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STRIPE-seq library construction V.3

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1 Works for me dx.doi.org/10.17504/protocols.io.bdtri6m6

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ABSTRACT

Accurate mapping of transcription start sites (TSSs) is key for understanding transcriptional regulation; however, current protocols for genome-wide TSS profiling are laborious and expensive. We present Survey of TRanscription Initiation at Promoter Elements with high-throughput sequencing (STRIPE-seq), a simple, rapid, and cost-effective protocol for sequencing capped RNA 5′ ends from as little as 50 ng total RNA. Including depletion of uncapped RNA and bead cleanups, a STRIPE-seq library can be constructed in approximately 5 hours.

MATERIALS

NAME	CATALOG #	VENDOR
Terminator 5-Phosphate-Dependent Exonuclease	TER51020	Lucigen
RNAClean XP	A63987	Beckman Coulter
5M Betain	AAJ77507UCR	Thermo Fisher Scientific
KAPA HiFi HotStart ReadyMix	KK2601	Roche
Sorbitol	DSS23080-500	Dot Scientific
Trehalose	0210309705	MP Biomedicals
dNTPs 10 µM each	97063-232	VWR Scientific
SuperScript II Reverse Transcriptase	18064014	Thermo Fisher Scientific
RNA ScreenTape	5067-5576	Agilent Technologies
High Sensitivity D5000 ScreenTape	5067-5592	Agilent Technologies

BEFORE STARTING

Prepare 3.3 M sorbitol/0.66 M trehalose solution as per Batut and Gingeras (PMID 24510412).

1. Add **2 ml RNase-free H2O** to a 50 mL tube.
2. Add **8.02 g trehalose** to the tube.
3. Add **3 ml RNase-free H2O** .
4. Add **17.8 g sorbitol** to the tube.
5. Add **5.5 ml RNase-free H2O**
6. Bring volume to 30 mL with **0 ml RNase-free H2O**
7. Transfer to an RNase-free glass bottle and autoclave at 121°C for 30 min.

Store **1.5 ml** aliquots at **Room temperature** protected from light.

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Prepare Total RNA

1 Check RNA quality and concentration on an Agilent TapeStation using a High-Sensitivity RNA ScreenTape. 15m

You should have at least 50 to 200 ng of total RNA at a concentration of at least 30 to 125 ng/µl. Your total RNA should also not be highly degraded, as measured by the quality of the rRNA peaks.

TapeStation

Agilent G2991AA

Terminator Exonuclease (TEX) Digestion of Uncapped RNA

2 **Prepare TEX Reaction.** TEX preferentially degrades uncapped RNA, thus reducing the amount of rRNA and degraded mRNA fragments in the sample.

TEX is magnesium-dependent, so ensure that the RNA storage buffer does not contain EDTA.

2.1 Create TEX master mix. Prepare a sufficient volume for the number of reactions to be performed + 1 to account for volume loss during pipetting. 3m

1. **0.2 µl Terminator Exonuclease** .
2. **0.2 µl Terminator Exonuclease Reaction Buffer A** .

Vortex to mix and spin down.

2.2 Prepare TEX reactions in 0.2 mL PCR tubes.

1. **0.4 µl TEX Master Mix**
2. Up to **1.6 µl Total RNA** .
3. Nuclease free water to **2 µl** total reaction volume.

Vortex to mix and spin down.

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3 Incubate the TEX reactions in thermal cycler. 1h

1. **30 °C** for **01:00:00** .
2. **4 °C Hold** .

This is a good time to prepare the Reverse Transcription Oligo (RTO) annealing and Template Switching Reverse Transcription (TSRT) reaction mixtures from steps 4.1 and 5.1.

Template Switching Reverse Transcription

4 **Anneal reverse transcription oligo (RTO) to RNA.** STRIPE-seq primes reverse transcription via a random pentamer adjacent to the full length TrueSeq R2 adapter (including the barcode) in the RTO.

4.1 Prepare one RTO annealing mix per sample in 0.2 mL PCR tubes. 5m

1. **1.5 µl Sorbitol/Trehalose Solution** .
2. **1 µl Reverse Transcription Oligo (RTO)** **10 Micromolar (µM)** . Each sample should have its own unique barcode.
3. **0.5 µl dNTPs** **10 Millimolar (mM) Each** .

Vortex to mix and spin down.

4.2 Add **2 µl TEX Reaction** (from step 3) to **3 µl RTO Annealing Mixture** (from step 4.1). Vortex to mix and spin down. 3m

4.3 Incubate RTO annealing mixture in thermal cycler. 7m

1. **65 °C** **00:05:00** .
2. **4 °C** **00:02:00** .
3. **4 °C Hold** .

5 **Prepare template switching reverse transcription (TSRT) reactions.** The process of TSRT enriches for the 5' ends of capped RNA in the final library.

5.1 Prepare TSRT reaction master mix (per sample). 5m

1. **2 µl Betaine** **5 Molarity (M)** .
2. **2 µl 5X SuperScript II First Strand Buffer** .
3. **0.5 µl DTT** **0.1 Molarity (M)** .
4. **0.5 µl SuperScript II Reverse Transcriptase** .

Vortex to mix and spin down.

Add reverse transcriptase to master mix just prior to adding to samples.

