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STRIPE-seq library construction V.3

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Protocol status: Working
We use this protocol and it's working

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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

MATERIALS

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

Troubleshooting

Before start

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.

Prepare Total RNA

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

Expected result

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.

Equipment

TapeStation	NAME
Agilent	BRAND
G2991AA	SKU
https://www.agilent.com/en/product/tapestation-automated-electrophoresis/tapestation-instruments/4200-tapestation-system-228263	LINK

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.

Note

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.

3 Incubate the TEX reactions in thermal cycler. 1h

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

> Note
>
> 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) [M] 10 micromolar (µM) . Each sample should have its own unique barcode.
3. 0.5 µL dNTPs [M] 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 [M] 5 Molarity (M) .
2. 2 µL 5X SuperScript II First Strand Buffer .
3. 0.5 µL DTT [M] 0.1 Molarity (M) .
4. 0.5 µL SuperScript II Reverse Transcriptase .

Vortex to mix and spin down.

Note

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

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 .

Note

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 [M] 400 micromolar (µM) .
2. Quickly vortex to mix, spin down, and immediately place tubes back in thermal cycler.

Note

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 .

Note

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

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) [M] 10 micromolar (µM).
3. 0.75 µL Reverse Library Oligo (RLO) [M] 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.

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

Expected result

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