Cloning of small RNAs with 5’ phosphate and 3’ OH ends (PROT40)

Ericka Havecker
Baulcombe Lab (The Sainsbury Laboratory, Cambridge), UK

email feedback to: erh45@cam.ac.uk

Last reviewed: 28 Apr 2008 by Eric Miska, Gurdon Institute, Cambridge

---

Introduction

The following protocol describes a procedure for the purification and cloning of miRNAs and other small RNAs in the 20-30 nucleotide size range from plant tissue. Figure 1 gives an overview of the cloning procedure.

Procedure

Purifying 20-30 Nucleotide lengths from 10µg of Total RNA (1 µg/µl).

Instead of gel purification, mirVANA isolation can replace this step following manufacturer?s instructions. We have used from 1.5 to 5 µg of small RNA (from 10 to 35 µg total RNA). Note, we also use Arabidopsis floral tissue, which has a high percentage of small RNAs (comment 1).

1. **Prepare the 15% 8M UREA gel.** Pour a 15% UREA gel in the Biorad MiniProtean 2, using 0.75mm spacers.

   12.5 ml makes two 0.75mm gels

   1. 5.25g Urea
   2. 625 µl 10X TBE
   3. 4.7 ml 40% acrylamide (19:1 acrylamide:bis-acrylamide)
   4. Add water to 12.5 ml and dissolve urea
   5. Add 87.5 µl 10% APS
   6. 4.4 µl TEMED
   7. Pour immediately (comment 2)

2. Pre-run the gel for 30 min at 200V and then wash the wells using 0.5X TBE.
3. Mix 10 µl (10 µg) of total RNA with 10 µl of 2x Gel Loading Buffer II (or equal volume 100% formamide with loading dye) in a 200 µl RNase-free microfuge tube. Heat the sample at 65°C for 5 min, then snap cool on ice. Centrifuge to collect volume to bottom of tube and load the sample into one well.
4. Load 10µl of the Baulcombe oligo ladder already prepared (sizes marked 20, 30, 40, 60 and 80)

5. Run the gel at 150V until the bromophenol blue reaches the bottom of the gel (about 2 hours). Stain the ladder portion of the gel with 0.5X EtBr for 2-5 min.
6. Cut out the gel slice corresponding to 20-30 nt (visualized with UV) with a clean razor blade and transfer to a 0.5 ml RNase-free microfuge tube whose bottom has been punctured 3 to 4 times by a 21 gauge needle.
7. Place this tube into a 2 ml RNase-free round-bottom microfuge tube and spin the gel through the holes into the 2 ml tube at full speed for 2 min (comment 3).
8. Add 500µl of sterile 0.3 M NaCl to the tube and elute the RNA by rotating the tube overnight at 4°C.
9. Transfer the eluate and the gel debris into a Spin-X Cellulose Acetate filter and spin at full speed for 2 minutes.
10. Wash the gel bits once more with 100µl 0.3M NaCl and spin for another 2 min. Collect the 100µl eluate in the same tube.
11. Add an equal volume (~600µl) of 100% Isopropanol and 3µl of glycogen (we used Ambion glycoblue) to the sample and incubate at ?80°C for 20 to 30 minutes.
12. Spin down at 14K rpm for 25 minutes in a 4°C microcentrifuge.
13. Carefully remove supernatant and wash pellet with 750µl of room temperature 75% EtOH. Allow the RNA pellet to air dry then dissolve the RNA in total of 5.7µl of DEPC-treated water (we used Ambion's nuclease-free water).

5' Adaptor Ligation and Purification

1. Heat the RNA for 30 seconds at 90°C and then snap cool on ice.
2. Set up the 5?Adaptor ligation reaction in a 1.5 ml RNase free silconized microfuge tube:

    - Purified miRNA from step 1.12 5.7µl
    - 5' RNA adaptor (5 µM) 1.3µl
    - 10X RNA ligation buffer 1µl
    - T4 RNA ligase (10 U/µl) 1µl
    - RNAguard? (40 U/µl) 1µl

