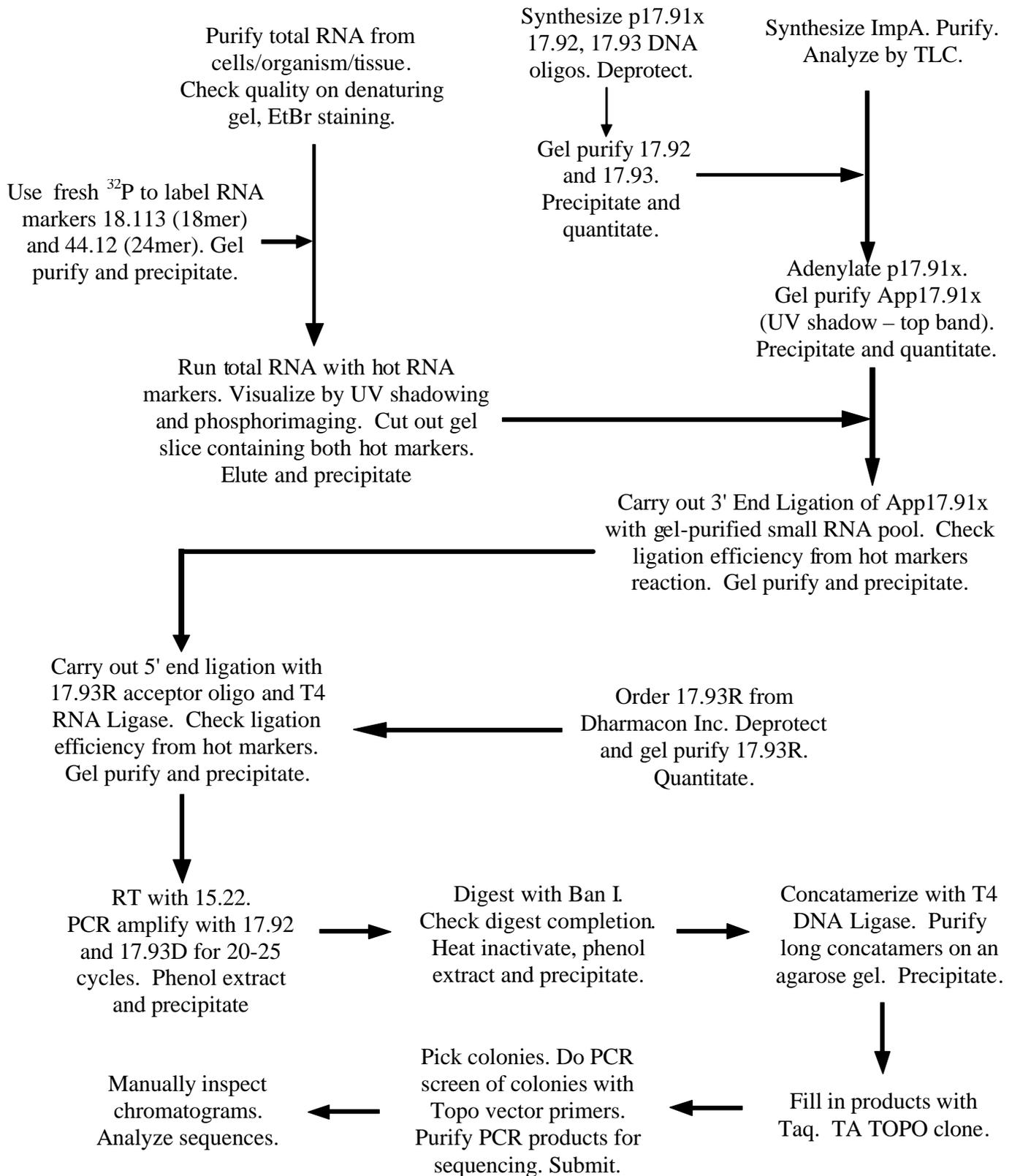


### RNA Cloning Method Flowchart



## Procedural Outline:

1. Oligo Design
2. ImpA Synthesis
3. Synthesizing Oligos
4. Oligonucleotide Adenylation
5. Small RNA Purification
6. 3' Adaptor Ligation
7. 5' Adaptor Ligation
8. RT-PCR
9. Concatamerization
10. Cloning into Topo vector
11. Screen and Sequence

