific optimization might be necessary to suit the needs and wants and final material required.

However as a point of reference:

- HEK cells (12xPCR cycles in preamplification PCR) we use a standard 12xPCR cycles in tagmentation PCR
- PBMCs (10-18xPCR cycles in preamplification PCR) we use 14xPCR cycles in tagmentation PCR.

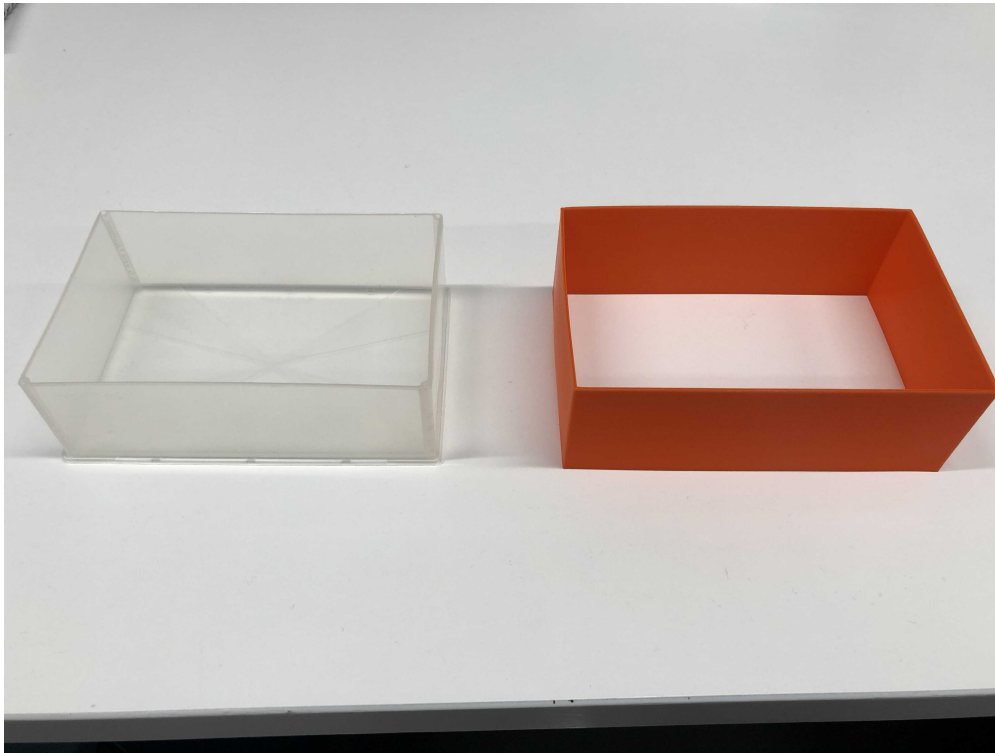
Library pooling by spin-out and bead clean-up

- 14 To quickly pool a plate or multiple plates together we designed a centrifugation holder heavily inspired from [Quartz-seq2](#), although more accessible. This holder can easily be 3D printed, and fits together with



Nalgene Disposable Polypropylene Robotic Reservoirs **Thermo Fisher Scientific Catalog #1200-1300**

and a standard SBS format 384 well plate of choice. Design and template can be found here <https://github.com/sandberg-lab/Smart-seq3xpress>.



Reservoir and 3D printed holder

Note

A couple of things to consider before jumping into it!

You need a swing bucket rotor centrifuge with enough clearance to allow the buckets to swing while holding the contraption (Reservoir + 3D printed adapter + plate).