3. Incubate at 37°C for 1 hour. (Or at +20°C for 6 hours then 4°C overnight in a PCR machine).
4. Stop reaction by adding either 10µl 2x Gel Loading Buffer II or 10µl 100% formamide and 3µl loading dye. Heat the ligated sample/loading buffer mixture at 65°C for 5 minutes prior to loading gel.
5. Prerun the 15% TBU gel (0.5X TBE 0.75mm Biorad Mini-Protean II gel) for 30 minutes at 200V. See recipe in step 1. Wash the wells with TBE buffer prior to loading.
6. Load the ligated samples and the **40-60nt** ladder (10µl of prepared ladder) in wells with at least 1 space in between ladder and samples. It is advisable to run each sample with its own ladder and cut the gel before staining OR run each sample on separate gels to avoid contamination amongst samples.
7. Run gel at 150V until the xylene cyanol is near the bottom of the gel (about 2hours: the xylene cyanol runs at about 40nt on this gel). Stain the ladder portion of the gel OR each sample with its own ladder using 0.5X TBE/ EtBr.
8. Cut out the gel band corresponding to 40-60 nt with a clean razor blade (after aligning the stained ladder back to the gel if ladder was cut away separately) and transfer the

gel slice to a 0.5 ml RNase-free microfuge tube whose bottom has been punctured 3 to 4 times by a 21 gauge needle.

9. Place this tube into a 2 ml Rnase-free round-bottom microtube and spin the gel through the hole into the 2 ml tube at full speed for 2 min.
10. Add 500µl of sterile 0.3 M NaCl to the tube and elute the RNA by rotating the tube overnight at 4°C.
11. Transfer the eluate and the gel debris into a Spin-X Cellulose Acetate filter and spin at full speed for 2 minutes.
12. Wash the gel bits once more with 100µl 0.3M NaCl and spin for another 2 min. Collect the 100µl eluate in the same tube.
13. Add an equal volume of 100% Isopropanol (~600µl) and 3µl of glycogen to the sample and incubate at ?80°C for 20-30 minutes.
14. Spin down at 14K rpm for 30 minutes in a 4°C microcentrifuge.
15. Carefully remove supernatant and wash pellet with 750µl of room temperature 75% EtOH. Allow the RNA pellet to air dry then dissolve the RNA in total of 6.4µl of DEPC-treated water (we used Ambion?s nuclease-free water).

3' Adaptor Ligation and Purification

1. Heat shock the RNA by putting at 90°C for 30 seconds. Snap cool on ice.
2. Set up the 3' Adaptor ligation reaction in a 1.5 ml siliconized tube:

    - Purified 5' ligation product from step 2.15 6.4µl
    - 3' RNA adaptor (10µM) 0.6µl
    - 10X RNA ligation buffer 1µl
    - T4 RNA ligase (10 U/µl) 1µl
    - RNAguard (40 U/µl) 1µl

3. Incubate at 37°C for 1 hour (or overnight at +20°C for 6 hours then 4°C incubation).
4. Stop reaction by adding 10µl 2x Gel Loading Buffer II OR 10µl 100% formamide plus loading dye. Heat ligated sample/loading buffer mixture at 65°C for 5 minutes prior to loading gel.
5. Prerun a 0.5X 10% Urea gel for 30 minutes at 200V. Wash the wells with 0.5X TBE.

    12.5 ml makes two 0.75mm gels

    1. 5.25g Urea
    2. 625µl 10X TBE
    3. 3.125 ml 40% acrylamide (19:1 acrylamide:bis-acrylamide)
    4. Dissolve urea in 12.5 ml water, then
    5. Add 87.5µl 10% APS
    6. 4.4µl TEMED
    7. Pour immediately

1. Load the ligated samples and 10µl of the **60-80 nt** ladder in wells. It is advisable to either run all samples on separate gels or run the ladder with each sample and cut the gel into individual pieces before staining to avoid contamination. Run gel at 150V until the xylene cyanol is above the bottom of the gel (on a 10% gel it runs around 55 nt). Stain the gel or the ladder portion of the gel with 0.5X TBE / EtBr.

2. Cut out the gel band corresponding to 60-80 nt with a clean razor blade and transfer to a 0.5 ml RNase-free microtube whose bottom has been punctured 3 to 4 times by a 21 gauge needle.
3. Place this tube into a 2 ml RNase-free round-bottom microtube and spin the gel through the hole into the 2 ml tube at full speed for 2 min.
4. Add 500µl of sterile 0.3 M NaCl to the tube and elute the RNA by rotating the tube overnight at 4°C.
5. Transfer the eluate and the gel debris into a Spin-X Cellulose Acetate filter and spin at full speed for 2 minutes.
6. Wash the gel bits once more with 100µl 0.3M NaCl and spin for another 2 min. Collect the 100µl eluate in the same tube.
7. Add an equal volume (~600µl) of 100% Isopropanol and 3µl of glycogen to the sample and incubate at ?80°C for 20 to 30 minutes.
8. Spin down at 14K rpm for 30 minutes in a 4°C microcentrifuge.
9. Carefully remove supernatant and wash pellet with 750µl of room temperature 75% EtOH. Allow the RNA pellet to air dry then dissolve the RNA in total of 4.5µl of DEPC-treated water (we used Ambion?s nuclease-free water).

