

# **Mello Lab Small RNA Cloning Protocol**

## **[a la Tuschl]**

**Edited by  
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### **Some notes before starting:**

**We have more or less retired the ligation-independent protocol that we initially posted on Nemo's website. Clontech's Powerscript enzyme is no longer available (due to patent issues) and we found that Invitrogen's Superscript II enzyme showed a very strong bias against full length miRNAs and piRNAs (for technical reasons that we will not get into here – if you really want to know you feel free to ask). To resolve this issue, we are using the approach that Tom Tuschl's lab uses: first dephosphorylate the gel purified small RNA, ligate an activated 3'linker, phosphorylate the small RNA/3'linker products and then ligate the 5'linker. The profile of these libraries is more representative of the actual population of small RNA in the sample as determined by molecular and biochemical analyses. Therefore, to help validate your data, we recommend looking at the 5'nt distribution of the gel purified small RNA sample biochemically (i.e. thin-layer chromatography). Provided your sample is not too limiting (we know that all samples are precious), it doesn't hurt to do Northern blot or other analyses to check sample quality as well. It can be difficult to ascertain the actual degree of degradation in the small RNA range. Furthermore, different organisms (or tissues/cell lines) have different banding patterns – get to know your organism. For worms, we know that two bands are visible at 21 and 22nt (piRNAs and siRNAs, respectively). Considerable smearing from 30nt down over these bands, making them difficult to see, is a likely indication that there is a lot of degradation. The amount of degradation should be apparent when you clone and sequence a portion of the library for validation. Hopefully, you won't have to go through the entire procedure to find out that you cloned 75 to 80% tRNA, rRNA or snRNA degradation product. Even so, 20% of your library can mean ~1 million useful reads (a lot of data) if your library is prepared well; that is: most, if not all, of the amplicons have inserts with the correct linkers and it is quantified carefully.**

**We are continually trying to improve this protocol and will update it periodically, 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.**

**As always, if you have particular questions, please don't hesitate to ask!**

### **Thanks to:**

**Pedro Batista and Julie Claycomb for input and experience  
Jim Carrington's lab for initial protocols and suggestions for Solexa sequencing  
Ellie and CFAR for patience and suggestions  
NEMO for great data...and lots of it!**

**Please read through sections prior to starting.**

We have successfully cloned small RNAs from <20ng enriched small RNA starting material, which is mostly structural RNA. If working with small amounts of material, you can use smaller wells for gel purification (i.e. do not use a prep well for initial gel purification).

## **I. Purification of 18-30nt RNA from *C. elegans***

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 may not be totally free of protein contamination with only one Trizol extraction, a second phenol extraction may be performed if necessary. We usually resuspend total RNA in TE buffer (10 mM Tris-Cl, pH 7.5, 1 mM EDTA) at 10-20  $\mu\text{g}/\mu\text{l}$ , and store at  $-20^{\circ}\text{C}$  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.

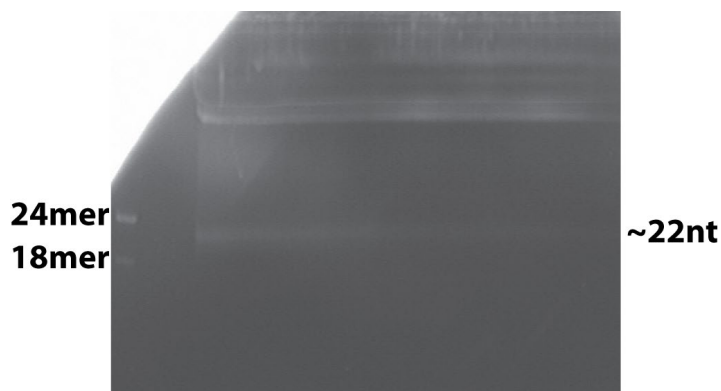
Total RNA	80 $\mu\text{l}$ ( $\leq 1\text{mg}$ )
MirVana lysis/binding buffer	400 $\mu\text{l}$
MirVana Homogenate buffer	48 $\mu\text{l}$
Mix well and incubate at RT for 5 min to denature RNA	
Add 1/3 vol of 100% ethanol and mix well	176 $\mu\text{l}$
Spin at 5000 rpm or 2500 x g for 4 min at RT to pellet large (>200nt) RNA	
Transfer the supernatant to a new eppendorf tube and fill it with isopropanol (~700 $\mu\text{l}$ )	
Precipitate at $-70^{\circ}\text{C}$ until it is frozen (~10 min) or at $-20^{\circ}\text{C}$ 30 min.	
Pellet small RNA at 20,000 x g at $4^{\circ}\text{C}$ 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*.

