Preparation of Single-Stranded Tailed Templates for 454 Sequencing
Citation: Ruby et al., *Cell* 127: 1193-1207 (2006)

This protocol adapts the method of Williams and Bartel (*Nucleic Acids Res*. 23:4220) to generate single-stranded DNA suitable to go directly into the emulsion PCR step of 454 pyrosequencing. The protocol was first performed by Wendy Johnston (Bartel lab).

PCR product with one tailed primer and one poly A-extended tailed primer

Sequence of 5´ primer= 5´GCCTCCCTCGCGCCATCAGTATCGTAGGCACCTGAGA

Sequence of 3´ primer= 5´AAAAAAAAAAAAAAAAAAAAAAAAAA-AAAA/iSp18/GCCTTGCCAGCCCGCTCAGTATTGATGGTGCCTACAG
(purchased from Integrated DNA Technologies, Inc)

0.75 uL	100 uM 5´ primer
0.75 uL	100 uM 3´ primer
10 uL	10X PCR buffer (1X = 10 mM TRIS, pH8.4, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% Gelatin)
10 uL	10X dNTPs (1X = 0.2 mM each dNTP)
0.5 ul	template (PCR reaction from the miRNA/siRNA cloning protocol)
1 uL	Taq
77 uL	H20
100uL	

Each cycle of PCR:
1 min 94 degrees
1 min 50 degrees
1 min 72 degrees

Do 12-16 cycles of PCR, according to how quickly band comes up. Lowest number of cycles is desired.

Stop PCR with addition of EDTA to 2 mM .
Add NaCl to final concentration of 300 mM.
Add 2 volumes of EtOH.
Place at –20° for at least 2 hours.

Prepare 90% Formamide, 8% Acrylamide gel (1.5 mm thick)

Use 7.5 in. x 7.5 in. plates and 20-well comb (each lane is 0.5 cm wide)

In beaker, combine 10 mL 10X TBE, 400 mg BIS acrylamide, 90 ml formamide, and 7.6 g acrylamide. Stir w/ magnet on stir plate until solution is dissolved.

Sterile suction filter to remove any particles and oxygen. Store in light-blocking bottle.

When pouring gel, use 3X amount of APS and Temed. For 50ml gel, use 500 uL APS, 150 uL Temed. Mix vigorously. Polymerization is very slow. Best to let it polymerize O/N.

1X Formamide loading dye: 900 uL formamide
100 uL 10X TBE
10 uL dye-BB, XC

Gel purify PCR product:

Spin down precipitations: 30 min, full speed.
Resuspend in 12-15 uL 1x Formamide loading dye.

For each marker lane add 10uL formamide loading dye to 2 uL of 0.1 ug/uL 10-bp ladder (Invitrogen) in water.

Pre-run gel at 20 watts for 45 min.
Heat samples for 10 min at 85 degrees, including ladder.

Load on gel with ladder next to sample.
Run gel at 20 watts until BB runs off gel.

Lay gel on saran wrap in a container.
Stain with a solution of 1X TBE/ethidium bromide (about 20uL 10mg/ml ethidium bromide in every 200mls 1X TBE). Add only enough so that solution touches only the gel and saran wrap- not the dish. Staining will take approximately 45 min without shaking (with shaking, about 25 min.).

(If concerned about cross-contamination, load each sample on different gel. If less concerned, load multiple samples on same gel, each with a marker lane, then cut gel into pieces and stain each piece separately.)

Look at staining on UV light box.
There should be two bands, one at 130 bp, and one at 100 bp.
Make a cut in between the two bands (about 115 bp) and one about 15-20 bp above the 130 band, and one about 15-20 bp below the 100 band.

Elute the two pieces separately in 300mM NaCl O/N.

Ethanol precipitate higher bands as for PCR product above.
Wash pellet in cold 70% EtOH, spin again.
Speed vac any leftover formamide (30-60 min).

Resuspend pellet in 20 uL water.
Send to 454 for sequencing.