Mello Lab Small RNA Cloning Protocol
(Edited by Weifeng Gu and Darryl Conte)

We are continually trying to improve the protocol and will update it as necessary. Please let us know if you find inconsistencies and/or problems with the protocol. Also, if you improve on methods or find an easier way to do something, we'd love to hear about it so that we can amend/append this protocol and share it with others…

I. Purification of 18-24nt RNA

Prepare total RNA from **fresh** worms using Trizol or equivalent, in order to obtain RNA of high quality with a minimum contamination of degraded RNA. To get rid of contamination from *E coli* RNA and to remove free eggs and newly hatched L1 worms, vortex worms with 1X M9 buffer, allow them to settle down by gravity for 5 min, and aspirate the supernatant. Repeat this process several times until only clean adult worms are left. Extract RNA with Trizol using a metal douncer according to the Trizol instruction. A phase-lock column could help recover all the supernatant and reduce protein contamination during extraction. Note: Since the supernatant is not totally free of protein contamination with only one Trizol extraction (a second phenol extraction may be preferred if necessary), we usually resuspend total RNA in TE buffer (10 mM Tris-Cl, pH 7.5, 1 mM EDTA) at 10-20 µg/µl, and store at -20°C until ready to prepare small RNA. A trick to dissolve RNA pellet at such a high concentration is to grind the RNA pellet against the eppendorf wall using a tip and pipet several times with TE buffer.

A. Purify small RNA from total RNA using MirVana (Ambion)

Note: We have modified the MirVana protocol to eliminate the columns, which we find is more convenient and has a comparable yield. This modification also allows ~200 samples to be purified with a single MirVana kit. The columns can be saved for other purposes. You can finish all the steps below in a single 1.5 ml eppendorf tube at **room temperature**.

Total RNA	80µl (~1mg)
MirVana lysis/binding buffer	400µl
MirVana Homogenate buffer	48µl

Mix well and incubate at RT for 5 min to denature RNA

Add 1/3 vol of 100% ethanol and mix well 176µl

Spin at 5000 rpm or 2500 x g for 4 min at RT to pellet large (>200nt) RNA

Transfer the supernatant to a new eppendorf tube and fill it with isopropanol (~700µl)

Precipitate at -70°C until it is frozen (~10 min) or at -20°C 30 min.

Pellet small RNA at 20,000 x g at 4°C for at least 10 minutes

Wash once with 70% cold ethanol

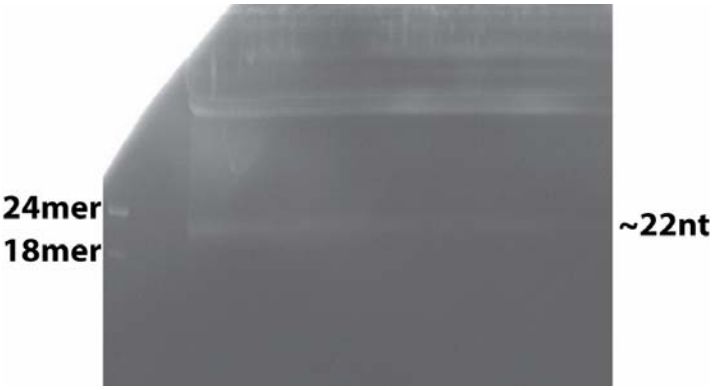
Dissolve with TE if your only purpose is to further purify small RNA by PAGE

The small RNA yield is typically ~8% of total RNA

B. Fractionate small RNA on a 15% PAGE (19:1 or 29:1) / 7M urea

Starting with ~400µg of mirVana purified small RNA, I usually obtain ~1µg of ~18-24nt RNA.