RT-PCR of small RNAs ligated with adaptors

1. Set up a reverse transcription reaction in a 1.5 ml RNase-free microfuge tube:

    - Purified ligated RNA from step 3.15 4.5 µl
    - Small RNA RT-primer (100 µM) 0.5 µl

2. Heat to 65°C for 10 minutes, spin down to cool.
3. Add following in order:

    1. 5X First-Strand buffer 2.0 µl
    2. 12.5 mM dNTP mix 0.5 µl
    3. 100 mM DTT 1 µl
    4. RNAguard(40U/µl) 0.5 µl

1. Heat to 48°C for 3 min and then add 1.0 µl of SuperScript? II RT (200U/µl).
2. Incubate at 42°C for 1 hour.
3. Set up pilot 20 µl PCR reactions from the Reverse Transcription samples to check and see if the cloning steps worked. I normally clone and manually sequence from this reaction. If everything looks good, then I scale up for the final PCR. Also, the number of PCR cycles can be optimized here. Start with 15 cycles and then go up or down depending on the strength of the product. **Another way is to do the large scale PCR, then take an aliquot of the PCR product and clone it into pgemT. The uncloned portion will then be ready for sequencing. Please go to section 6 if you wish to proceed in this way.**

    NOTE: If ready for the final PCR reaction, skip to section: Scaled-up PCR for final cloning.

    - RT reaction mix from step 4.5 1µl
    - 5X Phusion™ HF Buffer 4 µl

- Small RNA PCR Primer 1 (10uM) 0.25 µl
- Small RNA PCR Primer 2 (10uM) 0.25 µl
- 2.5 mM dNTP mix 2.0 µl
- Phusion™ DNA Polymerase 0.2 µl
- H_2O 12.3 µl

1. PCR Conditions:

   - 98°C 30 sec
   - 98°C 10 sec |
   - 58°C 30 sec |15 Cycles of PCR
   - 72°C 20 sec |
   - 72°C 5 min
   - 4°C forever

2. To manually clone the PCR product, add 1µl of Taq to the above PCR reaction after it has finished. Incubate at 72°C for 15 min. This adds A?s to the end and allows for pgem cloning. Phusion polymerase does not add As.
3. Pour a 1.5 mm 6% **TBE native** gel in the Biorad MiniProtean II to visualize the PCR products. It is advisable to use 1 ladder per sample or cut the ladder off and stain separately or run individual gels for each sample to avoid contamination.

   25 ml makes two 1.5 mm gels or four 0.75mm gels

   1. 1.25 ml 10X TBE
   2. 3.75 ml 40% acrylamide (19:1 acrylamide:bis-acrylamide)
   3. Water to 25 ml
   4. Add 175 µl 10% APS
   5. 8.75 µl TEMED
   6. Pour immediately

1. After the PCR is finished, (and after the As have been added if you chose to do so), add 4 µl of DNA loading buffer to 20µl of your PCR sample and load into 6% non-denaturing gel. Also, load 0.5µg (5µl) of the Fermentas 100bp ladder along with 4µl of the SIGMA 20bp low ladder dilution. It is not necessary to prerun this gel.
2. Run the gel until the xylene cyanol is 2/3 of the way down the gel. XC runs at 106nt on this gel.
3. Stain the gel in 0.5X TBE/EtBr buffer for 5 min.
4. Visualize the gel using UV. Cut out the 92bp band with a clean razor and put band into a 0.5ml microfuge tube whose bottom has been punctured 3 to 4 times by a 21 gauge needle. NOTE: The adapter-adapter band runs at 70 nt.
5. Place this tube in a 2ml round-bottom microfuge tube and spin the gel through the holes into the 2ml tube by centrifuging 2 min at top speed.
6. Add 300µl Elution Buffer (10mM Tris-HCl pH7.9; 10mM MgC_{l2}; 50mM NaCl) to the gel debris and shake a 30°C for 1 hour and then put to rotate at 4°C overnight (we have also done JUST 4°C overnight).