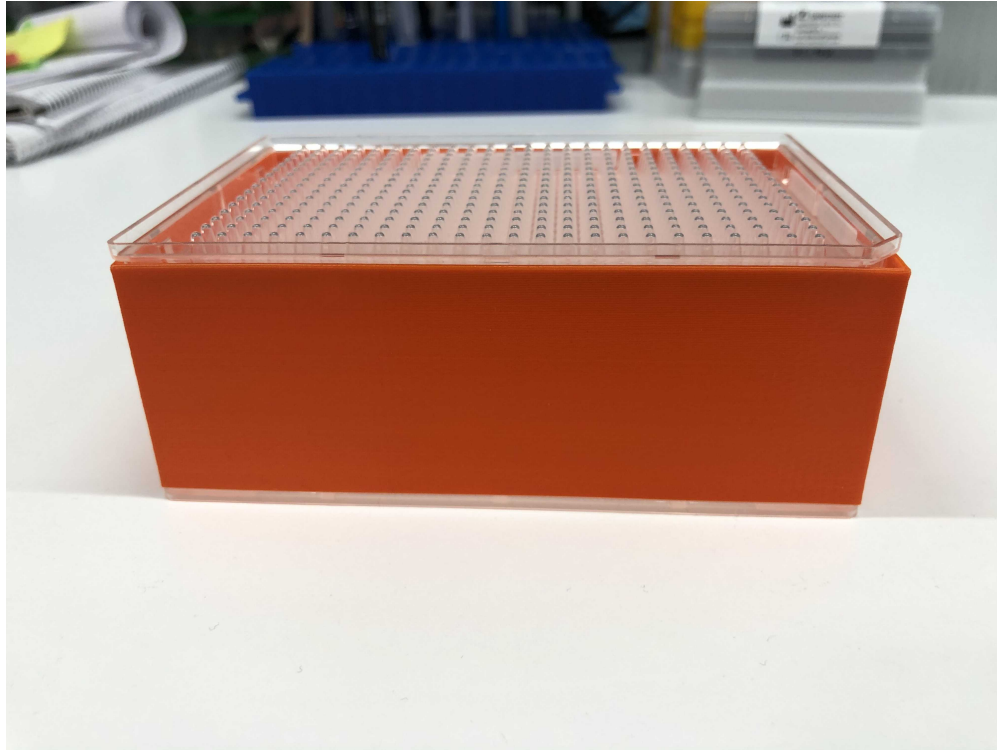
The plates need very little centrifugation force to spin out the contents, so just pulse the centrifuge gently, at high g-force your library will end up in the centrifuge!

Since these are polypropylene reservoirs some losses in terms of volume might occur. This does not affect overall amplified library quality.

Print two holder so you have a decent balance weight if you don't want to spin out two plates

Put the holder around the reservoir, and add onto the PCR plate containing the final tagged libraries upside down. Liquid should stay in the wells, by surface tension until centrifugation, but again do this with a slight amount of caution. See pictures if in doubt.





Once this is assembled, put in a centrifuge and pulse to max  100 x g .





Collect the pooled library in a fitting tube (depending on how many plates you choose to spin in the same reservoir) and **purify the library with 22% PEG Clean-up beads or similar at a ratio of 0.7 : 1 beads to sample.**

Mix the beads and sample gently by pipetting up and down and incubate for



00:08:00

at



Room temperature

Place on magnet and let beads settle for roughly  00:05:00 to  00:10:00 before discarding the supernatant

Wash twice with freshly made **80% Ethanol**

Remove Ethanol and let the bead pellet air dry for at least  00:05:00 (Until the pellet is no longer shiny)

Elute the beads in  40 μ L of UltraPure Water, resuspend beads and incubate for



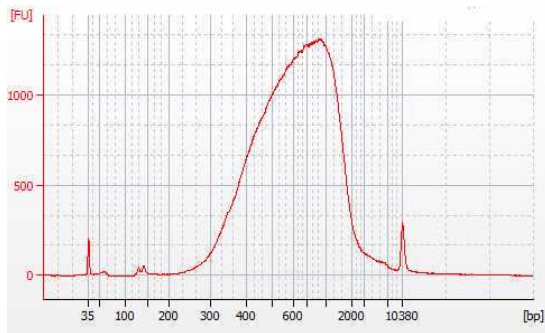
00:05:00

Final Library QC

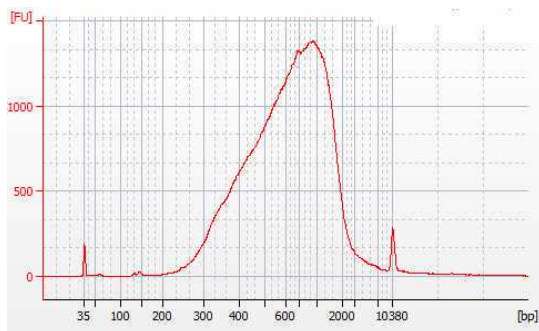
- 15 Use Qubit fluorometer (Qubit dsDNA HS Assay) or similar to quantify the final library pool / pools.

Run the final library on an Agilent Bioanalyzer (High Sensitivity DNA chip) to inspect quality and get median base-pair length information.

