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(TSS-MPRA Protocol

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Carlos Guzman

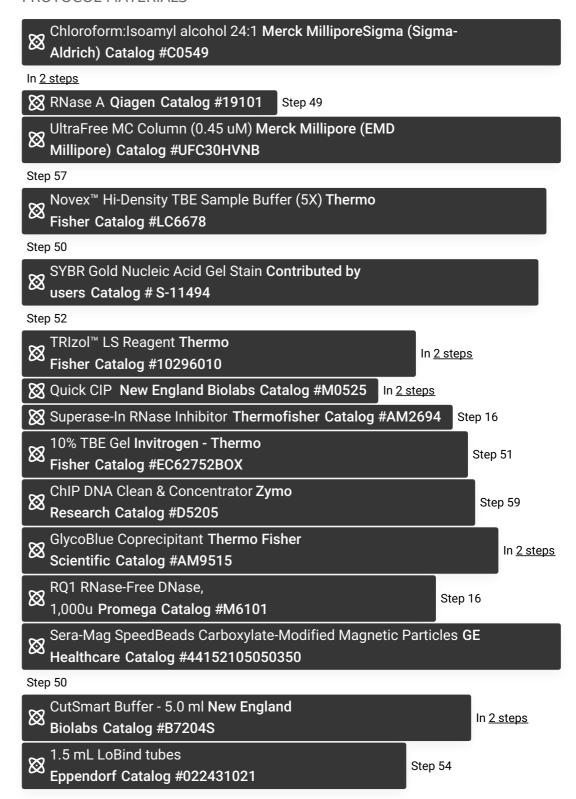
ABSTRACT

Cis-regulatory elements can be classified by the shapes of their transcription initiation patterns, which are indicative of distinct regulatory mechanisms. While massively parallel reporter assays (MPRAs) have enabled the functional study of sequence features within regulatory elements on an unprecedented scale, current MPRA approaches focus on quantifying transcript abundance, largely ignoring where transcription starts. This information however, could provide evidence that regulatory mechanisms in the context of the reporter assay resemble those active in the genome. Here we describe a transcription start site-capturing massively parallel reporter assay (TSS-MPRA) that simultaneously measures the location and frequency of transcription initiation. We characterize the degree to which plasmidbased MPRAs recapitulate endogenous initiation patterns ("TSS shapes") and transcription levels and evaluate the effects of increasing insert length and reporter chromatinization on plasmid-derived transcription initiation. Employing a new bioinformatic approach to compare TSS shapes, we find that shorter, episomal constructs most faithfully replicate endogenous initiation patterns and transcription levels. Finally, we illustrate how TSS-MPRA can be used to decode cis-regulatory grammar by assessing the effects of core promoter and transcription factor motif mutations and single nucleotide polymorphisms on transcription initiation. Taken together, TSS-MPRA reveals important caveats to consider when using MPRAs and enables high-resolution analysis of the sequence grammar underlying transcription initiation.

Oct 26 2023

PROTOCOL integer ID: 62444

PROTOCOL MATERIALS



BEFORE START INSTRUCTIONS

- * Make sure that cells are resuspended in media/PBS before TRIzol LS extraction (250 uL)
- * Wipe down work surface and pipettes with RNAse Zap
- * Cool down centrifuge to 4°C

RNA Extraction

58m 20s

TRIzol™ LS Reagent **Emd** Millipore Catalog #10296010

per

Δ 250 μL of sample volume (3x) and pipette up and down 5x (Optional: samples can be stored \$\colon 4 \colon C \quad ON or at \$\colon -20 \circ C \quad for up to a year).

2 Incubate at RT for 00:05:00

5m

3 Add Δ 230 μL

Chloroform:Isoamyl alcohol 24:1 Emd Millipore Catalog #C0549

5m

15m

and

shake vigorously by hand for 00:00:15

4 Incubate for 00:05:00 at RT.

5 Centrifuge samples for $\bigcirc 00:15:00$ (13,000 x g, 4°C).

6 Transfer the aqueous layer containing the RNA to a new 1.5 mL LoBind tube (~ 400 - 500 uL).

7 GlycoBlue Coprecipitant Emd Millipore Catalog #AM9515

to each

sample.

8 Add 1/10th volume of 3M NaOAc (to each sample.

- 9 Vortex 00:00:05 and add 1x volume of isopropanol to each sample. Mix by inverting 10 times and then spin down briefly.
- 5s

- 10 Incubate ON at [-20 °C (optionally 20 minutes at -20 °C).
- Centrifuge samples for 00:30:00 (MAX x g, 4°C).

30m

- Discard supernatant, wash with 1 mL 75% EtOH. Discard EtOH, quick-spin, remove rest of EtOH.

