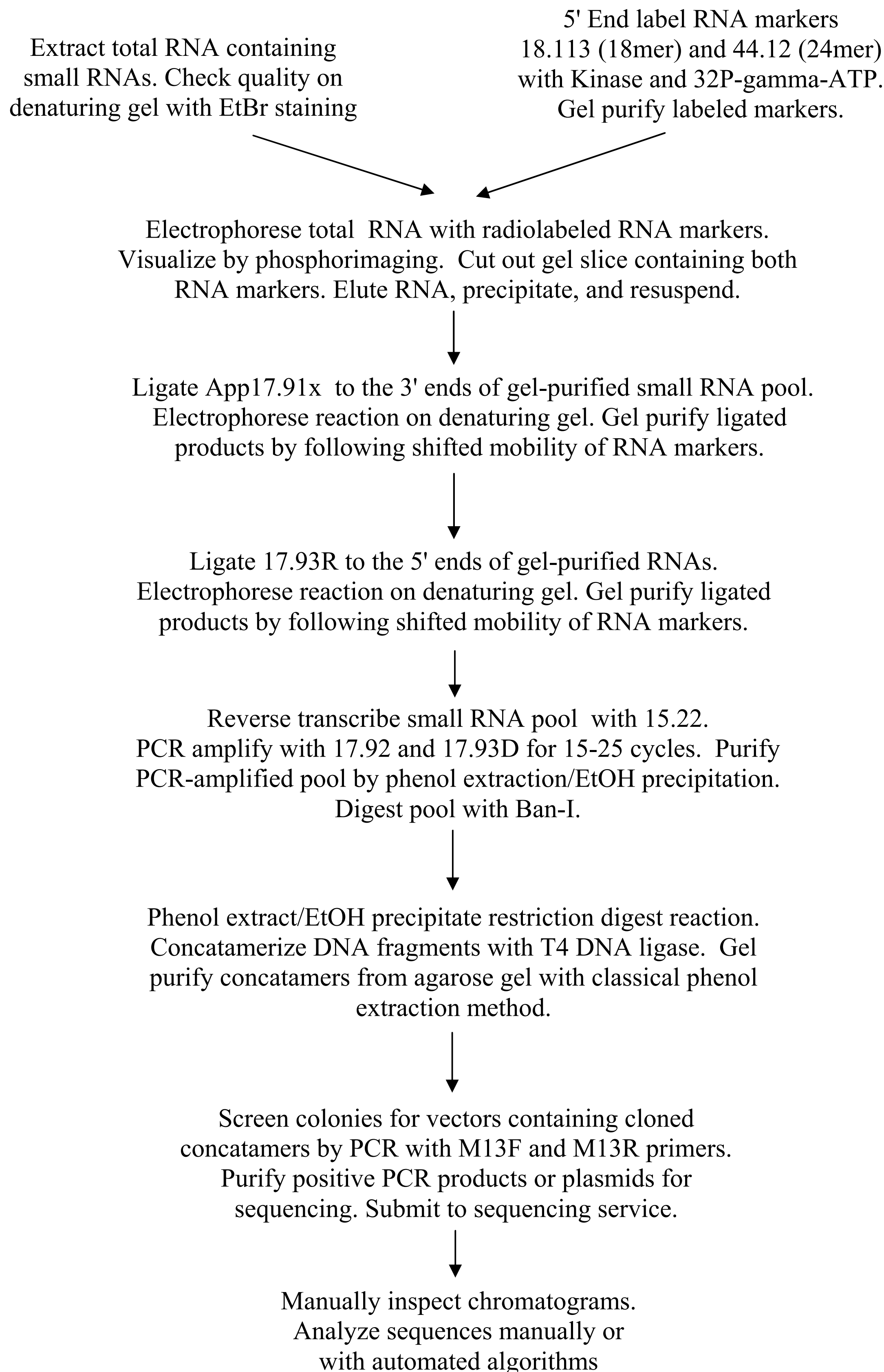
RNA Cloning Method Flowchart



OLIGOS FOR SMALL RNA CLONING

Underline nucleotides mark a Ban I restriction digest site (G↓GYRCC)

3' End Donor Oligo (available from IDT Inc. as the miRNA cloning linker)
App17.91x: AppCTGTAGGCACCATCAddA
Note: To synthesize this oligo in your lab, see the original 2001 version of the cloning protocol on the Bartel Lab website.