- **Oligos Design**

**Oligos for RNA Cloning** [Underline is Ban I site (G↓GYRCC)]

**3' End Donor Oligo** (requires 5' Phosphorylation and 3' Modification)

**17.91:** pCTGTAGGCACCATCAAx (x: DMT-O-C3 –CPG)

**5' End Acceptor Oligo**

**17.93R :** ATCGTaggcacctgaaa (RNA/DNA version, lowercase RNA)

**3' RT Primer Oligo**

**15.22:** ATTGATGGTGCCTAC

**5' PCR oligo**

**17.93D :** ATCGTAGGCACCTGAAA (DNA version, calcTm = 42°C)

**3' PCR oligo**

**17.92:** ATTGATGGTGCCTACAG (calcTm = 40°C)

**24mer and 18mer marker RNAs with restriction sites:**

**T7 template for 44.12R** (italicized sequence is T7 promoter)

**44.12D :** TCACTATTGTTGAGAACGTTGGCCTATAGT*GAGTCGTATTACGC*

**RNA Marker transcribed off 44.12D** (with Acl I site)

**44.12R:** GGCCAA↓CGUUCUCAACAAUAGUGA

**RNA marker purchased from Dharmacon** (contains BamH I site)

**18.113 :** AGCGUGUAGG↓GAUCCAAA

- **Synthesizing ImpA**

This protocol was modified from those of Mukaiyama and M. Hashimoto, *Bull. Chem. Soc. Japan* 1971 **44**: 2284-2289; Lohrmann and Orgel. *Tetrahedron* 1978 **34**: 853-855; and Unrau and Bartel. *Nature* 1998. **395**:260-263.

*Synthesis*: Prepare following separate mixes in clean beakers that are rinsed in acetonitrile to dry out the glass. Reagents for ImpA synthesis can be purchased from Sigma Chemical. Buy grades that are at least 99% pure (ie. imidazole order # I0125).

Mixture A:

0.5 mmol AMP (5' p) free acid (FW 347.2), 174mg  
15 ml Dimethylformamide

Mixture B:

1 mmol Triphenylphosphine (FW 262.3), 262 mg,  
1 mmol 2,2'-dipyridyldisulfide (FW 220.3), 220 mg,  
2.5 mmol Imidazole (FW 68.08), 170 mg  
  
0.90 ml Triethylamine (FW 101.2), d=0.726  
15 ml Dimethylformamide

Add mixture A slowly into mixture B while stirring with vigour (clean, dry stir bar). The mixtures initially are not very soluble, but the reaction begins to turn yellow-green, solids dissolve, some oil appears on bottom, and this also eventually dissolves in. Stir for 1-1.5 hr at room temp with a cover over beaker or reaction flask.

*Purification*: To precipitate and purify ImpA from reaction, add dropwise above reaction to vigorously stirred anhydrous solution containing:

9 mmol NaClO<sub>4</sub> (FW 122.4), 1.1g  
225 ml Acetone  
115 ml Ether (anhydrous ethyl ether, VWR)

The ImpA precipitates out as puffy aggregates. Let this settle, and then remove solvent phase down to about 60 ml. Save some of this solvent for analysis. Transfer precipitate to Corex tubes, rinse remaining chunks with acetone. Centrifuge at 5000 rpm (3000 g on ss34 rotor) for 10 min, pour off acetone, wash and spin down twice again in acetone. Do one final wash with just ether, and spin down at 3000g for 20 min. Removing all the ether will be difficult. Dry overnight in a vacuum vessel between 22.5°C and 45° C, scrape out white chunks with a clean spatula into a scintillation vial with cap for dry-storage at -20° C (stable for a couple weeks). Alternatively, you can dry out ether with Rotovap (need a chemist assistance but much faster).

