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Modified protocol for genome-wide mapping of uncapped transcripts (GMUCT) in eukaryotes V.3

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Protocol status: Working

We use this protocol and it's working

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Abstract

This is a modified protocol to construct a sequencing library for genome-wide mapping of uncapped and cleaved transcripts (GMUCT; Willmann et al, 2014 Methods, 10.1016/j.ymeth.2013.07.003).

Attachments



[MAN0015808_Dynabeads...](#)
ds...
117KB



[TruSeq-SmallRNA-libr...](#)
2.4MB



[NEBNext_mRNA_manual](#)
E...
285KB



[Illumina_index-adapt...](#)
1.7MB



[DNA library size sel...](#)
251KB

Guidelines

Make sure your workspace is nuclease-free and sterile, including following RNA-friendly practices such as using clean pipettes, filter tips, microtubes, and PCR tubes to minimize the chance of sample contamination, especially of RNases.

Keep all samples and enzyme mixes on ice in between steps. Store RNA or libraries at -20C at any pause steps and minimize length of storage.

Be careful with bead-based purification to avoid unwanted carryover.

Materials

A	B	C
Item	Manufacturer	Catalog Number
Dynabeads mRNA purification kit	Invitrogen	61006
NEBNext Poly(A) mRNA Magnetic Isolation module	NEB	E7490
Magnetic separation rack for microtubes	Sergi Lab Supplies	cat. No. 1005a
Magnetic separation rack for PCR tubes	Sergi Lab Supplies	cat. no. 1008
HPLC-purified random hexamer primer that includes the TruSeq RA3 3'-adapter on the 5' end: 5'-ctggagtccttggcaccgcagagaattccannnnnn-3'	IDT	ssDNA oligo order
TruSeq Small RNA adapters and index primers: RA5, RP1, RPI1-48 (based on TruSeq Small RNA Sample Prep Kit)	Illumina / IDT	RA5: ssRNA oligo; RP1: ssDNA oligo; RPI1-48: ssDNA oligo
Betaine 5 M	Thermo Scientific Chemicals	J77507.UCR
T4 RNA Ligase 1 (ssRNA Ligase)	NEB	M0204S
Phusion® High-Fidelity PCR Master Mix with HF Buffer	NEB	M0531S
Superscript II Reverse Transcriptase	Invitrogen	18064014
Deoxynucleotide (dNTP) Solution Set	NEB	N0446S
DEPC-treated water	Invitrogen	AM9906
RNaseOUT Recombinant Ribonuclease Inhibitor	Invitrogen	10777019
Gel Loading Buffer II	ThermoFisher	AM8546G
GlycoBlue™ Coprecipitant	ThermoFisher	AM9515
25-bp DNA Ladder	Invitrogen	931343
Spin-X Centrifuge Tube Filter, Sterile, 0.22 um	Fisher Scientific	07-200-385
GelStar® Nucleic Acid Gel Stain, 10,000X	Lonza	Catalog #: 50535
Adenosine 5'-Triphosphate (ATP)	NEB	P0756S
Qubit dsDNA HS Assay Kit	Invitrogen	Q32851
NEBuffer™ 2	NEB	B7002S

	A	B	C
	Magnetic beads for DNA purification	Sergi Lab Supplies	cat. no. 1040

Safety warnings

-  Gel purification entails risk of UV exposure, please ensure you use appropriate PPE (e.g. face shield, gloves, lab coat or clothes with arm and neck protection).

Before start

Purify DNA-free total RNA for all samples using desired method of choice. We use a TRIzol-based extraction with chloroform:IAA organic phase separation and isopropanol precipitation ([dx.doi.org/10.17504/protocols.io.bt8wnrx](https://doi.org/10.17504/protocols.io.bt8wnrx)).

Check out Sergi Lab Supplies for more accessible options to magnetic purification beads and magnetic separation racks for DNA/RNA (<https://sergilabsupplies.com/>).



Poly(A) selection

- 1 For each sample, dilute 5 - 30 µg total RNA in 50 µL of nuclease-free water, keeping the RNA amount as consistent as possible between samples.
- 2 Perform poly(A) RNA selection using Dynabeads mRNA purification kit as per the manufacturer's instructions (see attachments).