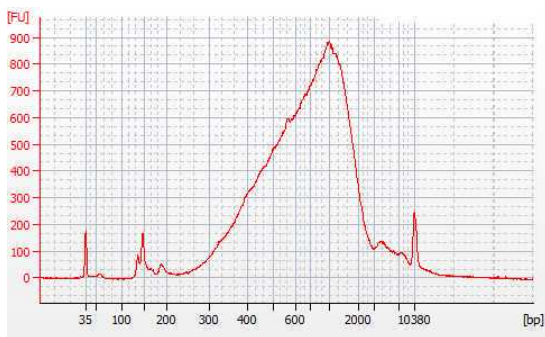
Expected result



Example 1: Pooled HEK cells



Example 2: Pooled HEK cells



Example 3: Pooled PBMCs



Example 4: Pooled PBMCs

Sequencing

- 16 The sequencing ready library should be sequenced on any MGI or Illumina compatible sequencer, either Single-end or Paired-end, depending on the question and need.

If sequencing on a MGI sequencer convert the final library into MGI compatible single stranded circles utilizing the Universal Library Conversion Kit (App-A). Follow the user manual.

	A	B	C	D	E
	Oligo Name	Vendor	Purification	Stock Concentration	Sequence
	Splint-Oligo (App-A)	IDT	HPLC	100uM	TCGCCGTATCATTCAAGCAGAAGACG
	MDA Primer	IDT	HPLC	100uM	CGTATGCCGTCTTCTGCTTGAATGATACGGCGAC
	Read1 Sequencing Primer	IDT	HPLC	100uM	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG
	Read2 Sequencing Primer	IDT	HPLC	100uM	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG

	A	B	C	D	E
	I5-index primer	IDT	HPLC	100uM	CTGTCTCTTATACACATCTGACGC TGCCGACGA
	I7-index primer	IDT	HPLC	100uM	CCGTATCATTCAAGCAGAAGACGG CATACGAGAT

Table of Sequencing primers and oligos needed for MGI sequencing of Nextera Style libraries.

Data processing

- 17 For primary data processing we **highly recommend** using the zUMIs pipeline. There are many specifics and features in the pipeline that are directly geared towards preparing and preprocessing Smart-seq3 data properly.

Software

zUMIs

NAME

Linux

OS

<https://github.com/sdparekh/zUMIs>

REPOSITORY

<https://www.biorxiv.org/content/early/2017/10/18/153940>

SOURCE LINK

For data sequenced on Illumina sequencers:

When sequencing has completed convert the binary base-call files (BCL) to fastq files. Use bcl2fastq (bcl2fastq v2.20).



Software

bcl2fastq

NAME

Illumina

DEVELOPER

<https://emea.support.illumina.com/downloads/bcl2fastq-conversion-software-v2-20.html>

SOURCE
LINK

Fastq files for zUMIs should be processed without demultiplexing in the following manner, instead of demultiplexing per cell.

Command

Example of preparing data from a 150bp paired-end sequencing run

```
bcl2fastq --use-bases-mask Y150N,I8,I8,Y150N --no-lane-splitting --  
create-fastq-for-index-reads -R  
/mnt/storage1/NextSeqNAS/191011_NB502120_0154_AHVG7JBGXB
```

Remove the flag `--no-lane-splitting` if the cell barcodes or index primers have been reused on the different lanes.

Fastq files are now ready and compatible with the zUMIs pipeline. An example of how config .yaml files should look like for zUMIs can be seen below.

Command

Smartseq3xpress.yaml (Illumina Paired-end 150bp)

```
project: Smartseq3xpress
sequence_files:
  file1:
    name: /Smartseq3xpress/fastq_files/Read1.fastq.gz
    base_definition:
      - cDNA(25-150)
      - UMI(12-21)
    find_pattern: ATTGCGCAATG;2
  file2:
    name: /Smartseq3xpress/fastq_files/Read2.fastq.gz
    base_definition:
      - cDNA(1-150)
  file3:
    name: /Smartseq3xpress/fastq_files/Index1.fastq.gz
    - BC(1-10)
  file4:
    name: /Smartseq3xpress/fastq_files/Index2.fastq.gz
    - BC(1-10)
reference:
  STAR_index: /genomes/Human/STAR7idx_noGTF/
  GTF_file: /genomes/Human/Homo_sapiens.GRCh38.95.chr.gtf
  additional_STAR_params: '--clip3pAdapterSeq CTGTCTCTTATACACATCT'
  additional_files:
out_dir: /Smartseq3xpress/zUMIs
num_threads: 20
mem_limit: 50
filter_cutoffs:
  BC_filter:
    num_bases: 4
    phred: 20
  UMI_filter:
    num_bases: 3
    phred: 20
barcodes:
  barcode_num: ~
  barcode_file: /Smartseq3xpress/expected_barcodes.txt
  automatic: no
  BarcodeBinning: 1
  demultiplex: no
  nReadsperCell: 100
```



```
discardReads: yes
counting_opts:
  introns: yes
  downsampling: '0'
  strand: 1
  Ham_Dist: 1
  velocityto: no
  primaryHit: yes
  twoPass: no
make_stats: yes
which_Stage: Filtering
samtools_exec: samtools
pigz_exec: pigz
STAR_exec: STAR
Rscript_exec: Rscript
```

