General Cloning Protocol:

Gel-purification

1. Pour 1mm thick, urea denaturing 10% or 15% polyacrylamide gels, with Sequagel kit.
2. Mix sample with 1.5+ volumes of 2x urea loading dye (with BB & XC), and heat the sample at 80°C for ~5 min to disrupt secondary structure, and spin down.
3. Load radiolabeled markers in separate lanes next to your primary sample lanes. Run the gel at 25W constant watts in 0.5X TBE, until BB dye just reaches bottom of gel.
4. Expose gel with phosphoimager plate. Scan plate on Typhoon. Print image at 100% zoom.
5. Lay image behind gel – draw out domains from marker lanes across lanes containing your sample
6. Cut out the zone corresponding to top and bottom hot markers and transfer gel slice carefully to 1.5ml low retention siliconized tubes.
7. Elute RNA from gel slice with 500ul of 0.3M NaCl per 1.5ml tube. Shake or quake overnight
8. Move supernatant to a fresh siliconized tube, add 2µL of glycogen and 2 volumes of 100% EtOH to the sample, vortex, and incubate at -20°C for >1hr.
9. Spin down at ~14K rpm for 25 minutes at 4°C in a microcentrifuge (for subsequent purifications).
10. Carefully remove all supernatant, try not disturbing RNA/glycogen pellet. Dissolve the RNA pellet in dH_2O (generally ~10ul).

Step 1. Purifying 18-32 nt area from 20ug of Total RNA (1ug/uL) (2days)

1.1 Run high specific activity radiolabeled marker (~20K count, ~0.5µL of mixed markers) and 2 volumes of 2x loading dye to 100µL (100µg) of total RNA sample.

1.2 Gel-purify total RNA on 15% gel (Expose for ~5-10 sec).

1.3 Dissolve RNA in 8 µL dH_2O.

Step 2. 3' Adaptor ligation and purification (2days)

2.1 Set up 3' Adaptor ligation reactions for your primary sample and for the radiolabeled 18mer and 32mer marker RNAs:

Reagent	Amount	Final
Purified 18-30nt RNA	8 µL	
100µM 3' Adaptor	0.5µL	50pmol
5X Ligation Buffer	2µL	1x
FPLC-pure T4 RNA Ligase (50U/µL)	1µL	
Total reaction volume (µL)	**10µL**	

2.2 Incubate the reaction at 18ºC for >2-4 hours.

2.3 Gel-purify on 15% gel (Run gel until BB dye is close to the bottom and expose for ~15-30 min). Run a lane of unligated 18mer and 32mer RNA along with the ligated 18mer and 32mer RNAs.

2.4 Note positions of shifted 18mer and 32mer with ligated adaptor.
SPECIAL NOTE: During this ligation step, there are side products generated from ligations of just the radiolabeled markers to themselves. It is important to run unligated markers in lanes separate from labeled markers. The side products will run too fast (faster than unligated marker), or will be too large (two markers concatamerizing).
Cut out gel zone with sample corresponding to positions of ligated radiolabeled RNA markers.

2.5 Elute all RNAs (hot markers too) and precipate and resuspend pellets into 10µL dH_2O.
For hot markers, resuspend that pellet in 20 ul dH_2O, using only half to set up 5' ligation reaction.

Step 3. 5' Adaptor ligation and purification (2 days)

3.1 Set up the 5' Adaptor ligation reaction:

Reagent	Amount	Final
Purified 3' ligation product	10µL	
100µM 5' Adaptor	4µL	400pmol
5X Ligation Buffer	4µL	1x
FPLC-pure T4 RNA Ligase (50U/µL)	1µL	20units
5mM ATP	1µL	5nmol
Total reaction volume (µL)	**20µL**	

3.2 Incubate at 22ºC for overnight.

3.3 Gel-purify on 10% gel (Run gel until BB dye runs out and expose 2 hours). This ligation reaction is more straightforward, very little side-products are generated. Be sure to run unligated markers from 3' ligation reaction to compare. Run markers on side lanes, near but separated by a lane from primary samples.

3.4 Elute ON in 0.3M NaCl, EtOH precipitate w/ glycogen, resuspend in 10µL dH_2O.

Step 4. RT and high-fidelity PCR of small RNAs with adaptors (1 day)

4.1 Set up a reverse transcription reaction:

Reagent	Amount
Purified ligated RNA	10µL
100uM RT-Primer (5' PCR primer)	1µL
dH_2O	5µL

4.2 Heat to 65°C for 5 minutes, spin down to cool.

4.3 Add following in order for a total 31ul reaction:

6µL 5x first strand buffer (Invitrogen)
6µL 10X dNTPs (2mM)
2µL 100mM DTT
1µL of Superscript III RT (200U/µL)

4.4 Incubate at 50°C for 1 hour, 75ºC for 15 min, cool to 4ºC to store.

4.5 After RT, immediately set up 50µL Single-amp PCR from the RT samples

Reagent	Amount
RT Reaction	5µL
5X PCR Buffer	10µL
2mM dNTP	6 µL
150nM 3' PCR primer	2µL (final 0.3pmol)
Phusion (NEB)	1µL
dH_2O	25µL

4.5 5 cycles of single-primer PCR (to let small RNAs extend before amplification):

98°C 3 min
94°C 45 sec
60°C 45 sec } 5 Cycles
72°C 45 sec
72°C 10 min

4.6 Add following 50 ul of additional solution to the PCR above for exponential library amplification :

Reagent	Amount
5X PCR Buffer	10µL
2mM dNTP	6 µL
25µM 3' PCR primer	0.5µL
25µM 5' PCR primer	0.5µL
Phusion (NEB)	1µL
dH_2O	25 µL

4.6 PCR conditions:

98°C 3 min
94°C 30 sec
60°C 30 sec } 15-20 Cycles of PCR
72°C 15 sec
72°C 10 min
4°C STORE

Step 5. Gel Purify PCR amplified cDNA libraries of small RNAs (2-3 days)

5.1 EtOH-precipitate PCR & resuspend in 15µL 1X glycerol loading dye.

5.2 Load samples on a 8% native acrylamide gel (0.8mm thick) (AccuGel). Run with a 10bp and 50bp DNA markers from Invitrogen

5.3 Stain gel with SYBR Gold and Ethidium Bromide. Scan gel on Typhoon in Fluorescence mode. Print out gel image, place behind gel & cut and elute 85-110nt gel piece.

