Library cloning protocol - shRNA context

Cloning shRNA libraries (short loop, perfect complementarity), into a modified pSicoR vector under the normal U6 promoter.
Materials:

insert PCR: oligo mix template, Phusion polymerase, Phusion GC buffer, dNTPs, DMSO, primers (see methods for details).
Insert and vector digests: XhoI, BstXI, NEB buffer 3, 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, gel-purification
PCR: lab notebook 3: 2,15,26 june 2008

Primers:
5'-BstXI 19bp CGGAGAACCACCTTGTTGG (BstXI site is CCANNNNN|NTGG)
3'-XhoI 18bp GGAATTCGCCAGCTCGAG

PCR REACTION	50µl
water	27µl
Phusion GC buffer	10µl
10 mM dNTPs	1µl
5 µM primer mix	5µl
template	5.5µl
DMSO	1µl
Phusion polymerase	0.5µl

PCR PROTOCOL:

* 1x : 98ºC for 30s
* 15x : 98ºC for 10s, 72ºC for 30s
* 1x : 72ºC for 10 min, 4ºC HOLD

I did three 50µl reactions per library (plus water control), combined each library and column-purified using the QIAGEN MinElute PCR Purification kit (eluted in 50µl). Expected product size – 97bp.

For some of the libraries I had to do 15 PCR cycles to get enough product.
Digest: lab notebook 3: 15 and 26 june 2008

60µl reaction: take the 50µl of column-purified PCR product, add 6µl NEB buffer 3, 0.6µl BSA, 2µl XhoI, 2µl BstXI. Incubate at 37ºC for 6h.

I don't actually quantify my PCR product accurately – I do run it through the NanoDrop, but that gives really inaccurate readings at such low concentrations and with short oligos. If you want to quantify, run it on the BioAnalyzer.

Expected product sizes: 96bp PCR product, 12bp cut off by BstXI, 15bp by XhoI – 70bp digest product.
Gel-purification: lab notebook 3: 16 and 30 june 2008

First run small samples (about 10%) of the PCR and digest on PAGE gel just to make sure it worked.

Then run all of the digest product on PAGE gel (two 25µl lanes) – I use 20% Invitrogen gels, run at 200V for 2h. Gel-purify using Novagen D-tubes (Qiagen columns don't work with PAGE gels) - especially important if you see any unwanted bands. It substantially increases the cloning efficiency.

Column-purify afterwards – D-tube electroelution leaves product in TAE, which would mess up the ligation.
2. Vector preparation: digest, gel purification, phenol-chloroform extraction

The vector used for this is MP177/pSnew, a variant on pSicoR with a sticky BstXI cloning site replacing the blunt HpaI site (design and modification done by Greg Ku), and with added puro-T2A (by Robert Lebbink).
Digest: lab notebook 3: 28 feb, 18 june 2008

NEB buffer 3 + BSA. 25µl reaction: 5µg MP177, 1µl of XhoI and BstXI, incubate in 37ºC for 24h (I left it for 24h by accident the first time and it worked, so I keep doing that – overnight may well work fine too).
Purification: lab notebook: 21 apr, 27 june 2008

First gel-purify: run digest on 1% agarose gel (80V for about 3h), cut out bands, use QIAquick 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: 7 and 22 apr, 6-8 and 22 may, 4-16 june 2008

Ligation – 10µl total: about 500ng vector, about 20ng insert, 1µl ligase buffer, 0.5µl T4 ligase, incubate at 16ºC for 15h (overnight in PCR machine). Also do a no-insert control.
Test transformation: lab notebook 3: 8 and 23 apr, 7-12 and 27 may, 5-17 june 2008

First I do a test transformation: 20µl 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 30-100 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 increase the perfect hairpin percentage). For low colony counts, try transforming with different amounts of the ligation (0.1-10µl per 100µl bacteria). The colony counts and uncut background also tend to vary between different vector preps.
Large-scale transformation and maxiprep: lab notebook 3: 6 may, 11-30 june, 1-4 july 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.