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5′ RACE-seq for RNA fragments with 5′ phosphate - library prep protocol

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We use this protocol and it's working

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Abstract

This protocol will allow identification of fragments of RNA that have a 5' phosphate on a transcriptome-wide scale using an Illumina sequencing platform. Library preparation relies on ligation of an RNA adapter to the 5' end of the fragment that matches the Illumina adapter sequence and contains a unique molecular identifier and reverse transcription with a random primer that adds Illumina adapter sequences. This is followed by amplification with forward and reverse primers that add Illumina adapter sequence inlcuding barcodes. We have used it to isolate fragments generated by viral RNases.

Materials

Reagents

RNeasy Mini kit (Qiagen – Cat #74104)
Ambion Turbo DNase (Thermofisher – Cat#AM2239)
Recombinant RNasin RNase inhibitor (Promega – Cat# N2515)
Phenol/chloroform with acidic pH (we use Fisher Cat#BP1752I-100 without adding the additional buffer, but others should work)
3 M sodium acetate (Thermofisher – Cat# AM9740)
Glycogen (Thermofisher – Cat# AM9510)
ERCC spike mix (Thermofisher – Cat# 4456739)
Illumina Ribo zero plus (Illumina – Cat# 20020598)
RNAClean XP beads (Beckman coulter – Cat# A63987)
100% Ethanol (we use VWR Cat#89125-170, but others will work)
Ultrapure DNase/RNase free dH_2O (Thermofisher – Cat# 10-977-015)
T4 RNA ligase (Thermofisher – Cat#AM2141)
RNA cleanup and concentrator (Zymo Research – Cat# R1015)
Invitrogen SuperScript III RT (Thermofisher – Cat # 18080044).
DL-Dithiothreitol (DTT) (Sigma – Cat #D0632-1G)
SPRIselect Reagent (Beckman Coulter– Cat# B23317)
Phusion polymerase (Thermofisher – Cat#F530S)
Illumina RSB buffer - sold with Illumina kits or see here for alternatives: https://knowledge.illumina.com/library-preparation/general-library-preparation/library-preparation-general-faq-list/000005040

Equipment

Thermocycler
Microcentrifuge tube magnet
Refrigerated microcentifuge

Primer/adapter sequences

-5′ adapter *(make 25 µM working stock)**:***
24 last bases of P5 section of Illumina adapter + UMI
5′ CCCUACACGACGCUCUUCCGAUCUNNNNNNNNN 3′

-RT *(make 100 ng/µl working stocks):*
23 first bases reverse complemented of P7 section of Illumina adapter + Ns for random priming
RT-short: 5′ TTCAGACGTGTGCTCTTCCGATCNNNNNNNN 3′
RT-long: 5′ TTCAGACGTGTGCTCTTCCGATCNNNNNNNNNNNNNN 3′

-PCR1 *(make 10 µM working stocks)**:***
Forward (*24 last bases of P5 section of Illumina adapter)* :
5′ CCCTACACGACGCTCTTCCGATCT 3′

Reverse (*23 first bases reverse complemented of P7 section of Illumina adapter)*:
5′ TTCAGACGTGTGCTCTTCCGATC 3′

-PCR2 *(make 10 µM working stocks)**:***
Forward *(Truseq Universal adapter, including P5 sequence)*:
5′ AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT3′

Reverse (*underlined sequence = barcodes for multiplexing samples; reverse complement of Truseq index adapters, including P7 sequence)*:
1) 5′CAAGCAGAAGACGGCATACGAGAT<u>CGTGAT</u>GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC 3′
2) 5′CAAGCAGAAGACGGCATACGAGAT<u>ACATCG</u>GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC 3′
3) 5′CAAGCAGAAGACGGCATACGAGAT<u>GCCTAA</u>GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC 3′
4) 5′CAAGCAGAAGACGGCATACGAGAT<u>TGGTCA</u>GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC 3′
5) 5′CAAGCAGAAGACGGCATACGAGAT<u>CACTGT</u>GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC 3′
6) 5′CAAGCAGAAGACGGCATACGAGAT<u>ATTGGC</u>GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC 3′
7) 5′CAAGCAGAAGACGGCATACGAGAT<u>GATCTG</u>GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC 3′
8) 5′CAAGCAGAAGACGGCATACGAGAT<u>TCAAGT</u>GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC 3′
9) 5′CAAGCAGAAGACGGCATACGAGAT<u>CTGATC</u>GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC 3′
10) 5′CAAGCAGAAGACGGCATACGAGAT<u>AAGCTA</u>GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC 3′
11) 5′CAAGCAGAAGACGGCATACGAGAT<u>GTAGCC</u>GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC 3′
12) 5′CAAGCAGAAGACGGCATACGAGAT<u>TACAAG</u>GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC 3′

Troubleshooting

Procedure

1 **RNA extraction**
Extract all RNA using RNeasy Qiagen kit.
Elute in 50 µl H_2O.