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5.2 Add **5 µl TSRT Master Mix** (from step 5.1) into the **5 µl RTO Annealing Reaction** from step 4.3. Vortex to mix and spin down. 3m

6 TSRT.

6.1 First half of TSRT reaction. 25m

1. **25 °C** **00:10:00** .
2. **42 °C** **00:05:00** .

Move on to step 6.2 immediately after the end of step 6.1.

6.2 Add TSO. Keep the samples in the thermal cycler while adding the TSO. 3m

1. **0.25 µl TSO** **400 Micromolar (µM)** .
2. Quickly vortex to mix, spin down, and immediately place tubes back in thermal cycler.

Move on to step 6.3 immediately after end of step 6.2.

6.3 Second half of TSRT reaction. 30m

1. **00:25:00** **42 °C** .
2. **00:10:00** **70 °C** .
3. **4 °C** **Hold** .

This is a good time to prepare the library PCR master mix in step 8.1.

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7 Cleanup of TSRT product. 20m

1. Transfer the TSRT product from step 6.3 into 0.5 mL tube.
2. Pipette **8 µl RNAClean XP Beads** up and down 10 times into **10 µl TSRT Reaction** from step 6.3.
3. Incubate for **00:05:00** at **Room temperature** .
4. Place tubes on magnetic rack and incubate for **00:05:00** at **Room temperature** .
5. Carefully aspirate supernatant, leaving ~ **2 µl** in tube to avoid sucking up beads.
6. While tube is still on rack, wash beads with **175 µl 70% Ethanol** , and immediatly discard wash without incubation.
7. Air dry beads for **00:05:00** at **Room temperature** .
8. Resuspend beads in **12 µl Nuclease Free Water** , and incubate on magnetic rack for **00:01:00** at **Room temperature** .
9. Transfer **11 µl Supernatant** into new 0.2 mL PCR tubes.

Library PCR

8 **Prepare library PCR reaction.**

8.1 Create library PCR master mix (per sample). 5m

1. **12.5 µl 2X KAPA HiFi HotStart ReadyMix** .
2. **0.75 µl Forward Library Oligo (FLO)** **10 Micromolar (µM)** .
3. **0.75 µl Reverse Library Oligo (RLO)** **10 Micromolar (µM)** .

Vortex to mix and spin down.

8.2 Add **14 µl Library PCR Master Mix** (from step 8.1) into **11 µl Cleaned TSRT Product** (from step 7). Vortex to mix and spin down. 2m

9 Run library PCR reaction. 45m

Initial Denaturation:

- **95 °C** **00:03:00**

16-20 cycles:

- **98 °C** **00:00:20**
- **63 °C** **00:00:15**
- **72 °C** **00:00:45**

Final Extension:

- **72 °C** **00:02:00**
- **4 °C Hold**

10 **Size selection of final library.** SPRI bead size selection is used to remove fragments that are outside the ideal size for Illumina sequencing.

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10.1 Removal of small fragments. 20m

1. Transfer library PCR product from step 9 into 0.5 mL tube.
2. Pipette **16.3 µl RNAClean XP Beads** up and down 10 times into **25 µl Library PCR Product** from step 9.
3. Incubate for **00:05:00** at **Room temperature** .
4. Place tubes on magnetic rack and incubate for **00:05:00** at **Room temperature** .
5. Carefully aspirate supernatant, leaving ~ **2 µl** in tube to avoid sucking up beads.
6. While tube is still on rack, wash beads with **175 µl 70% Ethanol** and immediately discard wash without incubation.
7. Air dry beads for **00:05:00** at **Room temperature** .
8. Resuspend beads in **17 µl Nuclease Free Water** and incubate on magnetic rack for **00:01:00** at **Room temperature** .
9. Transfer **15 µl Supernatant** to new 0.5 mL tube.
10. **Optional**: Reserve **1 µl Remaining Supernatant** from beads if you would like to see library size distribution after removing small fragments.

10.2 Removal of large fragments. 40m

1. Pipette **8.3 µl RNAClean XP Beads** up and down 10 times into **15 µl Cleaned Product** from step 10.1. Make sure to vortex the beads again prior to use.
2. Incubate for **00:10:00** at **Room temperature** .
3. Place tubes on magnetic rack and incubate for **00:10:00** at **Room temperature** .
4. Transfer **22 µl Supernatant** to new tube.
5. Pipette **22 µl RNAClean XP Beads** up and down 10 times into **22 µl Supernatant** from previous step.
6. Incubate for **00:05:00** at **Room temperature** .
7. Place tubes on magnetic rack and incubate for **00:05:00** at **Room temperature** .
8. Carefully aspirate supernatant, leaving ~ **2 µl** in tube to avoid sucking up beads.
9. While tube is still on rack, wash beads with **175 µl 70% Ethanol** , and immediately discard wash without incubation.
10. Air dry beads for **00:05:00** at **Room temperature** .
11. Resuspend beads in **16 µl Nuclease Free Water** , and incubate on magnetic rack for **00:01:00** at **Room temperature** .
12. Transfer **15 µl Supernatant** to new tube.

Library Quality Control

11 Run final libraries on the Agilent TapeStation using a High Sensitivity D5000 ScreenTape. 15m

Final libraries should be distributed between 250 to 750 bp with a total library amount of 25 to 100 ng.

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