

Smart-seq3 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

- Please note that the optimal SEQURNA concentration differs between Smart-seq3 and Smart-seq2.
- Using more RNase inhibitor than recommended does not improve results and may reduce cDNA library yield and quality.
- Different labs may use slight variations of the Smart-seq3 protocol, such as changes in lysis buffer detergent or concentration. Regardless of these modifications, use the recommended concentration of SEQURNA in the Smart-seq3 lysis buffer for all protocol versions.

Oligonucleotide Sequences (5' to 3')

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

SS3 TSO: 5'-/5Biosg/AGAGACAGATTGCGCAATGNNNNNNNNrGrGrG-3'

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

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

* Phosphorothioate bonds

1. Preparation of Lysis Plates

- 1.1 Prepare the lysis buffer mix according to Table 1. Optimal concentration range of SEQRNA in the Smart-seq3 protocol is between 0.15-0.3 mass units/ μ l in the lysis buffer, resulting in 0.11-0.23 mass units/ μ l in the RT reaction.

Reagent	Conc. in lysis buffer	μ l per reaction	96-well plate (110 rxns)	384-well plate (410 rxns)
Poly-ethylene Glycol 8000 (50% solution)	6.7%	0.40	44	164
Triton X-100 (10% solution)	0.1%	0.03	3.3	12.3
SEQRNA (50 mass units/ μ l)	0.2 mass units/ μ l	0.012	1.32	4.92
SS3 oligo dT (100 μ M)	0.67 μ M	0.02	2.2	8.2
dNTPs (25 mM/each)	0.67 mM/each	0.08	8.8	32.8
Nuclease Free Water	-	2.46	270.6	1008.6
ERCC spike-ins (Optional)	-	-	-	-
Total	-	3μl	330 μl	1230 μl

Table 1. Reagent preparation for Smart-seq3 lysis buffer: Volumes for 96- and 384-well plates

- 1.2 Add 3 μ l lysis buffer to each well of a 96/384-well plate, and centrifuge briefly to collect lysis buffer in the bottom of the wells.

2 Sample Collection

- 2.1 Sort single cells into 3 μ l of lysis buffer in 96- or 384-well plates.
- 2.2 Seal the plate with appropriate cover seals (tolerating -80 °C to +110 °C) and centrifuge the finished sorted plate immediately after. Transfer the plate to a -80 °C freezer if not processing the cells into cDNA libraries within one day (plates can be stored at ~4 °C for up to one day). Prompt processing is beneficial for retained RNA integrity.

3 Cell Lysis

- 3.1 Remove the plate of sorted cells from the -80° C freezer and incubate in a thermocycler with heated lid at 72 °C for 3 min, followed by a 4 °C hold. Ensure that the plate is properly sealed, to avoid evaporation (use thermal pads, depending on thermocycler model).

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4 Reverse Transcription

4.1 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	Reaction Conc.	μl per reaction	96-well plate (110 rxns)	384-well plate (410 rxns)
Tris-HCl pH 8.3 (1 M)	25 mM	0.1	11	41
NaCl (1 M)	30 mM	0.12	13.2	49.2
MgCl ₂ (100 mM)	2.5 mM	0.1	11	41
GTP (100 mM)	1 mM	0.04	4.4	16.4
DTT (100 mM)	8 mM	0.32	35.2	131.2
SS3 TSO (100 μM)	2 μM	0.08	8.8	32.8
Maxima H-minus RT enzyme (200 units/μl)	8 units	0.04	4.4	16.4
Nuclease Free Water	-	0.2	22	82
Total	-	1 μl	110 μl	410 μl

Table 2. Reagent preparation for reverse transcription reaction: Volumes for 96- and 384-well plates

4.2 Add 1 μl RT mix to each well of a 96/384-well plate without dipping pipette tips into the lysis buffer, avoiding loss of original RNA molecules (no mixing needed).

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

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

4.5 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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5 Pre-amplification PCR

5.1 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	Conc. in PCR	µl per reaction	96-well plate (110 rxns)	384-well plate (410 rxns)
Kapa HiFi HotStart buffer (5x)	1x	2.0	220	820
dNTPs (25 mM/each)	0.3 mM/each	0.12	13.2	49.2
MgCl ₂ (100 mM)	0.5 mM	0.05	5.5	20.5
Fwd Primer (100 µM)	0.5 µM	0.05	5.5	20.5
Rev Primer (100 µM)	0.1 µM	0.01	1.1	4.1
Kapa Polymerase (1 units/µl)	0.02 units/µl	0.2	22	82
Nuclease Free Water	–	3.57	392.7	1463.7
Total	–	6 µl	660 µl	2460 µl

Table 4. Reagent preparation for PCR amplification: Volumes for 96-well plates

5.2 Add 6 µl PCR mix to each well of the 96/384-well plate without dipping pipette tips into the first-strand cDNA mix, avoiding loss of original unamplified cDNA molecules (no mixing needed).