\* **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 (up to 700µl) 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).

Filter eluate through 100K Nanosep filters at 5000xg for 1-2min (or as long as 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<sub>2</sub>O.

## II. Dephosphorylate small RNAs

	Stock	Final	10 $\mu$ l RxN
Buffer 3	10 X	1 X	1 $\mu$ l
Supersasin (Ambion)	20 U/ $\mu$ l	1 U/ $\mu$ l	1 $\mu$ l
RNA (? ng)			7 $\mu$ l
CIP	10 U/ $\mu$ l	1 U/ $\mu$ l	1 $\mu$ l

Incubate reactions at 37°C for 1hr.

Bring volume to 100 $\mu$ l.

Phenol extract twice. Again Phase-lock columns are very helpful here.

**Note:** We usually reserve a small portion (5-10 $\mu$ l) of this dephosphorylated small RNA for TLC (thin-layer chromatography) analysis to examine the distribution of 5'nts in the small RNA sample and beta-elimination to determine whether a portion of small RNA is blocked at the 3'end. The result of TLC analysis should be consistent with the distribution of 5'nt among the sequence reads and serves as a nice validation of sample.

Precipitate with 4 volumes of ethanol -20°C for 30min.

Use dry pellet for ligation.

## III. 3' adapter ligation

### 10X Ligation Buffer – Prepare fresh

	Stock	Final	50 $\mu$ l
Tris-Cl pH7.5	1 M	0.5 M	25 $\mu$ l
MgCl <sub>2</sub>	1 M	0.1 M	5 $\mu$ l
DTT	1 M	0.1 M	5 $\mu$ l
H <sub>2</sub> O			12 $\mu$ l

### Ligation reaction

Prepare enough reaction mix for N+1 small RNA samples.

	Stock	Final	10 $\mu$ l RxN
Buffer	10 X	1 X	1 $\mu$ l
Supersasin (Ambion)	20 U/ $\mu$ l	1 U/ $\mu$ l	0.5 $\mu$ l
BSA	1 mg/ml	0.1 mg/ml	1 $\mu$ l
T4 RNA ligase	30 U/ $\mu$ l	3 U/ $\mu$ l	1 $\mu$ l
modban	100 $\mu$ M	10 $\mu$ M	1 $\mu$ l
DMSO	100 %	10 %	1 $\mu$ l
H <sub>2</sub> O			4.5 $\mu$ l

Resuspend RNA pellets in the ligation reaction mixture, pipetting up and down and rinsing wall of tube.

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

	<b>Stock</b>	<b>Final</b>	<b>10 <math>\mu</math>l RxN</b>
<b>Buffer</b>	10 X	1 X	0.5 $\mu$ l
<b>Supersasin (Ambion)</b>	20 U/ $\mu$ l	1 U/ $\mu$ l	0.5 $\mu$ l
<b>RNA oligo (18-, 24- or 26-mer)</b>	10 $\mu$ M	2 $\mu$ M	2 $\mu$ l
<b>modban</b>	100 $\mu$ M	10 $\mu$ M	0.5 $\mu$ l
<b>T4 RNA ligase</b>	30 U/ $\mu$ l	1.5 U/ $\mu$ l	0.5 $\mu$ l
<b>DMSO</b>	100 %	10 %	1 $\mu$ l
<b>H2O</b>			3.5 $\mu$ l

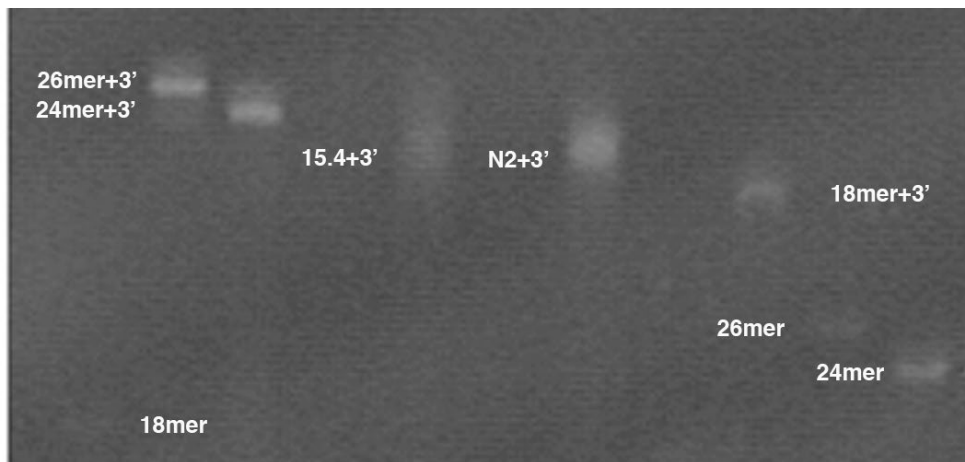
We use 18nt, 24nt and/or 26nt RNA oligos synthesized by IDT as size standards and ligate them separately.