Prepare enough Dynabeads/Binding Buffer suspension to mix with each total RNA sample at 1:1 ratio (i.e. 50 µL per sample).

Note, the capacity of 100 µL beads is 75 µg total RNA. Since we are starting with < 32.5 µg total RNA, we scale-down the reactions by one-half to conserve reagents.

- 3 Elute your poly(A)-selected RNA in 9 µL of DEPC-treated water.
- 4 Quantify the amount of poly(A) RNA recovered (e.g. Qubit or Nanodrop) and normalize the amount to be used as input for 5' adapter ligation (e.g. ~600 ng poly(A) RNA from 30 µg total RNA).



Ligate 5' adapter

- 5 For each sample, combine the following in PCR tubes and mix by pipetting 6 times.

A	B
Reagent	Volume (µL)
25 µM RNA 5' Adapter (RA5)	1
Poly(A) selected RNA	9

- 6 Incubate at 70 °C for 2 min, then 4 °C for at least 2 min.
- 7 Prepare the following ligation mix, multiplying the volumes for the number of samples being processed (+50% for pipetting errors). Mix with gentle pipetting then briefly centrifuge to collect.



A	B
Reagent	Volume (μL)
T4 RNA Ligase buffer	1.5
10 mM ATP	1
RNaseOUT	1
T4 RNA Ligase 1	1

5' ligation recipe

- 8 Add 4.5 μL of the mix to each sample, mix by pipetting 6 times, then briefly spin down.
- 9 Incubate at 28 °C for 1 h.
- 10 Proceed directly to the next section or store RNA at -20 °C overnight (-80 °C if longer).



Poly(A) selection

- 11 Perform second poly(A) selection to remove un-ligated adapter using NEBNext Poly(A) mRNA Magnetic Isolation Module (reduced RNA input). Alternatively, you can still use Dynabeads mRNA purification kit and scale the amount of beads used accordingly. The following steps are based on the NEBNext Poly(A) mRNA Magnetic Isolation Module.
- 12 In a clean PCR tube, aliquot 20 μL of resuspended NEBNext Magnetic Oligo d(T)₂₅ Beads.
- 13 Add 100 μL RNA Binding Buffer and mix by pipetting 6 times.
- 14 Place tubes containing beads on a magnetic rack until solution is clear.
- 15 Remove supernatant without disturbing the beads.



- 16 Remove tubes from the rack and repeat steps 13 - 15.
- 17 Resuspend beads in 50 μ L RNA Binding Buffer.
- 18 Add 35 μ L DEPC-treated water to all RNA samples to bring the final volume to 50 μ L.
- 19 Combine 50 μ L of ligated RNA sample with 50 μ L beads and mix by pipetting 6 times.
- 20 Incubate at 65 $^{\circ}$ C for 5 minutes then hold at 4 $^{\circ}$ C (denature RNA and facilitate binding of the poly(A) to oligo dT beads).
- 21 Remove tubes from thermal cycler when samples reach 4 $^{\circ}$ C.
- 22 Resuspend beads by pipetting 6 times, then incubate for 5 minutes at room temperature.
- 23 Repeat step 22.
- 24 Place tubes on magnetic rack until solution clear. Poly(A) RNA is now bound to beads.
- 25 Discard all supernatant without disturbing beads.
- 26 Remove tubes from magnetic rack.
- 27 Add 200 μ L Wash Buffer and mix by pipetting 6 times.
- 28 Place tubes on the rack and wait for the solution to become clear.
- 29 Discard the supernatant then remove tubes from the rack.



- 30 Repeat wash (steps 27 - 29).
- 31 Resuspend beads in 8 μ L of Tris Buffer and mix by pipetting 6 times.
- 32 Incubate samples at 80 $^{\circ}$ C for 2 minutes, then hold at 25 $^{\circ}$ C.
- 33 Once samples reach 25 $^{\circ}$ C, immediately place PCR tubes on the magnetic rack.
- 34 Once solution is completely clear, transfer the supernatant to a clean nuclease-free PCR Tube.
- 35 Continue with reverse transcription reaction or store cDNA at -20 $^{\circ}$ C.