5.4 Elute DNA from gel slice overnight with vigorous shaking at room temp in 0.3M NaCl. EtOH precipitate DNA with glycogen. Resuspend in 15µL of 10mM Tris (EB buffer). Quantitate DNA concentration, with desired concentration at >20 ng/ul, total of at >200ng DNA.

5.5 SPECIAL NOTES: If starting material from RT reaction is low, often a Primer Dimer artifact will amplify and dominate the reaction (~70 nt long). If this is the case, one can gel purify the desired product (85-110nt) and use half of this resuspend purified sample to go back to Step 4.6 for further PCR amplification.

Step 6. TOPO cloning for validation of small RNA library (2-4 days)

6.1 Purchase the Zero-Blunt TOPO kit (pCR4 vector from Invitrogen). Take 1ul of purified cDNA sample and follow instructons of TOPO kit manual.

6.2 Pick 12-24 colonies per library, prepare plasmid minipreps, sequence plasmids with Sanger sequencing with M13F primer at CCIB core.

6.3 Analyze trace data, extract sequences from linkers. Annotate sequences by Blast/Blat.

Final Construct

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5′ AATGAT ACGGCGACCA CCGACAGGTT CAGAGTTCTA CAGTCCGACG ATC NNNNNNNNNN NNNNNNNNN T CGTATGCCGT CTTCTGCTTG  3′
3′ TTACTA TGCCGCTGGT GGCTGTCCAA GTCTCAAGAT GTCAGGCTGC TAG NNNNNNNNNN NNNNNNNNN A GCATACGGCA GAAGACGAAC  5′
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Oligos (PAGE purified):

32merR: GGCAUUAACGCGGCCGCUCUACAAUAGUGA

18merR: AGCGUGUAGGGAUCCAAA

3' Adaptor
21.340x: pTCGTATGCCGTCTTCTGCTTGidT

5' Adaptor
26.71: GUUCAGAGUUCUACAGUCCGACGAUC

3'PCR primer
44.45: AATGATACGGCGACCACCGACAGGTTCAGAGTTCTACAGTCCGA

RT-primer & 5'PCR primer
18.206: CAAGCAGAAGACGGCATA

Materials:

Step 1. Purifying 18-32 nt area from 10ug of Total RNA (1ug/uL)

- **Making 5' end label marker RNAs**
  1. Set up the labeling reaction:

Reagent	Amount
10μM RNA (32merR or 18merR)	1μL
PNK buffer	2μL
PNK	1μL
^{32}P γ-ATP	2μL
dH_2O	14μL

  Incubate for 1 hr at 37°C.
  2. Gel-purify labeled RNA on a 15% gel until BB dye reaches 1" from the bottom to get rid of excess hot ATP.
  3. Resuspend labeled RNA in 20μL dH_2O.

Step 2. 3' Adaptor ligation and purification

- **Synthesizing ImpA**
  1. Rinse 2 beakers in acetonitrile and air dry.
  2. Make two mixtures:
     Mixture A:
     174mg AMP (FW347.2) (0.5mmol)
     15mL Dimethylformamide
     Mixture B:
     262mg Triphenylphosphine (FW262.3) (1mmol)
     220mg 2,2'-dipyridyldisulfide (FW220.3) (1mmol)
     170mg Imiddazole (FW 68.08) (2.5mmol)
     0.90mL Triethylamine (FW 101.2, d=0.726)
     15mL Dimethylformamide
  3. Add Mixture A slowly into Mixture B while stirring until precipitates dissolve.
  4. Stir for 1-1.5 hr at RT with cover over beaker.

5. Make Precipitation Mixture:
   1.1g $NaClO_4$ (FW 122.4) (9mmol)
   225mL Acetone
   115mL Anhydrous ethyl ether
6. Add Mixture A+B dropwise to Precipitation Mixture.
7. Remove solvent phase down to ~60mL.
8. Transfer precipitates to Corex tubes, rinse with acetone, centrifuge at 5000rpm (3000g on ss34 rotor) for 10 min and pour off acetone 3 times.
9. Do a final wash with just ether, and spin down for 20 min.
10. Dry overnight in a vacuum vessel between 22.5-45°C.
11. Store at -20°C.

- **Adenylation of 3' adaptor**

1. Set up reaction:

Reagents	Stock conc	Amount	Final conc
ImpA (FW 423)		9mg in 420μL dH_2O	50mM
$MgCl_2$	2M	7μL	25mM
3' adaptor (no App)	1.3mM	80μL	0.2mM

2. Incubate at 50°C for 3 hrs.
3. Gel purify on 20% gel.

- **Preparation of 5x ligase buffer**

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

250mM Hepes pH 8.3
50mM $MgCl_2$
16.5mM DTT
50μg/mL BSA
41.5% glycerol