

Smart-seq3xpress protocol with SEQURNA (Cat. No. 9028)

In Brief

SEQURNA is a synthetic thermostable RNase inhibitor for single-cell RNA-sequencing (scRNAseq), yielding single-cell libraries of equal or superior quality compared to ubiquitously used protein-based recombinant RNase inhibitors (RRIs).

SEQURNA provides additional unique improvements in reproducibility and throughput, enables new experimental workflows including retained RNase inhibition throughout heat cycles, and can reduce the need for dry-ice transports.

Important Information

- Using more RNase inhibitor than recommended does not improve results and may reduce cDNA library yield and quality.
- This protocol requires a liquid handler capable of dispensing nanoliter volumes.

Oligonucleotide Sequences (5' to 3')

SS3 oligo dT: 5'-/5Biosg/ACGAGCATCAGCAGCATACGAT30VN-3'

Smart-seq3xpress TSO: 5'- /5BiosG/AGAGACAGATTGCGCAATGNNNNNNNNWWrGrGrG-3'

SS3 Fwd Primer: 5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGATTGCGCAA*T*G-3'

SS3 Rev Primer: 5'-ACGAGCATCAGCAGCATAC*G*A-3'

* Phosphorothioate bonds

Considerations

- This protocol requires a liquid handler capable of performing 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, Cytena 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.

1. Preparation of Overlay Plates

- 1.1 Use Vapor-Lock, Silicone Oil 25 cSt, or Silicone Oil 100 cSt (the higher viscosity is preferred for shipping plates). Caution: Do not dispense these silicone oils/overlays using non-contact liquid handlers, as the solutions may spread uncontrollably. Instead, use manual multichannel pipettes or semi-manual/automated systems with tips (e.g., Integra ViaFlow, Agilent Bravo, Tecan Fluent). Prepare and store in bulk. Add 3 μ l of overlay to each well of a 384 well plate. The amount of overlay can be increased if desired.
- 1.2 Briefly centrifuge at 1000 $\times$ g to collect all content at the bottom of the wells.
- 1.3 Seal the plate and store it at room temperature until use.

2. Preparation of Lysis Plates

- 2.1 Prepare the lysis buffer mix according to Table 1. Optimal concentration range of SEQRNA in the Smart-seq3xpress protocol is between 0.2 mass units/ μ l in the lysis buffer, resulting in 0.15 mass units/ μ l in the RT reaction.

Reagent	Conc. in lysis buffer	μ l per reaction	384-well plate (500 rxns)
Poly-ethylene glycol 8000 (40% solution)	6.7%	0.05	25
Triton X-100 (10% solution)	0.1%	0.003	1.5
SEQRNA (50 mass units/ μ l)	0.2 mass units/ μ l	0.0012	0.6
SS3 oligo dT (10 μ M)	0.167 μ M	0.005	2.5
dNTPs (10mM/each)	0.66 mM/each	0.02	10
Nuclease-free water	-	0.221	110.5
ERCC spike-ins (Optional)	-	-	-
Total	-	0.3 μl	150 μl

Table 1. Reagent preparation for Smart-seq3xpress lysis buffer: Volumes for 384-well plates

- 2.2 Add 0.3 μ l lysis buffer to each well of 384-well plate containing overlay, and centrifuge briefly to collect lysis.

3. Sample Collection

- 3.1 Sort single cells into 0.3 μ l of lysis buffer with overlay in 384-well plates.
- 3.2 Seal the plate with appropriate cover seals (tolerating -80 $^{\circ}$ C to +110 $^{\circ}$ C) and centrifuge the finished sorted plate immediately after. Transfer the plate to a -80 $^{\circ}$ C freezer if not processing the cells into cDNA libraries within one day (or keep plates in ~4 $^{\circ}$ C for up to one day). Prompt processing is beneficial for retained RNA integrity.

4. Cell Lysis

- 4.1 Remove the plate of sorted cells from the -80 $^{\circ}$ C freezer and incubate in a thermocycler with heated lid at 72 $^{\circ}$ C for 10 min., followed by a 4 $^{\circ}$ C hold.

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5. Reverse Transcription

5.1 While the plate is incubating at the cell lysis step, prepare the reverse transcription master-mix as described in Table 2. **Do not add additional inhibitor in the RT reaction.** The SEQRNA from the lysis buffer stays effective throughout lysis and the following RT.