Reverse transcription and addition of 3' adapter

- 36 Set up the following program in a thermal cycler:
1. 65 $^{\circ}$ C for 5 min
 2. Hold at 4 $^{\circ}$ C
 3. 25 $^{\circ}$ C for 10 min
 4. 42 $^{\circ}$ C for 60 min
 5. 85 $^{\circ}$ C for 5 min
 6. Hold at 4 $^{\circ}$ C

Run the program and pause on step 1 to allow the thermal cycler to preheat.

- 37 Prepare the following in clean 200 μ L PCR tubes

A	B
Reagent	Volume (μ L)
5' adapter-ligated RNA	8
20 μ M random hexamer primer that includes TruSeq RA3 sequence on the 5' end of the ssDNA oligo (see Materials)	1



A	B
12.5 mM dNTPs	1

- 38 Mix contents of each tube by pipetting 6 times.
- 39 Place tubes in pre-heated thermal cycler and run steps 1-2, allows samples to sit at 4 °C for at least 2 minutes.
- 40 Prepare the following reverse transcription mix. Multiply volumes per sample (+50%) to account for pipetting errors. Mix gently by pipetting, then briefly centrifuge.

A	B
Reagent	Volume (uL)
DEPC-treated water	2
5x First Strand Buffer	4
100 mM DTT	2
SuperScript II	1
RNaseOUT	1

Reverse transcription recipe

- 41 Add 10 µL of the mix to each sample (keep on ice) and mix by pipetting up and down 6 times, then centrifuge briefly.
- 42 Return samples to the thermal cycler and continue with steps 3-6.

PCR amplification

- 43 Use the Illumina Index Adapter Pooling Guide (TruSeq Small RNA Library Prep Kit, see supported attachments) to select which unique RNA PCR Primer Index (RPI) will be used for each library.
- 44 Set up the following program in a thermal cycler:

1. 98 °C for 30 sec
2. 98 °C for 10 sec
3. 60 °C for 30 sec
4. 72 °C for 15 sec
5. Back to step 2 (10x = 11 cycles total)
6. 72 °C for 10 min
7. Hold at 4 °C

Run the program to allow the thermal cycler to preheat (pause on step 1).

N.b. For ideal library construction, PCR cycle number should be a compromise between over-amplification (reduces library complexity and may introduce artefacts) and under-amplification (low library yield). We aim for our PCRs to run into the early exponential phase (~11 cycles but this will vary depending on input).

- 45 Add 2 µL of a unique RPI (10 µM) to each sample and mix by pipetting 6 times.
- 46 Prepare the following PCR mix, multiplying the volumes for the number of samples (+50 % for pipetting errors). Mix by pipetting and briefly centrifuge.

	A	B
	Reagent	Volume (uL)
	2X Phusion Master Mix	50
	RNA PCR Primer 1 (RP1)	2
	1 M Betaine	26

PCR mix per library

- 47 Add 78 µL of the PCR mix to each sample and mix by pipetting up and down 6 times. Centrifuge briefly and keep samples on ice.
- 48 Distribute 100 µL across 4 PCR tubes (i.e. 4 × 25 µL reactions per sample). This is done to increase the PCR efficiency by reducing bias for shorter molecules.
- 49 Place all samples into the pre-heated thermal cycler and run the program.
- 50 Combine all 25 µL reactions into a single 1.5 mL microtube.



- 51 Library purification is required after this step to remove unwanted fragments including:
1. Large fragments that tend to cluster poorly on a flow cell
 2. Short fragments that are typically adapter-adapter clones (~135bp).



This can be done in one of two ways: gel-based or bead-based purification.

In short, while bead-based purification is less laborious, faster, and scalable, gel purification allows for greater control on fragment size selection. While both methods are robust, we recommend being consistent with the method used for any set of samples per experiment.

Gel Purification

- 52 Concentrate samples with ethanol purification
- 52.1 Add 500 μ L ethanol, 3 μ L GlycoBlue, and 10 μ L 3 M NaOAc (pH 5.5) to your PCR products.
- 52.2 Incubate samples at -80 $^{\circ}$ C for at least 2 hours.
- 52.3 Centrifuge samples at max speed for 45 min at 4 $^{\circ}$ C.
- 52.4 Remove supernatant and rinse with 750 μ L 80% ethanol.
- 52.5 Centrifuge at max speed for 5 min at 4 $^{\circ}$ C.
- 52.6 Remove supernatant without disturbing pellet and allow to air dry for 1-2 min.
- 52.7 Resuspend in 10 μ L of nuclease-free water and allow samples to resuspend for 20 minutes on ice.
- 53 Setup a 6 or 8% TBE-polyacrylamide gel (Invitrogen) in a gel running tank with 1x TBE buffer (89 mM Tris base, 89 mM boric acid, 2 mM EDTA pH 8).