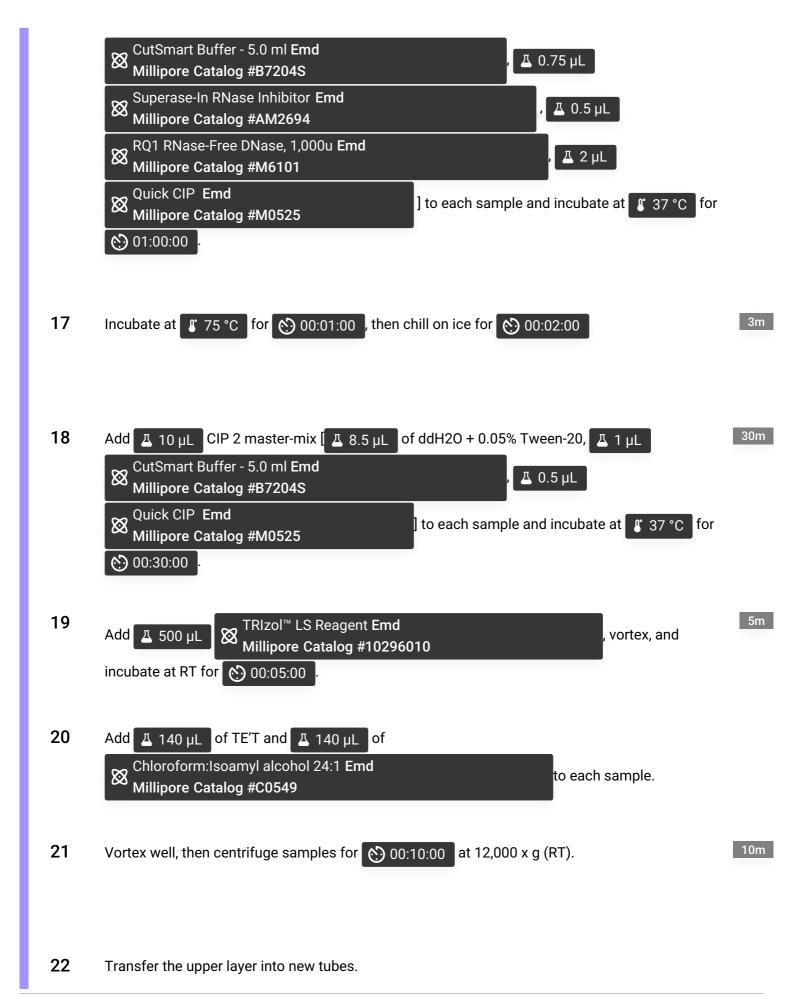
3m

Resuspend in Δ 30 μL TE'T [[M] 0.05 % volume Tween-20, [M] 0.1 millimolar (mM) EDTA [M] 10 millimolar (mM) Tris pH 7.5].

Capped MPRA 5' RNA-seq

1d 2h 45m 5s

- Aliquot Δ 15 μL of sample into 1.5 mL epi-tube and incubate at 75 °C for 00:02:00, then chill on ice for 00:02:00
 - _
- Add \triangle 35 μ L of CIP1 master-mix [\triangle 25.25 μ L of ddH2O + 0.05% Tween-20, \triangle 5 μ L





- Vortex 00:00:05 and add 1x volume of isopropanol to each sample. Mix by inverting 10 times and then spin down briefly.
- 26 Incubate Overnight at -20 °C (optionally 20 minutes at -20 °C).
- 27 Centrifuge samples for 00:30:00 (MAX x g, 4°C).
- Discard supernatant, wash with 1 mL 75% EtOH. Discard EtOH, quick-spin, remove rest of EtOH.
- Air dry pellet at RT until it is translucent (~ (>) 00:03:00). (Optional: pellet can be frozen at "-80 °C indefinitely)
- Resuspend RNA in A 6 µL TET [[M] 0.05 % volume Tween-20, [M] 1 millimolar (mM) EDTA [M] 10 millimolar (mM) Tris pH 7.5].

3m

5s

Add A 9 µL of RppH master-mix to each sample, mix really well and incubate at \$37 °C for

4m



Note

The PEG in the master-mix can be extremely viscous, so you need to be very careful when mixing both the master-mix together and when mixing it with the sample to ensure that it's properly mixed

Note

RppH master-mix (per sample):