> **Note:** A thinner PAGE gel (e.g. 0.8mm) with a 3-4 inch well will have better resolution at the ~22nt position while providing enough capacity for separating ~ 400 µg small RNA. Run the sample with 18mer and 24 mer RNA marker until the bromophenol blue migrates ~3 inches. Usually we are able to see a 22nt small RNA band from 8µg of small RNA on PAGE visualized by EtBr staining. A destaining process to remove the background staining will help obtain a good picture. Sometimes, I need to use short wavelength UV to visualize the band. Here we can also check the quality of purified small RNA. No more apparent bands than ~22mer RNA would be visualized if the RNA quality is good in *C. elegans*. A high quality small RNA is critical for preparing a cDNA library with a minimum contamination of degraded RNA (usually rRNA or tRNA).



Excise small ~18-24nt RNA.

Place the gel fragments into 1.5 or 2mL tubes and grind them using a 200µl pipet tip.

Add at least 2 gel volumes of 0.3M NaCl-TE (pH7.5) buffer and tumble overnight at RT

> **Note:** Using siliconized tubes during elution and precipitation may increase yield. A double elution will recover almost all the small RNA (not necessary).

Transfer eluted small RNA to fresh tubes (up to 700µl per tube).

Add 10µg glycogen and at least 1 volume of isopropanol.

Precipitate purified small RNA at -80°C until frozen or -20°C for 30 min.

Wash once with 70% Ethanol.

Resuspend pellets in 10µl H_2O or 10mM Tris-Cl (pH7.5).

II. For cloning 5' triphosphate small RNAs: Removal of miRNA, degraded RNA, and other RNAs

Skip this step if you are cloning miRNA or any RNA with 5' monophosphate

Treat small RNAs with polynucleotide kinase to ensure small RNAs have at least a 5'-monophosphate.

PNK reaction:

	Stock	Final	40 µl/RxN
H_2O			22.4 µl
T4 PNK buffer	10 X	1 X	4 µl
0.5 µg small RNA			9 µl
Superasin (Ambion)	20 U/µl	0.5 U/µl	1 µl
ATP	10 mM	0.4 mM	1.6 µl
T4 PNK	10 U/µl	0.5 U/µl	2 µl

Incubate 37°C for 1 hr

Add 60µl H2O and extract once with Phenol/Chloroform (We use phase-lock columns to recover entire aqueous phase)

Precipitate with at least 3 volumes of 100% Ethanol

Wash once with 70% Ethanol

Resuspend pellet in 44.6µl H_2O

Treat sample with Terminator exonuclease to remove small RNAs with 5' monophosphate:

	Stock	Final	50 ul/RxN
Small RNA			44.6 ul
Buffer	10 X	1 X	5 ul
Terminator Exonuclease	1 U/ul	0.008 U/ul	0.4 ul

Note: Terminator Exonuclease is available from Epicentre Biotechnologies (Cat# TER51020). Do not use more than1 Unit per µg of small RNA, since this enzyme may also degrade triphosphorylated RNA, albeit less efficiently. Less exonuclease can be used if desired (such as 0.2 µl here).

Incubate 30°C for 30-45 min

Add 50µl H_2O and extract once with Phenol/Chloroform

Precipitate with at least 3 volumes of 100% Ethanol

Wash once with 70% Ethanol

Resuspend triphosphate/5' modified small RNA in 5µl of H_2O

Store at -20°C.

III. 3' adapter ligation

10X Ligation Buffer

	Stock	Final	50 µl
Tris-Cl pH7.5	1 M	0.5 M	25 µl
MgCl2	1 M	0.1 M	5 µl
DTT	1 M	0.1 M	5 µl
BSA	10 mg/ml	0.6 mg/ml	3 µl
H2O			12 µl

Ligation reaction

	Stock	Final	10 µl RxN
Buffer	10 X	1 X	1 µl
DMSO (optional)	100%	10%	1 µl
Superasin (Ambion)	20 U/µl	1 U/µl	0.5 µl
Small RNA			4.5 µl
RNA 3' linker	100 µM	20 µM	2 µl
T4 RNA ligase	30 U/µl	3 U/µl	1 µl

Control ligation of small RNA markers (serve as size standards):

	Stock	Final	5 µl RxN
Buffer	10 X	1 X	0.5 µl
Superasin (Ambion)	20 U/ul	1 U/ul	0.25 µl
RNA oligo (18- or 24-mer)	10 µM	2 µM	1 µl
RNA 3' linker	100 uM	5 uM	0.25 µl
T4 RNA ligase	30 U/µl	3 U/µl	0.5 µl
H2O			2.5 µl

We use 18nt and 24nt RNA oligos synthesized by IDT as size standards and ligate them separately.