Alternatively, you could run a 4-20% gradient gel to maximize the separation of smaller fragments to better eliminate adapter-adapter clones while still capturing smaller fragments.

- 54 Prepare ladder and samples:
- Dilute 2.5 μ L of 25-bp DNA Ladder (Invitrogen) with 7.5 μ L nuclease-free water. Combine with 10 μ L Gel Loading Buffer II (ThermoFisher).
- Mix 10 μ L sample with 10 μ L Gel Loading Buffer II (ThermoFisher).
- 55 Load ladder and samples onto TBE gel. If possible, leave one lane free between each sample to avoid cross-contamination.
- 56 Run gel at $\sim$ 100 V for approximately 60 minutes (dye front should be just touching the bottom of the gel).
- 57 Prepare gel breaker tubes (or nuclease-free 0.5 mL tubes) for each sample.

Use a 21G needle to make holes in the bottom of the tubes (or, for gel breaker tubes, to increase their size) and place within a nuclease-free microtube.
- 58 Once gel run has completed, incubate gel for 10 minutes with 1X GelStar Nucleic Acid Stain in 100-200 mL 1X TBE in a nuclease-free tray.
- 59 Rinse gel with distilled water 3 times then image on a UV transilluminator.
- 60 For each library, excise the region corresponding to 150 - 500 bp (can include larger fragments if there is visible product) and place inside gel breaker tubes (nested in a 2 mL microtube).