<u>Д</u> 3.25 µL ddH20 + 0.1% Tween-20

 \perp 1.5 μ L

T4 RNA Ligase Reaction Buffer - 3.0 ml New England Biolabs Catalog #B0216L

50% PEG8000 New England **Δ** 3 μL Biolabs Catalog #B1004S

 \perp 0.25 μ L Superase-In RNase Inhibitor Thermofisher Catalog #AM2694

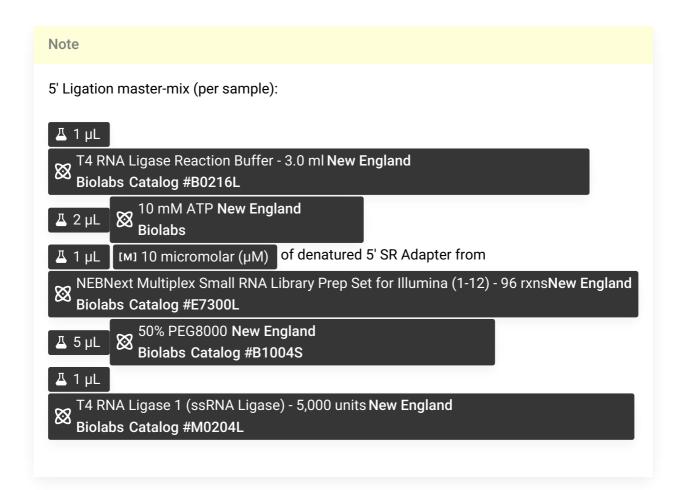
Δ 1 μL

RNA 5' Pyrophosphohydrolase (RppH) - 200 units New England Biolabs Catalog #M0356S

33

Add 🗸 10 µL 5' Ligation master-mix, mix well and incubate at 🕴 21 °C for 🚫 02:00:00

16 °C for **♦** 16:00:00



Note

It is **CRITICAL** that you denature the 5' SR Adapter at 75 °C for 00:02:00 prior to creating the master-mix.

- 34 <u>≡5 go to step #19</u>
- 35 go to step #20
- 36 <u>≡</u> go to step #21

37 <u>≡5 go to step #22</u>

38 <u>■5 go to step #23</u>

39 go to step #24

40 go to step #25

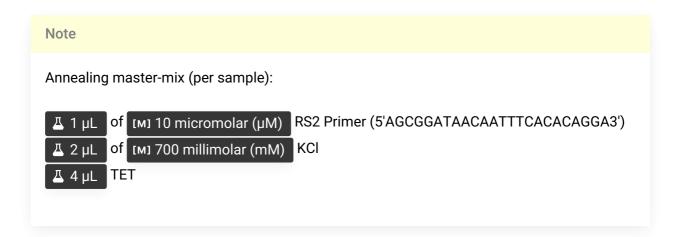
41 go to step #26

42 go to step #27

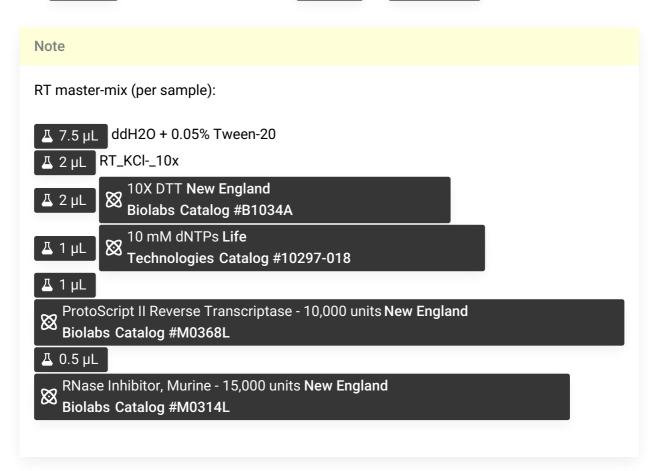
43 go to step #28

44 go to step #29

45 Resuspend pellet in $\boxed{\text{\em L}}$ 7 $\mu\text{\em L}$ of Annealing master-mix.



- Denature RNA at 75 °C for 00:02:00 , then incubate at 56 °C for 00:45:00 , then cool on ice.
- Add Δ 13 μL RT master-mix, incubate at 50 °C for 01:00:00



47m



Note

PCR master-mix (per sample):



LongAmp Taq 2X Master Mix - 100 rxns New England Biolabs Catalog #M0287S



Betaine 5M Sigma Aldrich Catalog #B0300



Δ 0.2 μL [M] 100 micromolar (μM) of blue cap SR Primer from

NEBNext Multiplex Small RNA Library Prep Set for Illumina (1-12) - 96 rxnsNew England Biolabs Catalog #E7300L



Д 2 μL of [м] 10 micromolar (μM) 3' barcode primer (we use TruSeq HT Primers D70х-

D7xx)

Note

The blue cap SR primer from the NEBNExt Small RNA Library Prep kit comes in 10 uM concentration. Therefore we order our own primer from IDT in order to use 100 uM concentration primers. Decrease the amount of Betaine used if using 10 uM concentrations.

	A	В	С
	PCR Program		
		94C	30 seconds
		94C	15 seconds
	15x cycles	63C	30 seconds
		70C	18 seconds***
		70C	5 minutes
		4C	hold infinity

the extension time depends on the length of your inserts! LongAmp copies DNA at 1kb per 50 seconds

