Library cloning protocol - mir30 context

Cloning shRNA libraries with a mir30 loop, into a modified pSicoR with a mir30 context.
Materials:

insert PCR: oligo mix template, Phusion polymerase, HF buffer, dNTPs, DMSO, primers (see methods for details).
Insert and vector digests: XhoI, EcoRI, NEB buffer 4, NEB buffer EcoRI, BSA
Insert and vector purification: QIAGEN MinElute PCR purification and gel-extraction kits, agarose gel, materials for a phenol-chloroform extraction.
Ligation and cloning: T4 DNA ligase and buffer, high-efficiency competent bacteria (I used library efficiency DH5a or MEGA-X DH10b), LB-Carb or LB-Amp plates (as large as possible).
Methods:
1. insert preparation: PCR, digest.
PCR: lab notebook 3: 19 feb – 20 march 2008

Primers:
5'-XhoI 40bp CAGAAGGCTCGAGAAGGTATATTGCTGTTGACAGTGAGCG
3'-EcoRI 35bp CTAAAGTAGCCCCTTGAATTCCGAGGCAGTAGGCA

PCR REACTION	50µl
water	31.5µl
Phusion HF buffer	10µl
10 mM dNTPs	1µl
5 µM primer mix	5µl
template	0.5µl
DMSO	1.5µl
Phusion polymerase	0.5µl

PCR PROTOCOL:
1x : 98ºC for 30s
15x : 98ºC for 10s, 72ºC for 3s
1x : 72ºC for 10 min, 4ºC HOLD

I usually do six 50µl reactions (plus water control), combine them and column-purify in one or two columns using the QIAGEN MinElute PCR Purification kit.
Expected product size – 140bp.
Digest: lab notebook 3: 19 feb – 20 march 2008

30µl reaction: 1µl of XhoI and EcoRI, 0.5-1µg PCR product, in NEB buffer 4 + BSA. Incubate at 37ºC overnight. This worked the first time I tried despite some mistakes, so there's probably room for improvement.

Expected products: 140bp PCR product, 8bp cut off by XhoI, 16bp by EcoRI – 116bp digest product.

Visualize on PAGE gel – I use Invitrogen 20% TBE gels, run at 200V for 2h. Again column-purify the product with QIAGEN MinElute kit.
2. vector preparation: digest, gel purification, phenol-chloroform extraction

The vector used for this is pSicoR-mir30, generated by Mike Bassik and me by PCRing the mir30 context from RHS1702 and cloning it downstream of mCherry in pSicoR-mCherry (using EcoRI and PciI sites, removing the EcoRI site in the process), and removing the shRNA cloning site from the pSicoR (XbaI and NotI digest, Klenow, ligation). See Lab Notebook 2, may 9 – june 25 2008.
Digest: lab notebook 3: 28 feb – 19 mar 2008

NEB buffer EcoRI + BSA. 150µl reaction with 5µg pSmir30, 5µl XhoI and 3µl EcoRI, overnight at 37ºC.

Careful – EcoRI tends to exhibit star activity. Could try adding less. Don't heat-inactivate!

No SAP – there's nearly no self-ligating background, and SAP decreases ligation efficiency.
Purification: lab notebook 3: 19-20 mar 2008

First gel-purify: run on 0.8% agarose gel (120V for about 2h, or 60V for about 4h for neater bands), cut out the bands, and use the QIAquick MinElute gel-extraction protocol.

Then do a phenol-chloroform extraction. I used phase-lock tubes: spin down the tubes for 2min, add 300µl sample and 300µl phenol, invert 20x, spin for 5min, remove top layer to a new tube. Then add 30µl 3M Na-Acetate pH 5.2, add 750µl 100% EtOH, invert several times to mix, incubate at -20ºC for 30min, spin for 2-10min at 4ºC, remove liquid (the pellet may be hard to see – to avoid disturbing it you can just leave the last 100µl of liquid and SpeedVac until it's gone), add 500µl 70% EtOH, spin for 2-10 min at 4ºC, remove liquid like above, resuspend in 20µl water or EB.
3. Cloning: ligation, transformation, maxiprep
Ligation: lab notebook 3: 28 feb and 20 mar 2008

Ligation – 10µl reaction: about 100-500ng vector (the purification yields are low, so I never have much vector – I try to use as much as I can), about 10ng insert, 1µl ligase buffer, 0.5µl T4 ligase, incubate at 16ºC overnight (in PCR machine).

Also do a –insert control: using the same amount of vector would be optimal, but I usually don't have enough vector, so I do 50-100ng vector in 10µl, 1µl buffer, 0.5µl ligase.
Test transformation: lab notebook 3: 29 feb and 24 mar 2008

First I do a test transformation: 20µl fresh DH5alpha bacteria, 0.5µl ligation, after heat-shock add 180µl SOC, incubate in shaker for 30min, plate 160µl on one pre-warmed 10cm plate and 16µl (diluted 10x) on another; do a +insert and control –insert transformation. Incubate at 37ºC overnight.

Count the colonies to check transformation efficiency (expect thousands) and uncut background (below 5%, or better below 1%). Sequence 100 colonies to make sure the results are good (the percentage of perfect sequence matches to the library is in the 60-80% range, all inserts are unique).

If everything looks good, go ahead with a large-scale transformation for a library maxiprep. Otherwise try changing the PCR conditions (reducing cycle number tends to help). The mir30-context hairpins tend to PCR and clone fairly easily though.
Large-scale transformation and maxiprep: lab notebook 3: 13 mar – 10 apr 2008

Do electrocompetent MegaX DH10b cell transformations – much higher efficiency. Decide how many colonies you need (usually 10-50x the size of the library; we did 30x for 55k libraries). The colony yield (number of colonies) from a 100µl DH10b transformation is 10-100x higher than a 20µl DH5a transformation, but it varies significantly, and estimates based on the test DH5a results aren't always accurate. It's frequently necessary to do a second transformation after getting a better yield estimate from the first one.

Use large plates (we have 24x24cm square ones). Dilute the transformation appropriately – you want about 20-100 thousand colonies per 24x24 plate in 0.3-0.5ml liquid, or about 5000 for a 10cm plate in 100-200µl of liquid. If you use too much liquid (which causes the colonies to "flow" into each other), or get too many colonies, your colonies will be touching instead of well-separated, which increases the bias – we want to avoid that. It's also frequently a good idea to plate a dilution series of the transformation on a few 10cm plates, to make colony counting easier, and to have good sequencing samples if we want sequence data from the same transformation as the final library (which is a good idea).

Do the transformations according to the protocol. Count them on the next day - make sure there are distinct colonies, not a lawn. Estimate the colony counts – if there are fewer than the desired coverage, do additional transformations, adjusting the amount plated for the colony yield.

Wash the colonies off the plates: add 5-10ml LB, gently scrape the colonies off the plate with a bent glass pipette (don't damage the agar!). When the bacteria look

well-suspended in the liquid, pipette it into a tube. Spin the tubes down, weigh the pellets, do a maxiprep or gigaprep depending on the yield.