5' End Acceptor Oligo (RNA/DNA version, lowercase RNA)
17.93R: ATCGTaggcaccugaaa

3' RT Primer Oligo (Shorter 5' end to minimize mispriming)
15.22: ATTGATGGTGCCTAC

3' Linker PCR oligo
17.92: ATTGATGGTGCCTACAG

5' Linker PCR oligo
17.93D: ATCGTAGGCACCTGAAA

TOPO-TA Screening/Sequencing Primers
M13F: GTAAAACGACGGCCAG
M13R: CAGGAAACAGCTATGAC

Small RNA Markers for following miRNAs and siRNAs
(markers should be 5' end labeled and gel-purified after labeling).

24 bp marker (synthetic sequence from an RNA ligase ribozyme; underlined bases mark an Acl-I site)
44.12R: GGCCAACGUUCUCAACAAUAGUGA

18 bp marker (underlined bases mark a BamH-I site)
18.113R: AGCGUGUAGGGAUCCAAA

Other recommended special items to purchase:

32P-γ-ATP (6000 ci/mmol)
T4 Polynucleotide Kinase
Vertical electrophoresis unit for acrylamide gels
Siliconized Tubes
Aerosol Filter tips
T4 RNA Ligase
Glycogen
Superscript III Reverse Transcriptase
Ban-I restriction enzyme
Metaphor GTG Agarose

- **5' End Label Marker RNAs**

Kinase RNA markers to very high specific activity by the following procedure:

1 µL of 1 µM RNA (44.12R or 18.113R)
2 µL PNK Buffer
1 µL PNK
5 µL 6000 Ci/mmol ^{32}P γ-ATP (3µM)
11 µL dH_2O

Incubate for 1 hr at 37 ^{o}C.
Gel-purify labeled RNA on a 20% denaturing acrylamide gel, using glycogen as a carrier to precipitate after eluting from gel slice.

Resuspend each labeled RNA in 40 µL dH_2O. Run 2 ul on a test acrylamide gel and wrap the wet test acrylamide gel. Expose to phosphorimage plate and see if you detect a strong signal after a 5 minute exposure. Generally, 3000 counts of labeled RNA is a good starting point to test. The goal is to determine the minimum amount of labeled RNA to add to your total RNA during the purification step of 18-26-mers. Minimizing the addition of marker RNAs will maximize the number of miRNA/siRNA clones in the final step.

- **Purifying 18-26mers from Total RNA**

Pour a 15% 1.5 mm denaturing polyacrylamide gel with wide wells (23mm). Prerun to warm up gel. Make sure the lane is quite flat for nice loading and resolution of markers.

Prepare an aliquot of total RNA (50-500 µg), adding trace but very high specific activity radiolabeled marker RNA and 1X volume of 8M Urea, 0.5 mM EDTA Loading Dye. Heat for 5 min in 80_o C heatblock and load entire volume in one lane. Electrophorese until the BB dye reaches the bottom. Expose gel, cut out gel slice that includes both top and bottom hot markers. Elute RNAs O/N in 0.3M NaCl, precipitate in 2X volume EtOH (>2 hrs) with glycogen (1 µg/ ml). Spin down (full speed, 30 min) and resuspend in 10 µl dH_2O.

A Note on Total RNAs: not all total RNA sources, particularly commercial total RNA sources, may contain small RNAs like miRNAs and siRNAs! If a sample of "total" RNA was purified by the popular silica matrix column procedure (i.e. Qiagen RNEasy columns), it will be significantly depleted in small RNAs. Extraction procedures like Trizol/TriReagent, however will purify all RNAs, large and small, and are the recommend methods for isolating total RNA from biological samples that will contain miRNAs/siRNAs.

- **3' Adaptor Ligation and Purification**

Prepare 5X T4 RNA Ligase Buffer (no ATP) taken from England et al. *PNAS*. 1977. **74**: 4839.