2 **DNase treatment:**
Add to each RNA sample:
1.2 µl Turbo DNase
1.5 µl RNasin
6 µl 10x turbo DNase buffer
1.3 µl H_2O.
(Total volume = 60 µl)
Incubate at 37°C for 20 min.

3 **RNA extraction using phenol/chloroform**:
Add 140 µl water to each tube + 200 µl phenol/chloroform.
Vortex samples for 20-30sec.
Spin down at 12,000xg at room temperature for 5 min.
Collect top layer and mix with 2 µl glycogen + 20 µl 3 M sodium acetate + 600 µl 100% ethanol.
Mix by inverting tubes a few times and incubate for 1 h at -20°C.
Spin down tubes for 20 min at max speed at 4°C.
Remove supernatant without dislodging the pellet and wash with 1 ml 70% ethanol.
Spin down for 5 min at max speed at 4°C.
Remove supernatant and spin again for 1 min.
Remove any liquid left and dry pellet for 1 min.
Resuspend pellet with 16 µl water.
Incubate at room temperature for 10 min, then place on ice and measure concentration.

Dilute RNA to have 3 tubes of 1 µg RNA in 9 µl water for each sample.

4 **Addition of spike-in controls (optional)**
If using spike-in controls, add 2 µl of 1:100 dilution of Mix 1 or Mix 2 RNA spike-in control mixes from ERCC to each 1 µg RNA tube (*these can be helpful to normalize total RNAseq data, but we do not use them in the 5' RACEseq analysis; using different mixes in samples that will be compared with each other can also help check for relative*)

5 **Ribosomal RNA removal**
Use Illumina Ribo zero plus kit following their protocol.
At the end of the protocol, you will have 30 µl of RNA solution per tube, and 3 tubes per sample.

6 **RNA purification with RNAClean XP beads**

Pool three tubes for each sample together (90 µl).
Add 180 µl RNAClean XP beads to the RNA sample.
Pipette up and down 10 times to mix.
Incubate for 5 min at RT.
Place tubes on magnet for 2 min.
When beads have separated and solution is completely clear, aspirate the clear solution and discard.

Add 400 µl 80% ethanol to each tube (still on magnet) and incubate for 30 sec at RT.
Remove ethanol and discard.
Wash again with 80% ethanol.
Remove ethanol and dry beads for 2 min.
Remove from magnet, add 19 µl water to each tube.
Mix well by pipetting up and down 10 times and to make sure all the beads are in solution, then incubate for 2 min at RT.
Place back on magnet and incubate for 1 min until beads are separated.
Remove 17 µl and place in new PCR tube.

7 **Total RNAseq sample (optional)**
If desired, take 10% of the sample (1.7 µl) to do total RNAseq (using Illumina TruSeq stranded kit protocol). Use the rest (~15 µl) of RNA to ligate 5′ adapter.

8 **Ligation of 5′ adapter using T4 RNA ligase**
Add to the 15 µl RNA:
- 2 µl T4 RNA ligase
- 1 µl of 25 µM adaptor
- 2 µl T4 RNA ligase buffer

Ligate at 25°C for 2h.

9 **RNA clean up**
Use Zymo RNA cleanup and concentrator kit using manufacturer's instructions to collect RNA fragments > 200nt.
Elute in 11 µl RNase/DNase free water.
Transfer 10 µl to a new PCR tube.

10 **Reverse transcription with SuperScript III RT**
Add to 10 µl of RNA:
- 1 µl primer RT-short (100 ng/µl)
- 1 µl primer RT-long (100 ng/µl)
- 0.4 µl 25 mM dNTP mix
- water up to 13 µl (including the 10 µl sample)

Incubate at 65°C for 5min, then place on ice for at least 1min.

Add:

- 4 µl 5X First-strand buffer
- 1 µl DTT (100 mM stock)
- 1 µl RNasin
- 1 µl SuperScript III RT (200 U/µl)

Incubate in thermocycler for RT reaction:

- 25°C for 5min
- 50°C for 1h
- 70°C for 15min.

11 **Purification of cDNA using RNAClean XP beads**

Add 36 µl RNAClean XP beads to the cDNA sample.
Pipette up and down 10 times to mix.
Incubate for 5 min at RT.
Place tubes on magnet for 2 min.
When beads have separated and solution is completely clear, aspirate the clear solution and discard (in practice, aspirate about 50 µl to leave 5 µl of supernatant behind and not aspirate beads).
Add 200 µl 80% ethanol to each tube (still on magnet) and incubate for 30 sec at RT.
Remove ethanol and discard.
Wash again with 80% ethanol (no need to leave some supernatant behind for this step, but can incubate for a few min for the ethanol to evaporate).
Remove from magnet, add 25 µl water to each well.
Mix well by pipetting up and down 10 times and to make sure all the beads are in solution, then incubate for 2 min at RT.
Place back on magnet and incubate for 1 min until beads are separated.
Remove 23 µl and place in new PCR tube.
(Can stop here and freeze DNA.)