Incubate ligations at 15°C for 2hr and then 4°C overnight in a PCR machine.

**Note:** We do a minimum 8hr ligation, but the enzyme is probably not very active after several hours.

**Note:** This is a good time to do the TLC and the beta-elimination analyses.

Fractionate the “small RNA – 3' adapter” ligation product on a 15% polyacrylamide / 7M urea gel. In this case, the ligated products were easily visualized by ethidium bromide staining. Cyber green can also be used. The size standards RNA should be visible and can be used to follow the reaction and as a point of reference. Alternatively, part of the sample RNAs (~10%-20%) can be dephosphorylated using CIP and then phosphorylated using PNK and radio-labeled ATP, and mixed with remaining unlabeled sample RNA.



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

Filter eluate through 0.22 $\mu$  Cellulose Acetate filter at 5000xg for 1min (or as long as necessary) to remove acrylamide pieces.

Transfer eluted small RNA to fresh tubes (up to 700 $\mu$ l per tube).

Add 10 $\mu$ g 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 and dry pellet. Use dry pellet for phosphorylation.

## IV. Phosphorylation with PNK

	Stock	Final	20 $\mu$ l RxN
Buffer	10 X	1 X	2 $\mu$ l
Supersasin (Ambion)	20 U/ $\mu$ l	1 U/ $\mu$ l	1 $\mu$ l
ATP	50 mM	2 mM	0.8 $\mu$ l
PNK	10 U/ $\mu$ l	1 U/ $\mu$ l	2 $\mu$ l
H <sub>2</sub> O			14.2 $\mu$ l

Resuspend each sample pellet in 20 $\mu$ l of reaction mix.

Incubate reactions at 37°C for 1hr.

Bring volume to 100 $\mu$ l.

Phenol extract once.

Precipitate with 4 volumes of ethanol -20°C for 30min.

Use dry pellet for ligation

## V. 5' adapter ligation

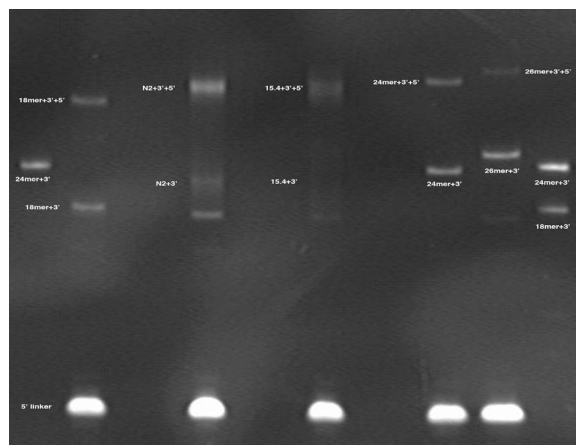
### Ligation reaction

	Stock	Final	10 $\mu$ l RxN
Buffer with ATP	10 X	1 X	1 $\mu$ l
Supersasin (Ambion)	20 U/ $\mu$ l	1 U/ $\mu$ l	0.5 $\mu$ l
BSA	1 $\mu$ g/ $\mu$ l	0.1 $\mu$ g/ $\mu$ l	1 $\mu$ l
T4 RNA ligase	30 U/ $\mu$ l	3 U/ $\mu$ l	1 $\mu$ l
CMo13281	75 $\mu$ M	30 $\mu$ M	4 $\mu$ l
DMSO			1 $\mu$ l
H <sub>2</sub> O			1.5 $\mu$ l

Incubate ligations at 15°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 RNA standards, which are processed in parallel.

Stain gel using Ethidium bromide or Cyber green.



Excise the ligation product, crush the gel slice in an eppendorf and elute overnight, as above.  
 Filter eluate through 0.22 $\mu$  Cellulose Acetate filter at 5000xg for 1min (or as long as necessary) to remove acrylamide pieces.  
 Transfer eluted small RNA to fresh tubes (up to 700 $\mu$ l per tube).  
 Add 10 $\mu$ g 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 10 $\mu$ l H<sub>2</sub>O.