250 mM Hepes pH 8.3
50 mM $MgCl_2$
16.5 mM DTT
50 ug/ml BSA
41.5% glycerol

Use RNase-free reagents and techniques. Store buffer at –20°C

Set a 3' Adaptor Ligation Reaction; Incubate at Room Temp for 2 hrs

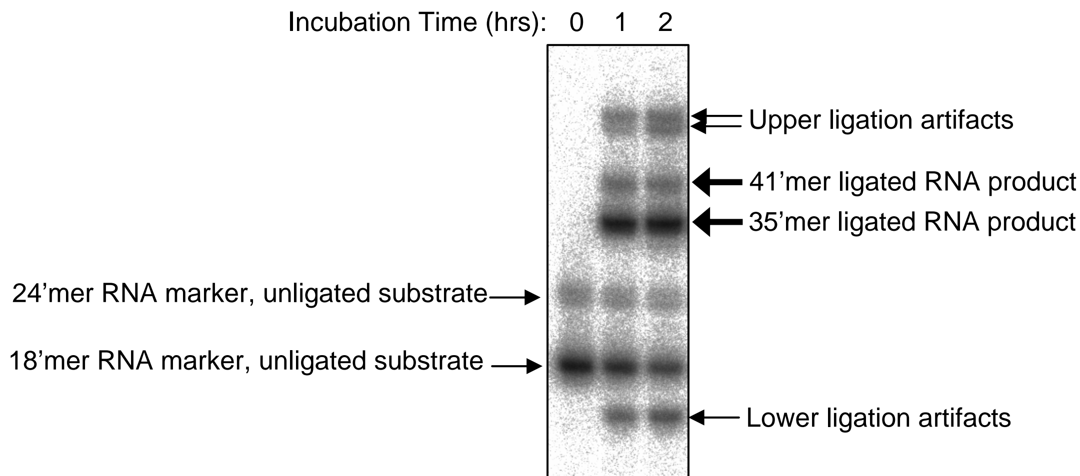
2 µL 5x Ligation Buffer
2 µL 100 µM App17.91x
1 µL T4 RNA Ligase (Promega or GE Amersham, FPLC pure)
5 µL purified small RNAs (containing hot labeled RNA markers)

Stop reaction with 15 µL 2X Urea Loading Dye.

Prepare a 10% (0.5 mm) denaturing polyacrylamide gel. Prerun, then load into 2-4 lanes (spread out the reaction to prevent overloading and to dilute the salt in the reaction). Run gel until good separation of BB and XC dyes (about 3-4 inches).

Separate one of the plates, keeping gel on other plate, and cover with Saran wrap. Expose on a phosphor plate, and locate ligated bands (higher mobility- see Figure 1.). Cut out the gel slice that includes the 35'mer and 41'mer ligation product and transfer into siliconized tubes. Avoid the upper and lower ligation artifacts (which occur due to the high ligation efficiency of the adenylated linker). Elute RNAs from gel slice, and ethanol precipitate with glycogen. Resuspend all pellets together into 10 µL dH_2O.

Figure 1. 3' Ligation Reaction Timecourse:



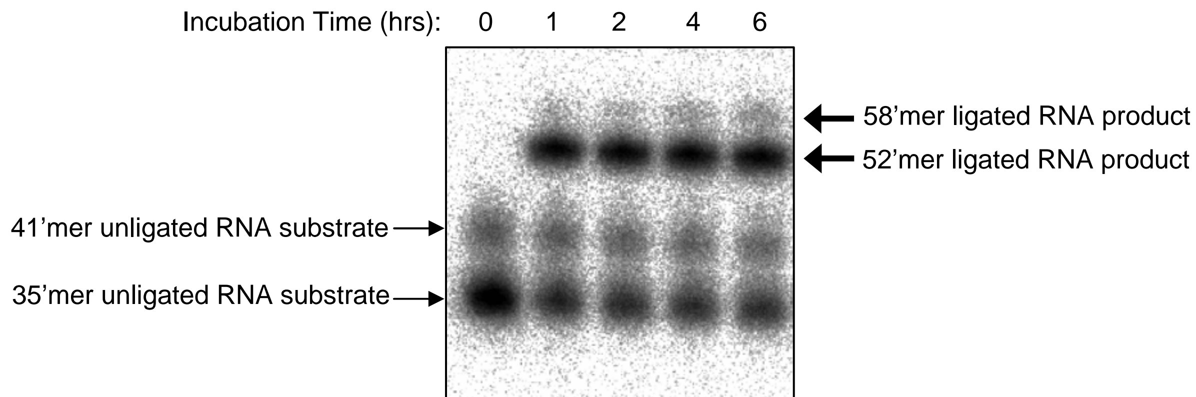
- **5' Adaptor Ligation and Purification**

Set a 5' Adaptor Ligation Reaction; Incubate at Room Temp for 6 hrs

2 µL 5x Ligation Buffer
2 µL 200 µM 17.93R
1 µL 4 mM ATP
1 µL T4 RNA Ligase
5 µL small RNAs from 3' Adaptor Ligation Reaction

Stop reaction with 10 µL 2X Urea Loading Dye. Prepare gel and purify 5' adaptor ligation products in the same way as for the 3' ligation products. For band identification, use freshly kinased 10bp ladder as a reference for size. Cut out the 52-60'mer products, and leave behind the unligated 35-43'mers (see Figure 2). Resuspend pellets in a total 10 µL dH_2O.

