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TSS-MPRA Protocol

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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 plasmid-based 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.

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62444

PROTOCOL MATERIALS

Chloroform:Isoamyl alcohol 24:1 **Merck MilliporeSigma (Sigma-Aldrich) Catalog #C0549**

In 2 steps

RNase A **Qiagen Catalog #19101** Step 49

UltraFree MC Column (0.45 uM) **Merck Millipore (EMD Millipore) Catalog #UFC30HVNB**

Step 57

Novex™ Hi-Density TBE Sample Buffer (5X) **Thermo Fisher Catalog #LC6678**

Step 50

SYBR Gold Nucleic Acid Gel Stain **Contributed by users Catalog # S-11494**

Step 52

TRIzol™ LS Reagent **Thermo Fisher Catalog #10296010** In 2 steps

Quick CIP **New England Biolabs Catalog #M0525** In 2 steps

Superase-In RNase Inhibitor **Thermofisher Catalog #AM2694** Step 16

10% TBE Gel **Invitrogen - Thermo Fisher Catalog #EC62752BOX** Step 51

ChIP DNA Clean & Concentrator **Zymo Research Catalog #D5205** Step 59

GlycoBlue Coprecipitant **Thermo Fisher Scientific Catalog #AM9515** In 2 steps

RQ1 RNase-Free DNase, 1,000u **Promega Catalog #M6101** Step 16

Sera-Mag SpeedBeads Carboxylate-Modified Magnetic Particles **GE Healthcare Catalog #44152105050350**

Step 50

CutSmart Buffer - 5.0 ml **New England Biolabs Catalog #B7204S** In 2 steps

1.5 mL LoBind tubes **Eppendorf Catalog #022431021** Step 54

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

1 Add 750 µL of TRIzol™ LS Reagent **Emd Millipore Catalog #10296010** per 250 µL of sample volume (3x) and pipette up and down 5x (**Optional:** samples can be stored at 4 °C ON or at -20 °C for up to a year).

2 Incubate at RT for 00:05:00. 5m

3 Add 230 µL of Chloroform:Isoamyl alcohol 24:1 **Emd Millipore Catalog #C0549** and shake vigorously by hand for 00:00:15. 15s

4 Incubate for 00:05:00 at RT. 5m

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

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

7 Add 1 µL of gly GlycoBlue Coprecipitant **Emd Millipore Catalog #AM9515** to each sample.

8 Add 1/10th volume of 3M NaOAc (pH 5.5) 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).

11 Centrifuge samples for 00:30:00 (MAX x g, 4°C). 30m

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

13 Air dry pellet at RT until it is translucent (~ 00:03:00). (**Optional:** pellet can be frozen at -80 °C indefinitely) 3m

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

Capped MPRA 5' RNA-seq

1d 2h 45m 5s

15 Aliquot 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 4m

16 Add 35 µL of CIP1 master-mix [25.25 µL of ddH2O + 0.05% Tween-20, 5 µL 1h

CutSmart Buffer - 5.0 ml **Emd Millipore Catalog #B7204S** , 0.75 µL
Superase-In RNase Inhibitor **Emd Millipore Catalog #AM2694** , 0.5 µL
RQ1 RNase-Free DNase, 1,000u **Emd Millipore Catalog #M6101** , 2 µL
Quick CIP **Emd Millipore Catalog #M0525**] to each sample and incubate at 37 °C for 01:00:00 .

17 Incubate at 75 °C for 00:01:00 , then chill on ice for 00:02:00 3m

18 Add 10 µL CIP 2 master-mix [8.5 µL of ddH2O + 0.05% Tween-20, 1 µL 30m
CutSmart Buffer - 5.0 ml **Emd Millipore Catalog #B7204S** , 0.5 µL
Quick CIP **Emd Millipore Catalog #M0525**] to each sample and incubate at 37 °C for 00:30:00 .

19 Add 500 µL TRIzol™ LS Reagent **Emd Millipore Catalog #10296010** , vortex, and incubate at RT for 00:05:00 . 5m

20 Add 140 µL of TE'T and 140 µL of Chloroform:Isoamyl alcohol 24:1 **Emd Millipore Catalog #C0549** to each sample.

21 Vortex well, then centrifuge samples for 00:10:00 at 12,000 x g (RT). 10m

22 Transfer the upper layer into new tubes.

23 Add 1 µL of gly GlycoBlue Coprecipitant **Emd Millipore Catalog #AM9515** to each sample.

24 Add 1/10th volume of 3M NaOAc (pH 5.5) to each sample.

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

26 Incubate Overnight at -20 °C (optionally 20 minutes at -20°C). 10m

27 Centrifuge samples for 00:30:00 (MAX x g, 4°C). 30m

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

29 Air dry pellet at RT until it is translucent (~ 00:03:00). (**Optional:** pellet can be frozen at -80 °C indefinitely) 3m

30 Resuspend RNA in 6 µL TET [[M] 0.05 % volume Tween-20, [M] 1 millimolar (mM) EDTA, [M] 10 millimolar (mM) Tris pH 7.5].