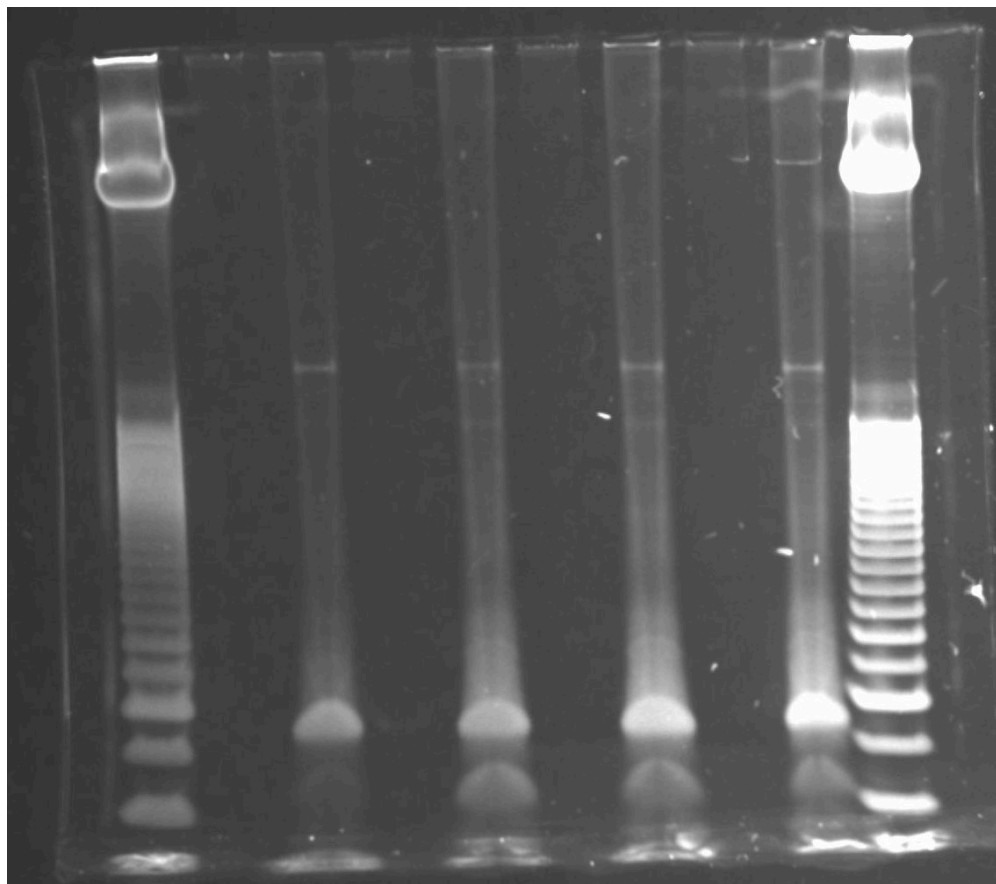


Excising regions under UV transilluminator entails the risk of exposure to UV. Ensure that you wear appropriate PPE.

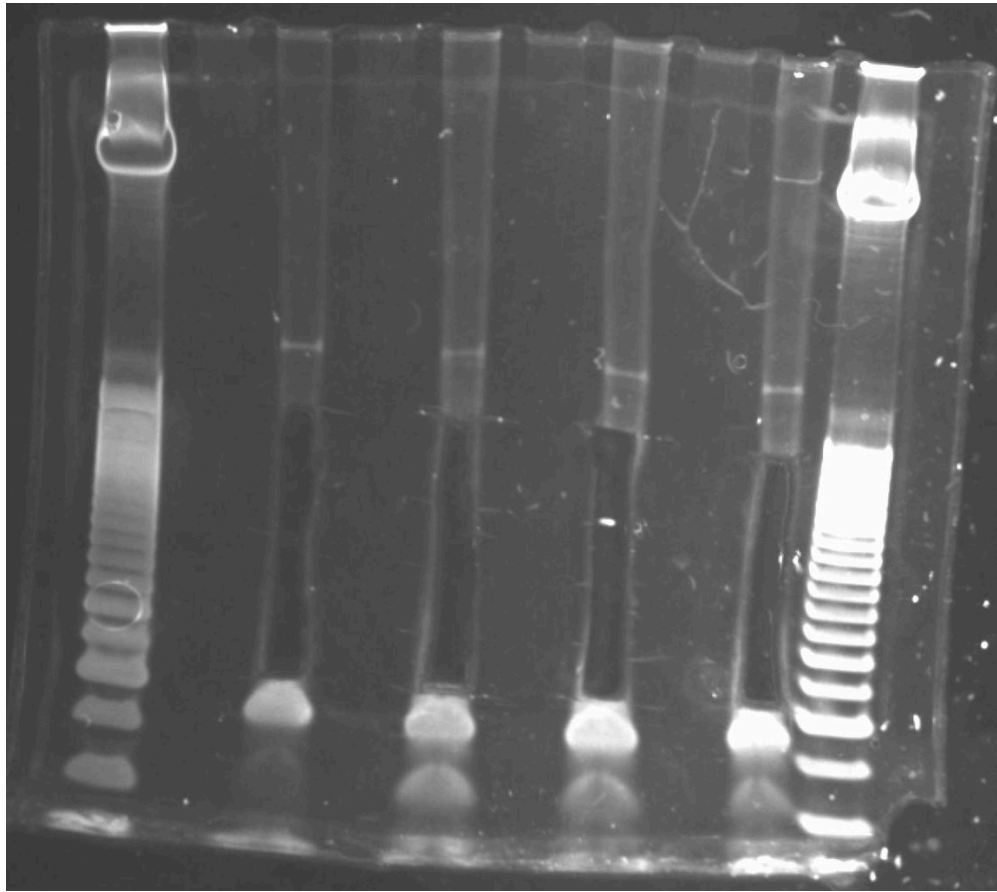
Note: a minimum size of 135 bp is recommended since this corresponds to a 17 bp insert and anything smaller becomes much more difficult to map uniquely to the genome. This also avoids adapter-adapter clones (produced from random hexamer binding to 5' adapter), the longest of which would be 118 bp. We prefer a slightly higher cut (150 bp) to minimize adapter-adapter clones.

The most important thing is to keep the excised region consistent among samples within an experiment.

61



Representative polyacrylamide gel before size selection.



Representative polyacrylamide gel after size selection.