*Analysis:* Note weight of recovered white chunks. I obtained ~80 mg of ImpA product.

Pre-soak Cellulose-F TLC (JT Baker) in 1:10 saturated  $(\text{NH}_4)_2\text{SO}_4$ . Let air-dry or blow dry.

Prepare much-scaled-down mini-samples of mixture A and B (stoichiometries no longer matter here, just need sample mixtures to compare before and after states of reaction). Dissolve AMP (mixture A) and ImpA in water instead of dimethylformamide for this analysis (should dissolve better).

Dot following samples with capillary tubes: AMP (mixture A), ImpA, Mix B, solvent from purification, and AMP again. Use 80% EtOH as solvent, run up about 9 cm. The AMP should run the slowest, ImpA should run out higher, Mix B and leftover solvent from purification should run with the EtOH solvent front. Visualize with hand-held UV lamp.

- **Synthesizing Oligos (making p17.91x)**

All regular oligos were synthesized by standard protocols for DNA synthesis on the Expedite 8900 DNA synthesizer (Applied Biosystems)

For making p17.91x, first purchase 3'-Spacer C3 CPG and chemical phosphorylating agent from Glen Research. Buy either the 4-pack 1 $\mu$ mole pre-packed columns, or buy the 0.1g powder and pack your own 4 columns for \$30 cheaper.

Prime the machine twice, then carry out a standard synthesis program of the 17.91 sequence for DNA synthesis at 1  $\mu$ mole scale. Note the trityl readings. Once done, remove the columns and set aside. Load on the phosphorylation reagent. Buy two 100  $\mu$ mole bottles of the phosphorylating agent, and dissolve into 3 mL acetonitrile. Load this into a clean amber bottle on the machine.

Before loading the columns back on, prime all reagents for each column (1 and 2). Be careful with the phosphorylating reagent because the small volume can be quickly primed away and wasted. Now configure the machine for RNA synthesis. This tells the machine to take longer coupling times. Program in the phosphorylating reagent as the last coupling, and monitor efficiency with the last trityl reading.

Deprotect all oligos by standard ammonia incubation, followed by butanol precipitation. Only oligos used in reverse transcription or T7 RNA transcription should be gel-purified. p17.91x does not need to be gel purified for the next step.

- **Adenylation – Synthesis of App17.91x**

Set up reaction in distilled H<sub>2</sub>O (i.e. in 500 µL reaction):

50 mM ImpA (FW 423)	420 µL dH <sub>2</sub> O with 9 mg
25 mM MgCl <sub>2</sub>	7 µL from 2M solution
0.2 mM p17.91x	80 µL of 1.3 mM solution

Incubate at 50° C for 3 hrs. Gel purify on a denaturing 20% polyacrylamide gel. Be careful not to overload the gel, or else it gets hard to visualize the App17.91x, which should run about 1 nt slower than the p17.91x (top band is App17.91x).

For gel purification, pour 1.5 mm thick gel (prep gel thickness) using a 6-lane comb (lane width 23 mm) and load about 25 nmoles per lane. Cut out gel piece, elute overnight in 0.3 M NaCl, precipitate with 2x volume EtOH at -20° C for more than 1hr. Spin at full speed for >15 minutes, and resuspend pellet in dH<sub>2</sub>O (want to make final stocks of 200 µM App17.91x).

In my opinion, I get ~10-20% reaction efficiency, because the phosphoanhydride bond is probably hydrolyzed soon after it is formed. As side note, ImpA is not very stable in aqueous solution (few days), so I used it up to adenylate all of my p17.91x. Once adenylated and gel purified, App17.91x can be stably stored in aliquots at -80° C for many months.

- **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° 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 ON 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 10-20 µl dH<sub>2</sub>O.