## VI. cDNA synthesis

Cocktail	Stock conc.	Final conc.	22 $\mu$ l/RxN
RNA			5 $\mu$ l
RT DNA oligo	100 $\mu$ M	2.5 $\mu$ M	0.55 $\mu$ l
dNTP	10 mM	0.5 mM	1.1 $\mu$ l
H <sub>2</sub> O			6.65 $\mu$ l

Incubate 5 min at 65°C

Incubate on ice for 2 min

Add the following components on ice:

Buffer	5 X	1 X	4.4 $\mu$ l
DTT	100 mM	10 mM	2.2 $\mu$ l
RNaseOut	20 U/ $\mu$ l	1 U/ $\mu$ l	1.1 $\mu$ l

Split samples into 2 reactions:

RxNs		Superscript III
1 (+RT)	19 $\mu$ l	1 $\mu$ l
2 (-RT)	2 $\mu$ l	

Incubate at 50°C for 1 hr

Inactivate at 85°C for 5 min

Add 1 and 0.1  $\mu$ l RNase H to the +RT and -RT reactions respectively

Incubate at 37°C for 20 min

## VII. Amplify Solexa amplicons

### 1<sup>st</sup> round

Shorter oligos are used during the 1<sup>st</sup> round PCR to reduce primer dimer 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 2<sup>nd</sup> round PCR. Other enzymes such as Phusion (NEB) or iProof (?) may help as well, because the annealing temp will be higher than for ExTaq.

Only the 5' oligo needs to be added for the first round, since there is excess RT oligo in cDNA reaction.

	Stock	Final	50 ul/RxN
H <sub>2</sub> O			37.65 µl
Buffer	10 X	1 X	5 µl
CMo13279	10 µM	0.15 µM	0.75 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
6 cycles	94°C	20sec
	50°C	20sec
	72°C	20sec
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 1<sup>st</sup> and 2<sup>nd</sup> rounds to obtain a clear band on a gel, you may want to increase the 1<sup>st</sup> round PCR No. to 15 or even 20. If the 1<sup>st</sup> round No. is less than 15, the above primer concentration will work. Adjust the primer concentration to 0.1-0.2 µM, if the 1<sup>st</sup> round No. is more than 20.

### 2<sup>nd</sup> round (addition of full Illumina adapter)

Add the following components to the above reaction:

	Stock	Final	10 µl/RxN
H <sub>2</sub> O			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:

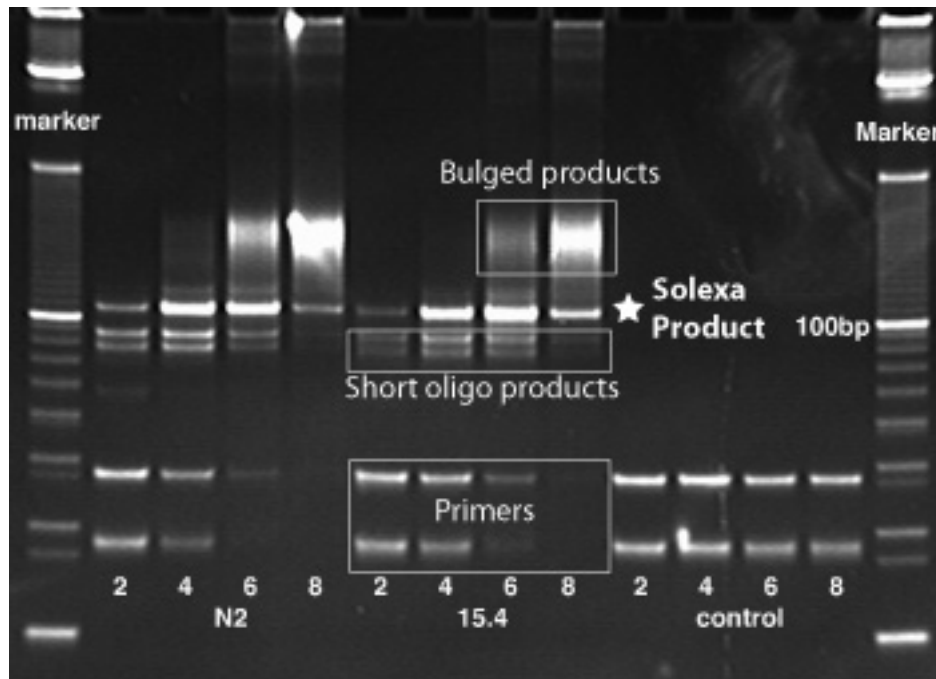
1 cycle	94°C	30sec
8 cycles	94°C	20sec
	50°C	20sec
	72°C	20sec
1 cycle	4°C	hold



During the 2<sup>nd</sup> round PCR, remove 4µl of the reaction after cycles 2, 4, 6, and 8. The reactions are short so you can stay by the machine, pause the PCR program at the end of each desired cycle, remove all tubes and place on ice while you take each sample for analysis. Place all tubes back into PCR machine and resume cycling.