7. Transfer the eluate and the gel debris into a Spin-X filter. Spin for 2 min at full speed. Add another 100µl of elution buffer to wash the gel debris and spin for another 2 min at full speed.
8. Precipitate the DNA with 40 µl 3M NaOAc pH 5.2; 1 ml 100% Ethanol, 3µl glycoblue.
9. Centrifuge at top speed for 25 min. Wash the glycoblue pellet with 1 ml 70% EtOH then let dry and resuspend in 6µl water.

pGEM T-cloning.

1. Mix the following components:

    - Pgem T vector 1µl
    - 2X ligation buffer 5µl
    - Small RNAs from step 4.18 3µl
    - T4 DNA ligase 1µl

2. Incubate at room temperature for 1 hour or 4°C overnight.
3. Transform competent E. coli using method of your choice.
4. PCR colonies using M13F/M13R primers. I sequence 48 inserts per cloning reaction to get the first idea if the small RNA cloning worked. If results look good, scale up the PCR reaction and repeat (comment 4).

Scaled-up PCR for final cloning.

1. After determining that your small RNA cloning worked or trying everything right from the beginning, set up a 200 µl PCR reaction from using your reverse transcription samples. The number of PCR cycles should have been empirically determined in step 4 (above).

    - RT reaction mix from step 4.5 4 µl
    - 5X Phusion? HF Buffer 40 µl
    - Small RNA PCR Primer 1 (10uM) 1 µl
    - Small RNA PCR Primer 2 (10uM) 1 µl
    - 2.5 mM dNTP mix 20 µl
    - Phusion? DNA Polymerase 2.0 µl
    - H_2O 132 µl

    PCR Conditions:

    - 98°C 30 sec
    - 98°C 10 sec |
    - 58°C 30 sec |15 Cycles of PCR (or whatever you want to use)(comment 5)
    - 72°C 20 sec |
    - 72°C 5 min
    - 4°C forever

2. Precipitate the PCR product with 1/10 volume 3M NaOAC, 2.5 volumes EtOH, 3µl glycoblue overnight at -20 or 15 min at -80. Resuspend in 20 µl water and then add 10 µl of DNA loading buffer. Your samples are ready to be loaded onto a 6% native gel.

Gel purification if doing high throughput sequencing

1. Pour a native 6% 1.5mm native gel (6% 0.75mm gels are VERY thin and difficult to handle). Prepare the ladder if necessary. We use 2µl of the Sigma 20bp ladder (4µl of dilution) and 0.5µg (5µl of the dilution) of the 100bp Generuler. The lowest size on the gene ruler ladder is 100bp so it helps to locate which band is 100bp on the SIGMA 20bp ladder.

   25 ml makes two 1.5mm gels

   - 1.25 ml 10X TBE
   - 3.75 ml 40% acrylamide (19:1 acrylamide:bis-acrylamide)
   - Water to 25 ml
   - Add 175 µl 10% APS
   - 8.8 µl TEMED
   - Pour immediately

2. Load your precipitated PCR samples into 2 consecutive lanes so as not to overload the lanes. For each different sample, I would run a separate ladder so that the gels can be cut and stained separately OR run individual gels for individual samples to avoid contamination.
3. Stain the gels in 0.5X TE /EtBr in a clean container for 4 to 5 minutes.
4. Cut out ~92 bp band with a clean razor blade and put band into a 0.5 ml microtube whose bottom has been punctured 3 to 4 times by a 21 gauge needle. The adaptor-adaptor band runs around 70nt.
5. Place this tube into a 2 ml round-bottom microtube and spin the gel through the holes into the 2 ml tube (2 min spin at full speed in microfuge).
6. Add 300 µl of elution buffer to the gel debris and elute the DNA by shaking the tube gently at 30°C for 1 hour and then rotating overnight at 4°C (we?ve also done 4°C overnight).
7. Transfer the eluate and the gel debris into a Spin-X filter. Spin the filter in the microfµge for 2 minutes at full speed.
8. Add another 100 µl of elution buffer to the gel debris and then spin for another 2 min at top speed to collect the extra eluate in the same tube.
9. Add 1 µl of Pellet Paint® (that has been brought to room temperature and vortexed very well), 40 µl of 3 M NaOAc and 1000ml of EtOH. Put at -20 for 1 hour to overnight or at -80 for 20 minutes. Spin at 14K for 25 mins to pellet.
10. Wash with 750 µl of RT 70% EtOH, speed vacuum dry, and resuspend in 15µl of sterile resuspension buffer (10mM Tris-HCl pH 8.5).

Quantification of small RNA PCR products.