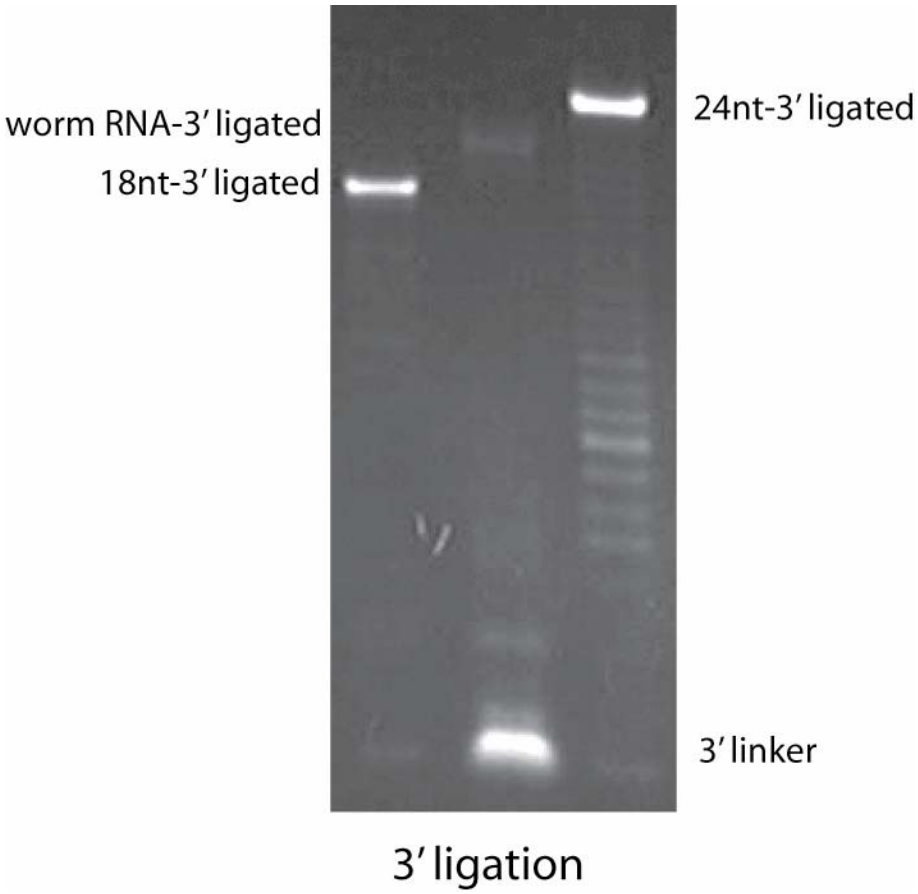
Incubate ligations at 20°C for 2 hr and then 4°C overnight in a PCR machine.

Fractionate the “small RNA – 3' adapter” ligation product on a 15% polyacrylamide / 7M urea gel. (In my case, I can easily see the ligated product using ethidium bromide staining.) Sometimes we used radio-labeled 18nt and 24nt RNA to follow the reaction in which part of the sample RNAs (~10%-20%) are dephosphorylated using CIP, phosphorylated using PNK and radio-labeled ATP, and mixed with unlabeled sample RNA (the rest 90% to 80%).

Excise the ligated product, crush the gel slice in an eppendorf and elute overnight, as above.

If cloning miRNAs or piRNAs, proceed to section VIII.

Alternatively, to clone a more diverse population of small RNAs including miRNAs, proceed directly to IV. This will also eliminate a gel purification step.



IV. 1st strand cDNA synthesis

Here we use invitrogen superscript II (Catalog No.11904-018) to synthesize 1st strand cDNA.