- 62 Centrifuge at max speed for 2 minutes and ensure that the gel completely passes through the holes (repeat spin and add more holes if necessary).
- 63 Discard gel breaker tubes.
- 64 Add 300 μ L NEB Buffer 2 (50 mM NaCl, 10 mM Tris-Cl, 10 mM MgCl₂, 1 mM DTT, pH 7.9) and rotate for at least 2 hours (or overnight at 4 °C).
- 65 Pipette entire sample onto a Spin-X column, and spin at 20,000 rcf for 2 min. Ensure all liquid (300 μ L) passes through column. Repeat spin if necessary.
- 66 Precipitate RNA by adding 5 μ L GlycoBlue, 30 μ L 3 M NaOAc pH 5.5, and 900 μ L ethanol.
- 67 Incubate at -80 °C for at least 2 hours (can leave overnight to maximize recovery).



- 68 Centrifuge at max speed for 1 hour at 4 °C.
- 69 Remove supernatant and wash with 750 µL 70% ethanol.
- 70 Centrifuge at max speed for 5 min at 4 °C.
- 71 Remove as much supernatant as possible, and allow tubes to air dry for 1 -2 minutes.
- 72 Resuspend libraries in nuclease-free water or Tris-Cl (10 mM, pH 8).
- 73 Store libraries at -20 °C or proceed to library quality-control.



Bead purification

- 74 Calculate the volume of magnetic beads required to absorb the desired size range of DNA molecules. For example, we aim for our libraries to be composed of 200 - 700 bp DNA fragments.

	A	B	C
	DNA molecule size (bp)	Bead : Sample	Volume for 100 uL
	100	1.5x	150
	200	1.0x	100
	300	0.8x	80
	400	0.7x	70
	600	0.6x	60
	700	0.5x	50
	1300	0.4x	40

Bead purification ratios based on SergiLab Supplies (#1040)



- 75 Bring the magnetic bead suspension to ambient temperature and vortex thoroughly until homogenous.
- 76 Add volume of magnetic beads to precipitate large DNA fragments (e.g. 0.5X) to sample and mix by pipetting 6 times.
- 77 Incubate at room temperature for 10 minutes.
- 78 Place tube on magnetic rack and allow solution to become clear.
- 79 **Retain supernatant** in new microtubes. The supernatant contains DNA smaller than 700 bp.
- 80 Discard tubes containing beads (DNA > 700 bp).
- 81 To the supernatant, add magnetic bead suspension *up to 1X original* sample volume.

For example, if 50 µL was added at step 76 (0.5X ratio), and the original sample volume was 100 µL, then add 50 µL here.
- 82 Mix by pipetting at least 6 times.
- 83 Incubate at room temperature for 10 minutes. This time, short DNA (<200 bp) remains in solution.
- 84 Place the sample onto the magnetic rack and wait for the solution to clear.
- 85 Discard supernatant and **retain beads**. DNA of size 200 - 700 bp should be precipitated onto beads.
- 86 Add 180 (0.2 mL PCR tubes) or 250 (1.5 mL microtube) µL of 80 % (v/v) ethanol and mix by pipetting 6 times.
- 87 Place tube on magnetic rack and allow solution to become clear.



- 88 Discard supernatant and repeat wash.
- 89 Discard supernatant (use p20 to remove as much ethanol as possible) and air-dry for 1 minute. Do not allow beads to dry completely as this will reduce DNA yield.
- 90 Resuspend beads thoroughly in 13 μ L nuclease-free water or Tris-Cl (10 mM, pH 8).
- 91 Incubate at ambient temperature for 10 minutes.
- 92 Place tubes on magnetic rack and wait for solution to become clear.
- 93 Transfer supernatant into a new clean tube. Be sure to avoid any bead carryover.
- 94 Store libraries at -20 °C.

Library quality-control

- 95 Quantify library concentration using a Qubit fluorometer with Qubit dsDNA HS Assay Kit (Invitrogen).
- 96 If available, inspect libraries on a LabChip GXII or Bioanalyzer to give information on fragment size distribution and molarity.
- 97 Pool libraries equal molar and submit to sequencing facility for Illumina-based sequencing.

Protocol references

M. R. Willmann, N. D. Berkowitz, B. D. Gregory, Improved genome-wide mapping of uncapped and cleaved transcripts in eukaryotes—GMUCT 2.0. *Methods*. **67**, 64–73 (2014).