1. Mix 1 µl of the resuspended small RNA PCR product with 2 µl of water for nanodrop determination. Do the same with 1µl of resuspension buffer + 2µl of water for the blank.
2. Take the following Nanodrop readings. Remember that your sample is a 3X dilution, so multiply the final concentration by 3.

   - 230
   - 260

- 280
- 260/280 (want 1.8 for pure DNA)
- 260/230
- ng/µl (see section "Materials & Reagents" for correction factor used for the Pellet Paint)

3. Mix 1 µl of your PCR product with 4 µl water and 5 µl DNA loading dye. Run your samples on a 10% native gel along with 0.5 µg (5µl of the dilution) of the 100bp Generuler Fermentas Ladder. If 0.5µg (5µl) is loaded, the 100bp band is 40 ng. Use the Quantity One software to determine the concentration of your PCR products.
4. Take an average reading between the nanodrop and the BioRad quantification. You will use this number to determine the Molarity of your library (comment 6).
5. Determine the molarity of your library

    - Size of cloned small RNAs (average is 93bp for Arabidopsis small RNAs)
    - Molecular mass = 93bp x 650 = 60450
    - 60450 g = 1 mol or 60450 µg = 1 µmol
    - Concentration of library in µmol/ml = [ng/µl]/60450

      i. Example, if PCR product is 16ng/µl. The concentration of the library is 16/60450 = 0.000264 µmol/ml = 0.264 µmol/L or 264nM

Materials & Reagents

SuperScript™ II Reverse Transcription Kit (10,000 Units)	Supplier: Invitrogen Catalog #: 18064-014
RNAguard 5000U	Supplier: GE Healthcare Catalog #: 27-0815-01
Generuler 100bp ladder	Supplier: Fermentas
20bp ladder low ladder	Supplier: SIGMA Catalog #: P1598-40UG
Phusion™ High Fidelity DNA Polymerase (100U)	Supplier: NEB Catalog #: F-530S
dNTP Solution Set (4x25µm)	Supplier: Invitrogen Catalog #: 10297-018
T4 RNA Ligase (50 U)+10X buffer	Supplier: Promega Catalog #: M1051
Glycoblue 15mg/ml	Supplier: Ambion Catalog #: AM9516
Nuclease Free Water	Supplier: Ambion Catalog #: AM9938
RNase-free 2 mL microfµge tube	Supplier: Ambion Catalog #: AM12425
Non-stick RNase-free 0.5 mL microfµge tube	Supplier: Ambion Catalog #: 12350
Pellet Paint®NF Co-precipitant	Supplier: VWR Catalog #: 70748-4
Spin-X Cellulose Acetate Filter (2 mL, 0.45 µm)	Supplier: Fisher/SIGMA Catalog #: MPA-150-

	040Q/CLS8162
mirVANA microRNA isolation kit	Supplier: Ambion Catalog #: AM1561
1.5ml Siliconized eppendorf tubes	Supplier: SIGMA Catalog #: T4816-250EA
2x Gel Loading Buffer II	Supplier: Ambion Catalog #: AM8546G
pGEM T-easy cloning vector kit	Supplier: Promega Catalog #: A1360
Big Dye v. 3.1	Supplier: ABI
EtOH	
Isopropanol	
0.5X TBE diluted from 10X TBE stock	
0.3 M NaCL diluted from 5M stock	
3 M NaOAC pH 5.2	
Resuspension Buffer (10 mM Tris-HCl, pH 8.5)	
Biorad Mini Protean II gel apparatus	
Deionized formamide	

Author Notes

1. Adapter specifications used in the Baulcombe lab. Please note, the sizes used in this protocol are based on the sizes of the adapter molecules and PCR primers. If you use primers/adapters of other sizes, then you need to adjust the sizes cut out of the gels accordingly.
    - 5? RNA adaptor = 26bp of an RNA:DNA hybrid molecule. The three 3? bases are RNA and the rest is DNA (ordered through Dharmacon). We dephosphorylated before use.
    - 3? RNA adaptor = 23bp of RNA/DNA hybrid molecule. The 5? base is phosphorylated. The three 5? bases are RNA the rest is DNA. The 3? end of the of the adapter has an inverted thymine moiety (iDT) (ordered through Dharmacon)
    - Small RNA RT-primer: DNA oligo complementary to the 3? adapter
    - Small RNA PCR Primer 1: identical to the RT primer
    - Small RNA PCR Primer 2: We use a DNA oligo 44bp long that incorporates the 5? adapter but adds a tail at the 5? end to increase the length of the PCR product.