Cocktail	Stock conc.	Final conc.	22 µl/RxN
RNA-3' linker pellet			0 µl
3' RT oligo	10 µM	0.25 µm	0.55 µl
H2O			11.2 µl

Incubate 5 min at 65°C

Incubate on ice for 2 min

Add the following components on ice:

CMo13277 oligo	10 µM	0.25 µM	0.55 µl
First strand buffer	5 X	1 X	4.4 µl
DTT	100 mM	10 mM	2.2 µl
dNTP	10 mM	0.5 mM	1.1 µl
RNaseout	20 U/µl	1 U/µl	1.1 µl

Note: CMo13277 is the 5' primer for the 2nd strand cDNA synthesis. Superscript II has terminal transferase activity, adding three un-templated dC nucleotides to the first-strand cDNA.

CMo13277 containing dGdGdG at its 3' end will anneal to the terminal dC residues on the first strand, thus providing a template to further extend the first-strand cDNA according to the 5' adaptor sequence. It will also serve as a primer for concurrent second-strand cDNA synthesis.

Incubate at 42°C for 2 min (not necessary, but recommended by the instruction).

Split samples into 2 reactions:

RxNs		Superscript II
1 (+RT)	19 µl	1 µl
2 (-RT)	2 µl	

Incubate at 42°C for 1 hr

Inactivate at 85°C for 5 min

Add 1 and 0.1 µl RNase H to the +RT and –RT reactions respectively

Incubate at 37 °C for 10 min

V. cDNA Amplification

1st round

Shorter oligos are used during the 1st round PCR to reduce primer dimmer formation that seems to be an issue with the longer primers under our PCR condition using ExTaq (TaKaRa catalog No. RR001A). Primers containing the full Illumina adapter sequences are added in the 2nd round PCR. Other enzymes such as Phusion (NEB) or iProof (?) may help as well, because the annealing temp will be higher than for ExTaq.

	Stock	Final	50 ul/RxN
H2O			37.9 µl
Buffer	10 X	1 X	5 µl
RT primer	10 µM	0.05 µM	0.25 µl
CMo13277	10 µM	0.05 µM	0.25 µl
cDNA Template			2 µl
dNTP	2.5 mM	0.2 mM	4 µl
ExTaq	2.5 U/µl	0.03 U/µl	0.6 µl

Reaction conditions:

1 cycle	94°C	30sec
8 cycles	94°C	15sec
	50°C	15sec
	72°C	15sec
1 cycle	4°C	hold

Note:

1. You may use 1µl of the –RT control template in a 25 µl Rxn in case you need to repeat it.
2. Depending on the total PCR cycles including the 1st and 2nd rounds to obtain a clear band on a gel, you may want to increase the 1st round PCR No. to 15 or even 20. If the1st round No. is less than 15, the above primer concentration will work. Adjust the primer concentration to 0.1-0.2 µM, if the 1st round No. is more than 20.

2nd round (addition of full Illumina adapter)

Add the following components to the above reaction:

	Stock	Final	10 µl/RxN
H2O			4 µl
Buffer	10 X	1 X	1 µl
CMo13278	10 µM	2.5 µM	2.5 µl
CMo13170	10 µM	2.5 µM	2.5 µl

Reaction conditions:

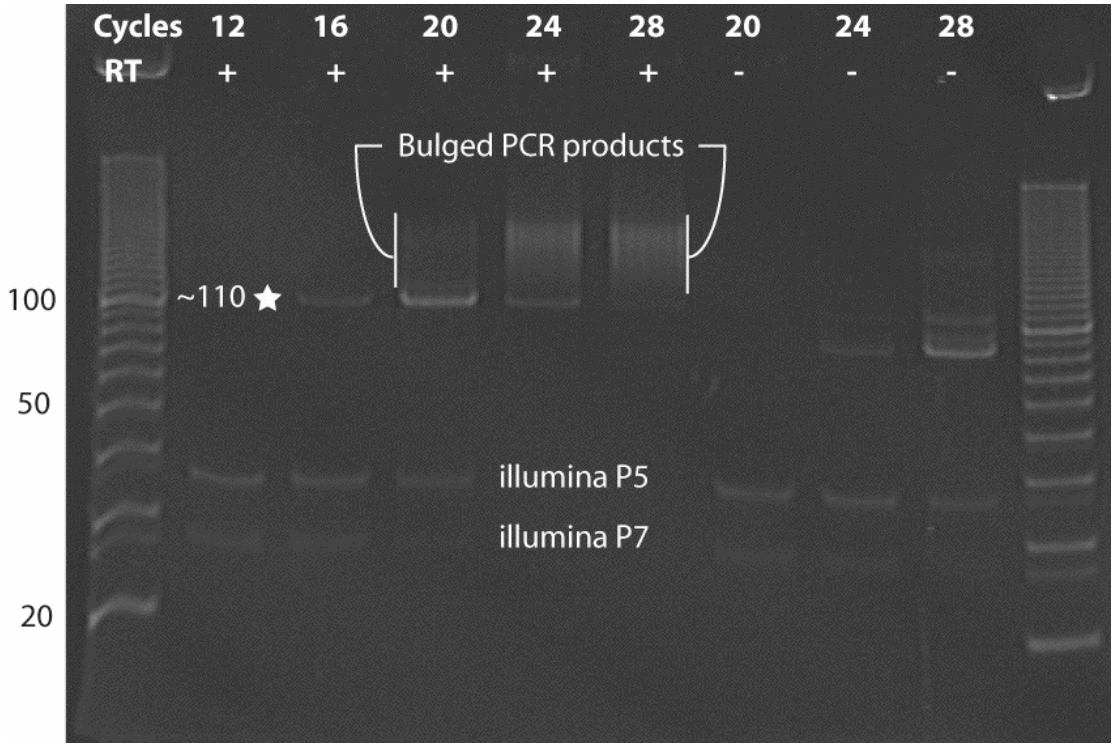
1 cycle	94°C	30sec
20 cycles	94°C	15sec
	50°C	15sec
	72°C	15sec
1 cycle	4°C	hold

During the 2nd round PCR, remove 3µl of the reaction at cycles 4, 8, 12, 16, and 20 to analyze on a non-denaturing 10% polyacrylamide gel (29:1).

Stain and photograph the products to determine the optimal number of cycles.

To obtain the final PCR products which are going to be loaded to Solexa machine, prepare 3 x 50 µl PCR reaction but double the +RT template concentration, and amplify the reaction at the determined cycle number **minus 1**.

The example gel below shows a specific product that was detected at total cycle 16 (ie cycle 8 of the second round). With increasing cycles, primers begin to deplete and bulged products accumulate because products denature and anneal at the adapters but not in the middle.



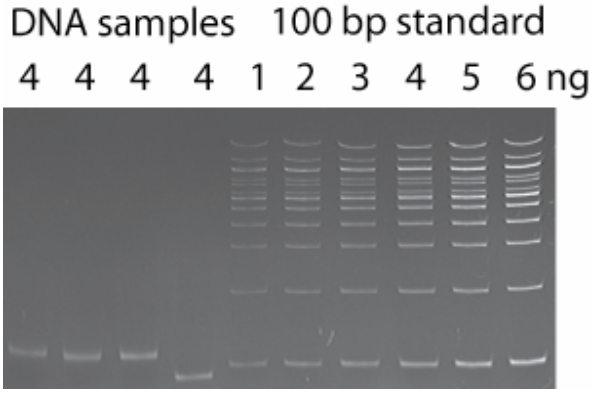
VI. Final purification steps, quality control and quantification

The 150 µl PCR product is concentrated to ~50 µl using a Microcon YM-10 (Millipore catalog No. 42407), mixed with 10 µl 6X DNA loading buffer, and then loaded to a 10% **native** PAGE gel (29:1), ~1mm thick with 30 mm or bigger wells. You may concentrate DNA by precipitation or using a Qiagen PCR purification kit. However, do every step quickly and never allow the DNA to dry or expose to air for long time.