Figure 2. 5' Ligation Reaction Timecourse:



- **RT-PCR of small RNAs with Adaptors**

Using siliconized tubes, set up a reverse transcription reaction:

5 µL of ligated RNAs
1 µL 100 µM 15.22
10 µL dH_2O
} Heat to 80°C for 2 min. Spin down to cool

6 µL 5X First Strand Buffer (Invitrogen)
7 µL 10X dNTP's
3 µL 100mM DTT
1 µL SuperScript III RT (200U/µL) final
} Heat to 48°C for 2 min before adding RT. Take out 3 µL for a (-)RT control.

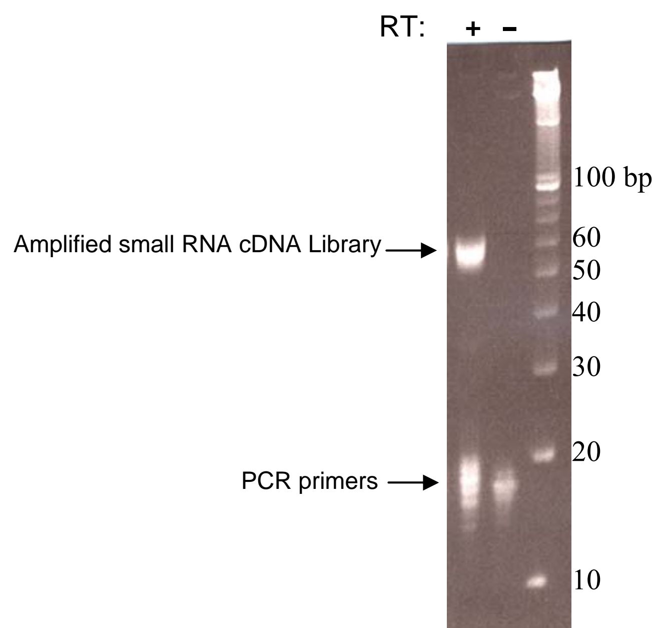
Incubate reverse transcription reaction at 48°C for 1 hour. Next, add 1 µL RNase H and incubate at 37°C for 30 minutes. Do all steps in parallel with the (-)RT control. Remaining RT reaction may be stored long term at -20°C.

Set up 100 µL reactions for the RT(+) and RT(-) samples for PCR.

Reaction mix	PCR conditions	Buffer composition
5 µL of RT reaction 10 µL 10X PCR Buffer 10 µL 10X dNTPs 1 µL 100 µM 17.92 1 µL 100 µM 17.93D 2 µL Taq Polymerase 71 µL dH_2O	15 to 25 cycles of PCR (hot start optional) 94° C – 30 sec 50° C – 30 sec 72° C – 30 sec	10X Bartel Lab PCR Buffer 100 mM Tris pH 8.3 500 mM KCl 15 mM $MgCl_2$ 0.1% Gelatin 1X dNTPs contain 0.2mM of each dNTP

Analyze reactions with a 15% denaturing polyacrylamide gel. Take 3 µL from each RT-PCR reaction, add loading dye, heat well before loading, and load onto a pre-run midi-thickness gel. Run using the 10bp ladder to follow bands. Do not use EtBr for staining, because the sensitivity is very weak for these small DNAs. Use the SYBR Gold stain from Molecular Dynamics. You should see a good smear in the size range of small RNAs ligated with linkers. Use filter tips. Two times phenol extract. Two times chloroform extract. Add NaCl to make 0.3M / EtOH precipitate (glycogen optional). Spin down pellet and resuspend the RT(+) reaction in 40 µL.

Figure 3. RT-PCR of Small RNA Library.

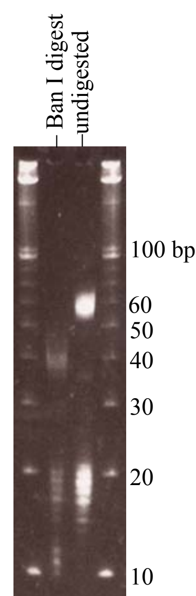


- **Concatamerization**

Set up a Ban I digest of PCR products - 4 hrs incubation at 37°C

40 µL of RT-PCR products (Pool 2 tubes)
30 µL NEBuffer 4
10 µL Ban I 20U/µL → 0.67 U final
220 µL dH_2O

Figure 4. Ban I Digest of PCR Pool of Small RNAs



Check 10 µL from digestion on a 15% denaturing polyacrylamide gel. Use 1 µL from the PCR and the 10 bp ladder as markers, then stain the gel with SYBR Gold. See Figure 4. Two times phenol extract. Two times chloroform extract. Add NaCl to make 0.3M and EtOH precipitate (glycogen optional).