5.3 Briefly centrifuge to collect reaction at the bottom of the plate. Seal with a new PCR seal. Ensure that the plate is properly sealed, to avoid evaporation (use thermal pads, depending on thermocycler model).

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

Step	Temp	Time	Cycles
Initial denaturation	98 °C	3 min	1×
Denaturation	98 °C	20 s	18-25×*
Annealing	65° C	30 s	
Elongation	72 °C	4 min	
Final Elongation	72 °C	5 min	1×
Hold	4 °C	Hold	

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

Table 5. Thermocycling conditions for PCR amplification

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6 cDNA Purification

Purification of cDNA is performed using Ampure XP beads or equivalent, e.g., 22% PEG Clean-up Beads (<https://www.protocols.io/view/smart-seq3-protocol-36wgq5rjxgk5>).

- 6.1 To purify cDNA, add 0.8:1 ratio of beads to sample (8 μ l) and mix by gently pipetting up and down. At this step, the PCR products can also be transferred to a round-bottom 96-plate for easier bead purification.
- 6.2 Incubate at room temperature for 8 min.
- 6.3 Place on magnet and allow beads to settle for ~5 min.
- 6.4 Discard the supernatant and wash with 20 μ l/100 μ l of freshly prepared 80% ethanol for 384/96-well plates respectively, keeping the plate on the magnet.
- 6.5 Remove the ethanol and let the beads dry for 2-5 min (do not over-dry the pellets).
- 6.6 Elute cDNA in 12 μ l UltraPure Water or other suitable elution buffer (e.g., 10mM Tris-HCl, pH 8.5) onto the pellets. Do not remove the plate from the magnet before adding the elution solution, as the magnetic pellets may then “jump”.
- 6.7 Remove the plate from the magnet and resuspend beads by pipetting up and down. Incubate for 8 min.
- 6.8 Place on magnet until clear (~3 min) and collect the eluate, containing the purified cDNA, to fresh plates or tubes.

7 Quality Control

- 7.1 Inspect the cDNA library yield and size distribution of a few randomly selected samples by capillary electrophoresis, e.g., on an Agilent Bioanalyzer High Sensitivity DNA Analysis chip.

A representative Bioanalyzer image of successfully amplified Smart-seq3 cDNA from a HEK cell using SEQURNA is shown in Figure 1.

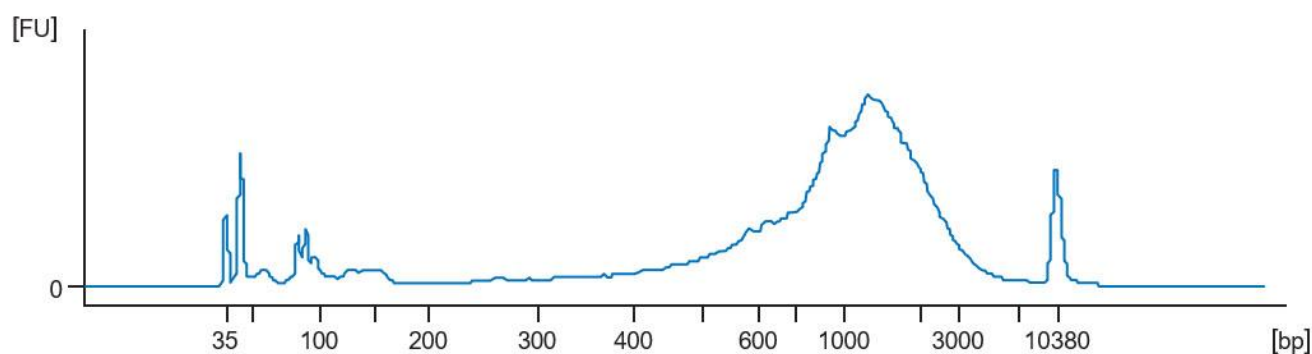


Figure 1. Trace of Smart-seq3 cDNA trace from a HEK cell, using an Agilent Bioanalyzer High Sensitivity DNA Analysis chip.

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8 Additional Literature

For detailed instructions on preparing indexed sequencing libraries from Smart-seq3 cDNA using tagmentation and PCR, as well as the subsequent steps for library generation and indexing, refer to the original Smart-seq3 protocol:

- **Hagemann-Jensen** *et al.*, Protocols.io – Smart-seq3 Protocol
<https://dx.doi.org/10.17504/protocols.io.bcq4ivyw>

For further details on the development of Smart-seq3:

- **Hagemann-Jensen** *et al.* (2020) [Single-cell RNA counting at allele and isoform resolution using Smart-seq3](#). Nature Biotechnology **38**, 708

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

PEG: Polyethylene glycol RT: Reverse transcription

SS3: Smart-seq3

TSO: Template-switching oligo

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