Reagent	Conc. in RT	μl per reaction	384-well plate (500 rxns)
Tris-HCl pH 8.3 (1 M)	25 mM	0.01	5
NaCl (2.5 M)	30 mM	0.0048	2.4
MgCl ₂ (100 mM)	2.5 mM	0.01	5
GTP (100 mM)	1 mM	0.004	2
DTT (100 mM)	8 mM	0.032	16
Smart-seq3xpress TSO (100 μM)	0.75 μM	0.003	1.5
Maxima H-minus RT enzyme (200 units/μl)	2 units	0.004	2
Nuclease-free water	-	0.0325	16.25
Total	-	0.1μl	50μl

Table 2. Reagent preparation for reverse transcription reaction: Volumes for 384-well plates

5.2 Add 0.1 μl RT mix to each well of a 384-well plate.

5.3 Replace the storage seal with a PCR seal. Ensure that the plate is properly sealed to avoid evaporation (use thermal pads, depending on thermocycler model).

5.4 Briefly centrifuge to collect reaction at the bottom of the tube.

5.5 Incubate the plate in a thermocycler at conditions listed in Table 3.

Temperature	Time	Cycles
42 °C	90 min	1×
50 °C	2 min	10×
42 °C	2 min	
85 °C	5 min	1×
4 °C	Hold	Hold

Table 3. Thermocycling conditions for reverse transcription

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6. Pre-amplification PCR

6.1 Start preparing the PCR mix when the incubation of the reverse transcription reaction is near completion, by combining the reagents listed in Table 4.

Reagent	Reaction conc.	μl per reaction	384-well plate (410 rxns)
SeqAmp PCR buffer (2×)	1x	0.5	250
Fwd Primer (100 μM)	0.5 μM	0.005	2.5
Rev Primer (100 μM)	0.5 μM	0.005	2.5
SeqAmp DNA polymerase (1.25 units/ul)	0.025 units/μl	0.02	10
Nuclease-free water	–	0.07	35
Total	–	0.6 μl	300 μl

Table 4. Reagent preparation for PCR amplification: Volumes for 384-well plates

6.2 Add 0.6 μl PCR mix to each well of a 384-well plate.

6.3 Briefly centrifuge to collect reaction at the bottom.

6.4 Incubate the plate in a thermocycler at the conditions listed in Table 5.

Step	Temperature	Time	Cycles
Initial denaturation	98 °C	1 min	1×
Denaturation	98 °C	10 s	12-16×*
Annealing	65° C	30 s	
Elongation	72 °C	4 min	
Final Elongation	72 °C	10 min	1×
Hold	4 °C	Hold	

* Depending on cell type (reflecting RNA content per cell)

Table 5. Thermocycling conditions for PCR amplification

7. Quality Control

7.1 Check the final library concentration and size distribution after tagmentation, PCR, and clean-up, by capillary electrophoresis such as an Agilent Bioanalyzer High Sensitivity DNA Analysis chip.

A representative Bioanalyzer image of successfully tagmented and pooled library using SEQRNA is shown in Figure 1.

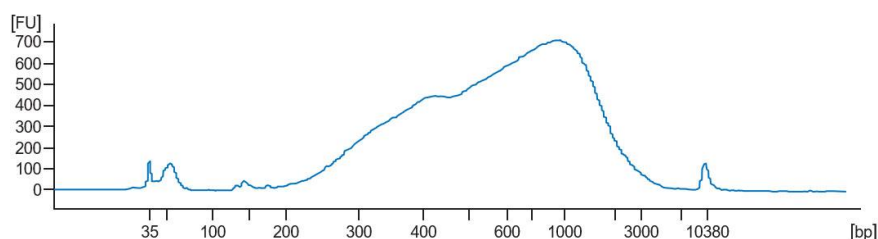


Figure 1. Trace of Smart-seq3xpress pooled library (after tagmentation and PCR amplification) from sorted HEK cells, using an Agilent Bioanalyzer High Sensitivity DNA Analysis chip.

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8. Additional Literature

For detailed instructions on preparing indexed sequencing libraries from Smart-seq3express cDNA using tagmentation and PCR, as well as the subsequent sequencing library generation steps, please refer to the online protocol:

- **Hagemann-Jensen** *et al.*, Protocols.io – Smart-seq3express Protocol
<https://www.protocols.io/view/smart-seq3express-yxmvmk1yng3p>

For further details on the development of Smart-seq3express:

- **Hagemann-Jensen** *et al.* (2022) [Scalable single-cell RNA sequencing from full transcripts with Smart-seq3express](#). Nature Biotechnology **40**, 1452

For more information on SEQRNA:

- **Noble** *et al.* (2024) [Introducing synthetic thermostable RNase inhibitors to single-cell RNA-seq](#). Nature Communications **15**, 8373

Abbreviations

dNTP: Deoxynucleotide triphosphate

DTT: Dithiothreitol

ERCC: External RNA Controls Consortium

HEK cell: Human embryonic kidney cell

PCR: Polymerase chain reaction

RT: Reverse transcription

SS3: Smart-seq3

TSO: Template-switching oligo

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