- **3' Adaptor Ligation and Purification**

Prepare 5X T4 RNA Ligase Buffer taken from England et al. *PNAS*. 1977. **74**: 4839.

250 mM Hepes pH 8.3	Use RNase-free reagents and techniques. Store buffer at -20°C.
50 mM MgCl <sub>2</sub>	
16.5 mM DTT	
50 µg/ml BSA	
41.5% glycerol	

Set a 3' Adaptor Ligation Reaction. Incubate at Room Temp for 2-6 hrs.

2  $\mu$ L 5x Ligation Buffer  
2  $\mu$ L 200  $\mu$ M App17.91x  
1  $\mu$ L T4 RNA Ligase (Amersham Pharmacia, high concentration)  
5  $\mu$ L small RNAs with Hot Markers

Stop reaction with 10  $\mu$ L 2X 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). Run gel until good deal separation of BB and XC (about 3-4 inches).

Separate one of the plates, keeping gel on other plate, cover with saran wrap. Expose on a phosphor plate, locate ligated bands (higher mobility), cut out gel fragments and transfer into siliconized tubes. You should expect to only cut out bands between 35-43 nt long. There shouldn't be too many artifacts, but you may see a 53mer artifact (18+18+17) and <16mer artifact (degradation or minor circularization of 18mer). Elute, EtOH precipitate with glycogen, spin down. Resuspend all pellets together into 10-20  $\mu$ L dH<sub>2</sub>O.

- **5' Adaptor Ligation and Purification**

Set a 5' Adaptor Ligation Reaction. Incubate at Room Temp for 2-6 hrs.

2  $\mu$ L 5x Ligation Buffer  
2  $\mu$ L 200  $\mu$ M 17.93R  
1  $\mu$ L 4 mM ATP  
1  $\mu$ L T4 RNA Ligase (Amersham Pharmacia, high concentration)  
5  $\mu$ L small RNAs from 3' Adaptor ligation reaction

Stop reaction with 10  $\mu$ L 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, just use the freshly kinased 10 bp ladder as a reference for size. You should cut out the 52-60mer bands, and you may see 35-43mers remaining from ligation. Resuspend pellets in a total 10-20  $\mu$ L dH<sub>2</sub>O.

- **RT-PCR of small RNAs ligated with adaptors**

Using siliconized tubes, set up a reverse transcription reaction:

5 $\mu$ L of ligated RNAs	}	Heat to 80° C for 2 min Spin down to cool
3 $\mu$ L 100 $\mu$ M 15.22		
5 $\mu$ L dH <sub>2</sub> O		
5 $\mu$ L 5X First Strand Buffer (from Invitrogen)	}	Heat to 48° C for 3 min before adding RT. Take out 2 $\mu$ L for a (-)RT control.
7 $\mu$ L 10X dNTP's		
3 $\mu$ L 100mM DTT		
3 $\mu$ L SuperScript II RT (200U/ $\mu$ L) final		

Incubate at 48° C for 1 hour, then add 1  $\mu$ L 0.1M EDTA and 3.8  $\mu$ L 1 M KOH. Incubate at 90° C for 10 minutes to degrade all the RNA. Do all steps in parallel with the (-) RT control.

Neutralize the reaction by adding 4  $\mu$ L 1M HCl-Tris pH 1, and 1  $\mu$ L 0.2M MgCl<sub>2</sub>. Use the entire RT(+) reaction for six 100  $\mu$ L PCR amplifications.

Set up 100  $\mu$ L PCR reaction from the RT(+) [six rxns] and RT(-) [one rxn] samples. Use fresh tubes. Use up all of the RT reaction – it does not store well.