31 Denature RNA at 75 °C for 00:02:00, then chill on ice for 00:02:00. 4m

32 Add 9 µL of RppH master-mix to each sample, mix **really** well and incubate at 37 °C for 01:00:00 1h

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):

3.25 µL ddH2O + 0.1% Tween-20

1.5 µL

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

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

0.25 µL Superase-In RNase Inhibitor **Thermofisher Catalog #AM2694**

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 or 16 °C for 16:00:00. 18h

Note

5' Ligation master-mix (per sample):

1 µL

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

2 µL 10 mM ATP **New England Biolabs**

1 µL [M] 10 micromolar (µM) of denatured 5' SR Adapter from

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

5 µL 50% PEG8000 **New England Biolabs Catalog #B1004S**

1 µL

T4 RNA Ligase 1 (ssRNA Ligase) - 5,000 units **New England Biolabs Catalog #M0204L**

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 go to step #19

35 go to step #20

36 go to step #21

37 go to step #22

38 go to step #23

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 7 µL of Annealing master-mix.

Note

Annealing master-mix (per sample):

1 µL of [M] 10 micromolar (µM) RS2 Primer (5'AGCGGATAACAATTTCACACAGGA3')
2 µL of [M] 700 millimolar (mM) KCl
4 µL TET

46 Denature RNA at 75 °C for 00:02:00, then incubate at 56 °C for 00:45:00, then cool on ice. 47m

47 Add 13 µL RT master-mix, incubate at 50 °C for 01:00:00. 1h

Note

RT master-mix (per sample):

7.5 µL ddH2O + 0.05% Tween-20
2 µL RT_KCl-_10x
2 µL 10X DTT **New England Biolabs Catalog #B1034A**
1 µL 10 mM dNTPs **Life Technologies Catalog #10297-018**
1 µL
ProtoScript II Reverse Transcriptase - 10,000 units **New England Biolabs Catalog #M0368L**
0.5 µL
RNase Inhibitor, Murine - 15,000 units **New England Biolabs Catalog #M0314L**

48 Do PCR

Note

PCR master-mix (per sample):

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

2.8 µL Betaine 5M **Sigma Aldrich Catalog #B0300**

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 rxns**New England Biolabs Catalog #E7300L**

2 µL of [M] 10 micromolar (µM) 3' barcode primer (we use TruSeq HT Primers D70x-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	B	C
	PCR Program		
		94C	30 seconds
	15x cycles	94C	15 seconds
		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

49 Add 1 µL RNase A **Emd Millipore Catalog #19101**, incubate for 00:15:00 at 37 °C. 15m

50 Add 47 µL 20% PEG / 2.5M NaCl + 3 µL **Sera-Mag SpeedBeads Carboxylate-Modified Magnetic Particles Emd Millipore Catalog #44152105050350** to each sample. Incubate at RT for 0 µL. Wash 2x with 80% EtOH. Air-dry beads until cracked (~12 minutes). Resuspend in 15 µL of 1x **Novex™ Hi-Density TBE Sample Buffer (5X) Emd Millipore Catalog #LC6678**. Incubate at RT for 00:05:00. 5m

51 Run the entire sample on **10% TBE Gel Emd Millipore Catalog #EC62752BOX** with 0.5 µL of any 25bp ladder (80V for 00:15:00, then 180V for 01:15:00). Perforate 0.5 mL qubit tubes 4x with 22g needle, and place qubit tubes inside 1.5 mL LoBind tube. 1h 30m

52 Stain the gel with 20 mL of 1x TBE. Buffer + 3 µL of **SYBR Gold Nucleic Acid Gel Stain Emd Millipore Catalog # S-11494** for 00:05:00 inside a RNase free container in the dark. 5m

53 Visualize gel and cut gel depending on desired size (adapters are ~118 bp, add to size you expect).

> **Note**
>
> This is highly dependent on the adapters you chose to use and the design of your inserts. In our case our adapters add up to 118 bp and we expect an average transcript length of 60 bp, so we are looking for bands in the ~170-180 bp range.

54 Centrifuge samples for 00:05:00 (20,000 x g , RT). Ensure that all the gel is crushed and 5m

spun down into 1.5 mL LoBind tubes **Emd Millipore Catalog #022431021**.

55 Add 160 µL of TET to each.

56 Shake samples for 00:45:00 at RT (do not shake too quickly). 45m

57 Transfer slurry to UltraFree MC Column (0.45 uM) **Emd Millipore Catalog #UFC30HVNB** and 2m
spin for 00:02:00 at 1000 x g.

58 Add 100 µL of TET + 300 mM NaCl to column and incubate for 00:30:00 at RT, then spin for 32m
00:02:00 at 1000 x g.

59 Clean up in ChIP DNA Clean & Concentrator **Emd Millipore Catalog #D5205** columns
according to instructions. Elute in 10 µL EB.