For data sequenced on a MGI sequencer:

Fastq files should be available after completed sequencing run, in the following format, that is directly compatible with zUMIs pipeline; Read_1.fastq, Read_2.fastq. The index barcodes are located as the last bases of read2.

Command

Smartseq3xpress.yaml (MGI, Paired-end 150bp)

```
project: Smartseq3xpress
sequence_files:
  file1:
    name: /Smartseq3xpress/fastq_files/Read1.fastq.gz
    base_definition:
      - cDNA(25-150)
      - UMI(12-21)
    find_pattern: ATTGCGCAATG;2
  file2:
    name: /Smartseq3xpress/fastq_files/Read2.fastq.gz
    base_definition:
      - cDNA(1-150)
      - BC(151-170)
reference:
  STAR_index: /genomes/Human/STAR7idx_noGTF/
  GTF_file: /genomes/Human/Homo_sapiens.GRCh38.95.chr.gtf
  additional_STAR_params: '--clip3pAdapterSeq CTGTCTCTTATACACATCT'
  additional_files:
out_dir: /Smartseq3xpress/zUMIs
num_threads: 20
mem_limit: 50
filter_cutoffs:
  BC_filter:
    num_bases: 4
    phred: 20
  UMI_filter:
    num_bases: 3
    phred: 20
barcodes:
  barcode_num: ~
  barcode_file: /Smartseq3xpress/expected_barcodes.txt
  automatic: no
  BarcodeBinning: 1
  demultiplex: no
  nReadsperCell: 100
  discardReads: yes
counting_opts:
  introns: yes
  downsampling: '0'
  strand: 1
```



```
Ham_Dist: 1
velocityto: no
primaryHit: yes
twoPass: no
make_stats: yes
which_Stage: Filtering
samtools_exec: samtools
pigz_exec: pigz
STAR_exec: STAR
Rscript_exec: Rscript
```

FOR MORE INFO:**Note**

For further descriptions and generally more info about the zUMIs pipelines and how to run or change setting please visit [zUMIs github repository](#).

Preprocessing (Basic look at zUMIs output)

- 18 To get a quick look and generate a small stats file with the most common metrics from the zUMIs output data, one can do as following in R.

Command

Example of how to quickly generate an overview of zUMIs output data in R

```
library(data.table)
dge <-
readRDS("/Smartseq3xpress/zUMIs/zUMIs_output/expression/Smartseq3xpress.dgecounts.rds")
stats <-
fread("/Smartseq3xpress/zUMIs/zUMIs_output/stats/Smartseq3xpress.reads.percell.txt")[!RG %in% "bad"]
stats <- dcast(stats, RG~type, value.var = "N")
stats[, nreadpairs := Ambiguity+Exon+Intergenic+Intron+Unmapped]
reads <-
fread("/Smartseq3xpress/zUMIs/zUMIs_output/Smartseq3xpresskept_barcode_s_binned.txt.BCUMIstats.txt")
reads[, UMIfraction := nUMItag/(nNontagged+nUMItag)]
stats <- merge(stats,reads, by.x="RG", by.y="XC")
exonic_UMIs <- as.matrix(dge$umicount$exon$all)
exonic_Reads <- as.matrix(dge$readcount$exon$all)
complexity <- data.table( RG = colnames(exonic_UMIs),
                        nGenes = colSums(exonic_Reads>0),
                        nGenesUMIs = colSums(exonic_UMIs>0),
                        nUMIs = colSums(exonic_UMIs))
stats <- merge(stats,complexity, by = "RG")
stats[,pct_coding := ((Exon+Intron)/nreadpairs)*100]
```



Citations

Step 2

Noble JC, Lentini A, Hagemann-Jensen M, Sandberg R, Reinius B. Introducing synthetic thermostable RNase inhibitors to single-cell RNA-seq.

<https://doi.org/10.1038/s41467-024-52717-4>