12 **First DNA amplification using Phusion**

Add to the 23 µl sample:

- 10 µl 5x Phusion buffer
- 0.5 µl Phusion enzyme
- 2.5 µl 10 µM PCR1 primers
- 0.4 µl 25 mM dNTPs
- 11.1 µl H2O

(50 µl total reaction)

PCR1 reaction:
-98°C 30s
-7 cycles of:
98°C 10 s
67°C 30 s
72°C 30 s

-72°C 5 min

13 **DNA Clean up and size selection using SPRIselect Reagent**

Shake SPRIselect bottle.
Add 35 µl SPRI beads to the cDNA sample.
Pipette up and down 10 times to mix.
Incubate for 1 min at RT.
Place tubes magnet until settled.
When beads have separated and solution is completely clear, transfer clear solution to a new tube and discard first tube with beads.

Add 55 µl SPRI beads.
Pipette up and down 10 times to mix.
Incubate for 1 min at RT.
Place on magnet and allow to settle.
When solution is completely clear, aspirate the clear solution and discard.

Add 180 µl 80% ethanol to each tube (still on magnet) and incubate for 30 sec at RT.
Remove ethanol and discard.
Remove from magnet, add 25 µl water to each well.
Mix well by pipetting up and down 10 times and to make sure all the beads are in solution.
Place back on magnet and incubate until beads are separated.
Remove 23 µl and place in new PCR tube.

14 **Second DNA amplification using Phusion**

Add to the 23 µl sample:

- 10 µl 5x Phusion buffer
- 0.5 µl Phusion enzyme
- 2.5 µl 10 µM PCR2 primers
- 0.4 µl 25 mM dNTPs
- 11.1 µl H2O

(50 µl total reaction)

PCR2 reaction: Same as PCR1 reaction.

15 **DNA clean up using RNAClean XP Beads**.

Add 90 µl RNAClean XP beads to the cDNA sample.
Pipette up and down 10 times to mix.
Incubate for 5 min at RT.
Place tubes on magnet for 2 min.
When beads have separated and solution is completely clear, aspirate the clear solution and discard.

Add 200 µl 80% ethanol to each tube (still on magnet) and incubate for 30 sec at RT.
Remove ethanol and discard.
Wash again with 80% ethanol (no need to leave some supernatant behind for this step, but can incubate for a few min for the ethanol to evaporate).
Air dry beads for 2min, remove from magnetic stand and add 22.5 µl Illumina RSB buffer directly onto the beads.
Slowly pipette up and down 10 times and to make sure all the beads are in solution, then incubate for 2 min at RT.
Place back on magnet and incubate for 1 min until beads are separated.
Remove 20 µl and place in new PCR tube.

Note: before running on Illumina sequencer, test the library on an Agilent Bioanalyzer or equivalent. Depending on the purity an additional Pippin run maybe needed to eliminate primer dimers and primer peaks.

*PyDegradome can be used to analyze the resulting data (*https://github.com/mgaglia81/PyDegradome)

Example of sequence

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Example of how sequences of adapter and primers work:

1. Hypothetical segment of RNA: 5′ – TGATGGCCGCAGTGCTGACCGTGACTGGAGCAGTTCCTGTCG – 3′

2. Ligation of adapter at 5′ of RNA:

5′ – CCCTACACGACGCTCTTCCGATCTNNNNNNNNNTGATGGCCGCAGTGCTGACCGTGACTGGAGCAGTTCCTGTCG – 3′

3. Reverse transcription with adapter on primer:

5′ – CCCTACACGACGCTCTTCCGATCTNNNNNNNNNTGATGGCCGCAGTGCTGACCGTGACTGGAGCAGTTCCTGTCG – 3′
3′ – GGGATGTGCTGCGAGAAGGCTAGANNNNNNNNNACTACCGGCGTCACGACTGGCACTGACCTCGTCAAGGACAGCCTAGCCTTCTCGTGTGCAGACTT – 5′

4. PCR1:

5′ – CCCTACACGACGCTCTTCCGATCTNNNNNNNNNTGATGGCCGCAGTGCTGACCGTGACTGGAGCAGTTCCTGTCGGATCGGAAGAGCACACGTCTGAA – 3′
3′ – GGGATGTGCTGCGAGAAGGCTAGANNNNNNNNNACTACCGGCGTCACGACTGGCACTGACCTCGTCAAGGACAGCCTAGCCTTCTCGTGTGCAGACTT – 5′

5. PCR2:

5′ – AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNNNNNNTGATGGCCGCAGTGCTGACCGTGACTGGAGCAGTTCCT
3′ – TTACTATGCCGCTGGTGGCTCTAGATGTGAGAAAGGGATGTGCTGCGAGAAGGCTAGANNNNNNNNNACTACCGGCGTCACGACTGGCACTGACCTCGTCAAGGA

GTCGGATCGGAAGAGCACACGTCTGAACTCCAGTCACATCACGATCTCGTATGCCGTCTTCTGCTTG – 3′
CAGCCTAGCCTTCTCGTGTGCAGACTTGAGGTCAGTGTAGTGCTAGAGCATACGGCAGAAGACGAAC – 5′