5 $\mu$ L of RT reaction	}	20-25 cycles of PCR (no hot start)	10X Bartel Lab PCR Buffer 100 mM Tris pH 8.3 500 mM KCl 15 mM MgCl <sub>2</sub> 0.1% Gelatin
10 $\mu$ L 10X PCR Buffer			
10 $\mu$ L 10X dNTPs			
1 $\mu$ L 100 $\mu$ M 17.92			
1 $\mu$ L 100 $\mu$ M 17.93D			
2 $\mu$ L Taq Polymerase			
71 $\mu$ L dH <sub>2</sub> O			
		94° C – 1 min	
		50° C – 1 min	
		72° C – 1 min	
			1X dNTPs contain 0.2mM of each dNTP

Analyze reactions with a 15% denaturing polyacrylamide gel. Take 3  $\mu$ L 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 each RT(+) reaction in 40  $\mu$ L.

- **Concatamerization**

Set up a Ban I digest of PCR products

40  $\mu\text{L}$  of PCR products  
30  $\mu\text{L}$  NEBuffer 4  
10  $\mu\text{L}$  Ban I 20U/ $\mu\text{L}$   $\rightarrow$  0.67 U/ $\mu\text{L}$  final  
220  $\mu\text{L}$  dH<sub>2</sub>O

4 hrs incubation at 37°C

Check 15  $\mu\text{L}$  from digestion on a 15% denaturing polyacrylamide gel. Use 1  $\mu\text{L}$  from the PCR and the 10 bp ladder as markers, then stain the gel with SYBR Gold. Two times phenol extract. Two times chloroform extract. Add NaCl to make 0.3M and EtOH precipitate (glycogen optional). Spin down pellet.

Take the entire pellet from a single Ban I digestion and add the following for concatamerization:

8  $\mu\text{L}$  dH<sub>2</sub>O  
1  $\mu\text{L}$  10X T4 Ligase Buffer (USB or NEB brand is fine)  
1  $\mu\text{L}$  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 the 100bp marker (MBI Fermentas). Run a short time, when the ladder can be visualized.

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, reextract with a 1:1 phenol, chloroform mix, and reextract 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.

- **Cloning into TOPO vector**

Spin down pellet and resuspend in the following Taq Fill-In Reaction

11.5  $\mu\text{L}$  dH<sub>2</sub>O  
1.5  $\mu\text{L}$  10x PCR Buffer  
1.5  $\mu\text{L}$  dNTPs  
0.5  $\mu\text{L}$  Taq

Heat to 72° C for 5 min. Have the TOPO TA cloning kit reaction tube set up . Freeze after taking 5  $\mu\text{L}$  from the Fill-In Reaction and adding to 1  $\mu\text{L}$  salt solution and 1  $\mu\text{L}$  TA Cloning vector from TOPO cloning kit. Use all of Topo reaction for the transformation, add 500  $\mu\text{l}$  SOC, let the cells grow for only 45 min (no longer), and plate out as 75  $\mu\text{L}$  , 150  $\mu\text{L}$ , 300  $\mu\text{L}$  (remaining) on LB Amp S-Gal plates. Grow ON at 37° C.

- **Screen and Sequence**

Pick white colonies, and restreak on a master plate. Let this master plate grow ON. Screen only the white colonies by PCR in microplate format – 30  $\mu\text{L}$  reactions enough.

3 $\mu\text{L}$ 10X PCR Buffer	}	25 cycles of PCR using the COLONY Protocol
3 $\mu\text{L}$ 10X dNTPs		
0.2 $\mu\text{L}$ 100 $\mu\text{M}$ M13F		94° C – 3 min (burst open cells)
0.2 $\mu\text{L}$ 100 $\mu\text{M}$ M13R		94° C – 40 sec
0.5 $\mu\text{L}$ Taq Polymerase		50° C – 40 sec
23 $\mu\text{L}$ dH <sub>2</sub> O		72° C – 40 sec

Pick a colonies from master plates with a pipette tip, swish around in a PCR reaction well. Check on 2% agarose gel, look for inserts greater than 220 bp (expect 500-800 bp inserts).

You can now either purify remaining PCRs and submit directly, or regrow colonies to extract plasmids. Submit to commercial sequencing facility, using M13F or M13R as sequencing primers.