Add the following for concatamerization to the entire pellet from the digest:

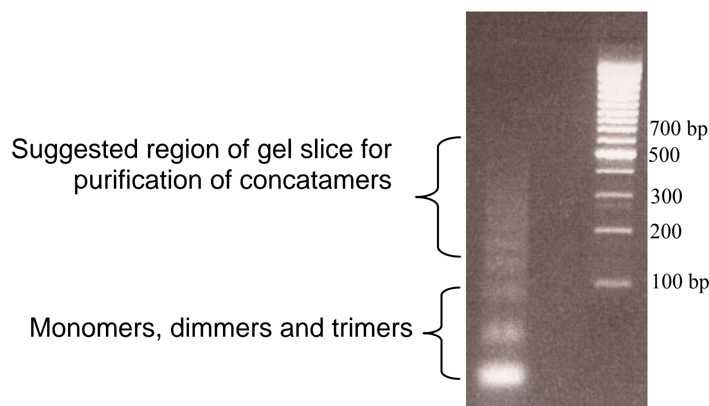
8 µL dH_2O
1 µL 10X T4 Ligase Buffer (USB or NEB brand is fine)
1 µL T4 DNA Ligase

Incubate at room temp for 30 min. Take a mini-gel casting tray for agarose and rinse thoroughly. Prepare a 2% GTG Nusieve Agarose Gel with 1x TAE, pre-stained with EtBr. Load entire concatamerization reaction with glycerol loading dye into a lane, run with 100bp marker. Run a short time, when the ladder can be visualized. See Figure 5 below.

Using the low energy, high wavelength setting on transilluminator, locate smear corresponding >300 bp concatamers and cut out with a clean razor blade. Add 10 volumes gel melting solution (20 mM TrisHCl pH 8, 1 mM EDTA pH 8) and melt for 5 minutes at 65°C. You may need to distribute this to a couple of siliconized tubes.

Add an equal volume of phenol, vortex for 20 seconds, chill on ice for 5 min, then spin at 5000 g for 10 min (4° C). Remove aqueous phase, re-extract with a 1:1 phenol, chloroform mix, and re-extract again finally with just chloroform. Add 0.06 volume of 5M NaCl and 2.5 volume EtOH and precipitate at –20°C with glycogen for >2 hrs.

Figure 5. Agarose Gel Resolution of Concatamerized DNA Fragments



- **Cloning into TOPO vector**

Resuspend concatamers in the following Taq Fill In Reaction. Incubate at 72°C for 5 min.

11.5 µL dH_2O
1.5 µL 10x PCR Buffer
1.5 µL dNTPs
0.5 µL Taq polymerase

Have the TOPO TA cloning kit reaction tube set up. Use 5 µL from the fill in reaction for a TOPO-TA Cloning reaction, and freeze the remaining fill-in reaction for storage. Use all of Topo reaction for transformation into chemical competent cells, add 500 µl SOC media, and let the cells grow for only 45 min (not longer) before plating out 50 µL , 150 µL, and 300 µL of the culture on to LB Amp S-Gal plates. Grow overnight at 37°C.

- **Screen and Sequence**

Pick white colonies, and restreak on a master plate. Let this master plate grow ON. Screen only white colonies by PCR in a 96-well microplate format – 30 µL reactions per well.

Reaction	25 cycles of PCR using the COLONY Protocol
3 µL 10X PCR Buffer 3 µL 10X dNTPs 0.2 µL 100 µM M13F 0.2 µL 100 µM M13R 0.5 µL Taq Polymerase 23 µL dH_2O	94° C – 3 min (burst open cells) 94° C – 30 sec 50° C – 30 sec 72° C – 30 sec

Pick colonies from master plates with a pipette tip, swish around in a PCR reaction well. Check completed reactions on a 2% agarose gel, and look for inserts greater than 220 bp (expect 500-800 bp inserts, see Figure 6). You can now either purify remaining PCRs and sequence directly, or regrow colonies to extract plasmids. Submit to commercial sequencing facility, using M13F or M13R as sequencing primers.

Figure 6. PCR Screening of Topo Colonies.