Run the gel until the xylene cyanol FF dye is 10 mm away from the front edge of the gel, stain the gel and cut the DNA band at ~ 110 position.

Elute in 500 µl buffer using the same protocol as described above. Concentrate the elute to ~100 µl using YM-10, and purify DNA using a Qiagen PCR purification kit. During PCR purification, finish every centrifugation step within 30 second and elute immediately using 30 µl of Tris (10 mM, pH 8.0).

You may quantify the DNA concentration using a NanoDrop machine. However, the reading is not accurate if it is below 10 ng/µl. Even if it is above 20 ng/µl, you still need to quantify the concentration using a 10 % PAGE gel and a DNA standard (100bp marker from NEB, catalog No. N3231S). Below is a quantification gel in which each lane contains a determined amount of DNA at 110 or 90 (DNA samples) or 100 nt (100bp standard) position.



DNA quantification using PAGE

VII Oligonucleotides:

3' End Adaptor (modban): (miRNA Cloning Linker-1 from IDT)
AppCTGTAGGCACCATCAAT/ddC/

RT DNA oligo
ATTGATGGTGCCTACAG

CMo13277 DNA oligo
TCTACAGTCCGACGATCGGG

CMo13279 DNA oligo
GTTCTACAGTCCGACGATC

CMo13278 DNA oligo [5' PCR primer (Illumina P5)]
AATGATACGGCGACCACCGACAGGTTCAGAGTTCTACAGTCCGACGATC

Cmo13170 DNA oligo [3' PCR Primer (P7-modban)]
CAAGCAGAAGACGGCATACGAATTGATGGTGCCTACAG

Clones should have the following sequence structure:

5'-AATGATACGGCGACCACCGACAGGTTCAGAGTTCTACAGTCCGACGATCGGG

...small_RNA…

CTGTAGGCACCATCAATTCGTATGCCGTCTTCTGCTTG-3'

VIII. 5' adapter ligation – for cloning miRNAs and piRNAs

After 3' ligation and gel purification as described above for 5' ligation-independent cloning, we carry out the following 5' ligation.

Ligation reaction

	Stock	Final	20 µl RxN
Buffer with ATP	10 X	1 X	2 µl
Superasin (Ambion)	20 U/µl	1 U/µl	1 µl
BSA	1 µg/µl	0.06 µg/µl	1.2 µl
Small RNA-3'ligated			12.8 µl
RNA 5' linker	100 µM	10 µM	2 µl
T4 RNA ligase	30 U/µl	1.5 U/µl	1 µl

Incubate ligations at 20°C for 2 hr and then 4°C overnight.

Fractionate the ligated product using a 15% polyacrylamide / 7M urea gel. (In my case, I can easily see the ligated product using ethidium bromide staining.). You may follow the position using 18mer and 24mer RNA standards, which are processed the same way as for the sample RNA. Alternatively, you can follow the position by radio-labelling or single strand RNA standard.

Excise the ligation product, crush the gel slice in an eppendorf and elute overnight, as above.

1st cDNA sythesis

The same as above, but only with the 3' RT primer (without CMo13277) and with superscript III instead of superscript II. You can use a higher concentration of 3' RT primer, e.g. 0.5 µM or even higher. Please follow the instructions or determine it empirically.

PCR

In the 1st round PCR, use the 3' RT primer and **CMo13279.**
Others are all the same as above.

IX Using Solexa kit to do the 5' ligation dependent cloning

We followed the protocol as described in the instruction. However here we want to point out a few differences between our own protocols and Soxlexa protocol:
1. The PCR product is ~90 nt since the 3' end adaptor (modban) is not used to do the 3' ligation. Instead, CAAGCAGAAGACGGCATACGA (the rest of CMo13170) is ligated to the 3' end of sample RNA. Therefore, the 3' ligation is not efficient.
2. RNA is first ligated at 5' end and then at 3' end. Ligated RNA is purified according to a RNA size marker.
3. We also followed the RT-PCR conditions.