Analyze on a non-denaturing 10% polyacrylamide gel (29:1), stain and photograph the products to determine the optimal number of cycles.

The example gel shows a specific product that was detected at total cycle 8 (ie cycle 2 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.



To obtain the final PCR products which are going to be loaded to Solexa machine:

Prepare 4 x 50µl PCR reactions and amplify at the determined total cycle number. For example, the samples in the experiment above were amplified for 10 cycles total.

## VIII. Final purification steps, quality control and quantification

Extract the 200 $\mu$ l (pooled) PCR product once with Phenol::Chloroform and spin in a phase-lock column.

**Note: Perform the remaining steps as quickly as possible and never allow the DNA to dry or expose to air for long time as this may result in bulged products from denatured and re-annealed amplicons making final quantification difficult.**

Concentrate the aqueous phase to  $\sim$ 50 $\mu$ l using a Microcon YM-10 (Millipore catalog No. 42407) at 4°C at 12,000xg for 20min.

Mix concentrated amplicon(s) with 10 $\mu$ l 6X DNA loading buffer and load onto a 10% **native** PAGE gel (29:1),  $\sim$ 1mm thick with 30mm or bigger wells.

Run the gel until the Xylene Cyanol FF dye is 10mm away from the bottom of the gel (10cm Pharmacia gel), stain the gel and isolate the DNA band at  $\sim$ 110bp position.

Elute in 750 $\mu$ l buffer O/N. **Crush one gel slice at a time and immediately add elution buffer before proceeding to the next sample.**

Precipitate the small RNA Solexa products with at least 1 volume isopropanol (fill tube) and 10 $\mu$ g glycogen at -20°C for 30min.

**Working with one sample at a time**, remove isopropanol and immediately add 70% ethanol to the pellet. Repeat for each sample.

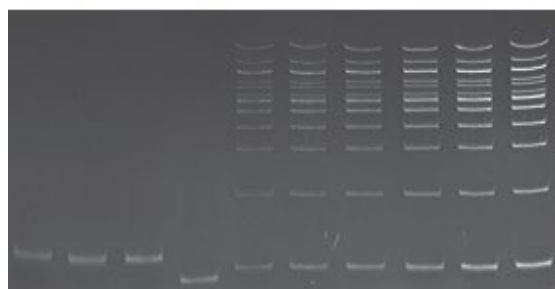
Spin to pellet again.

Again **working with one sample at a time**, remove all of the 70% ethanol using a gel loading tip and immediately add H<sub>2</sub>O. Repeat for each sample.

**Do not allow the pellets to dry!**

Quantify the DNA concentration using a NanoDrop spectrophotometer. The reading is not accurate below 10 ng/ $\mu$ l. Even if you obtain a concentration above 20 ng/ $\mu$ l, it is essential to quantify the concentration using a 10% PAGE gel and a known DNA standard (e.g. we use the 100bp marker from NEB, catalog No. N3231S). Below is a quantification gel in which each lane contains a determined amount of 110nt or 90nt (Solexa kit) products compared to dilutions of the 100bp standard.

DNA samples    100 bp standard  
4   4   4   4   1   2   3   4   5   6 ng



DNA quantification using PAGE

## **IX. Oligonucleotides:**

**3' Adaptor (=modban):** (miRNA Cloning Linker-1 from IDT)

AppCTGTAGGCACCATCAAT/ddC/

**5' Adaptor (=CMo13281): DNA/RNA hybrid oligo (black DNA and red RNA)**

TCTACrArGrUrCrCrGrArCrGrArUrC

**RT DNA oligo**

ATTGATGGTGCCTACAG

**CMo13279 DNA oligo**

**GTTCTACAGTCCGACGATC**

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

AATGATACGGCGACCACCGACAGGTTTCAGAGT**TCTACAGTCCGACGATC**

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

CAAGCAGAAGACGGCATAACGAATTGATGGTGCCTACAG

**Clones should have the following sequence structure:**

5'-AATGATACGGCGACCACCGACAGGTTTCAGAGT**TCTACAGTCCGACGATC**

...small\_RNA...

CTGTAGGCACCATCAATTCGTATGCCGTCTTCTGCTTG-3'