Reviewer Comments

Reviewed by: Eric Miska, Gurdon Institute, Cambridge

1. We use mirVana kit followed by gel purification. This allows precise size selection of small RNAs by preventing any smears from overloaded lanes on the gel.
2. Alternatively, the Criterion system can be used. We find it most important to exclude any cross-contamination of samples at this point. One sample per gel would be ideal.
3. We find eluting from an intact gel slice as effective and easier to handle. It is important to prevent any gel ?bits? to be included in the precipitation step.

4. We find it useful to pick colonies onto a rectangular agarose plate in an 8x12 grid. We the set up the PCR reactions using a 96 well replicator. Positive clonies can then easily be identified for sequencing.
5. More than 17 cycles will likely just result in empty amplification: you will get many identical reads instead of increased sequencing depth.
6. As concentration is absolutely critical, it would be useful to check samples additionally on a BioAnalyzer.

Figures

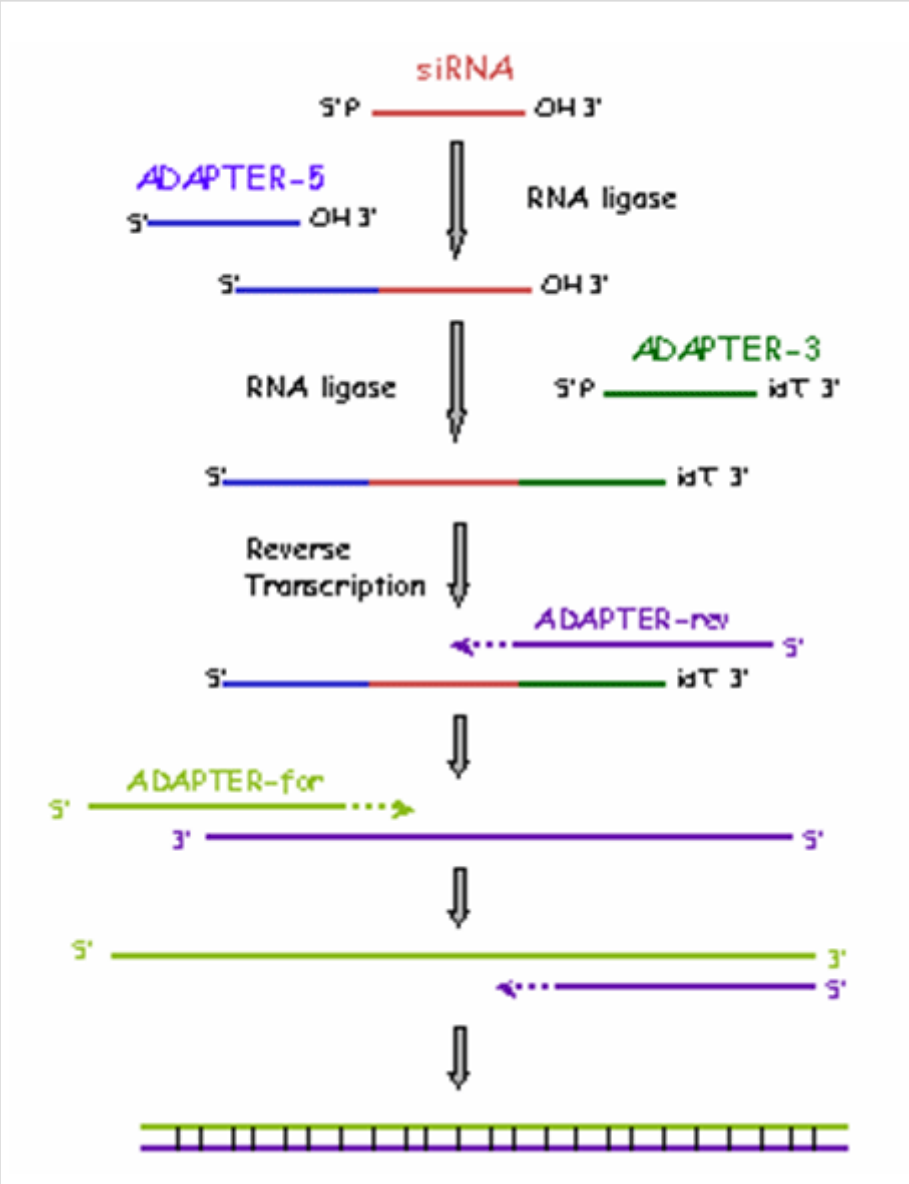

Figure 1. Overview over the flow of the cloning procedure