Making RNA spike-ins, linearizing and in-vitro transcription

as created/adapted/written by Benjy

Linearized DNA, template for txn

1. Grow DH5alpha cells overnight with pSP64 poly(A) plasmid containing inserted spike-in sequence. Currently, this is DGP 104-6.
2. Miniprep plasmids, quantify.
3. Set up the reaction, at least two reactions per spike-in:

<table>
<thead>
<tr>
<th></th>
<th>10x EcoRI compatible buffer</th>
<th>EcoRI</th>
<th>3ug pSP64-INSERT-poly(A)</th>
<th>Water</th>
<th>TOTAL</th>
</tr>
</thead>
<tbody>
<tr>
<td>5ul</td>
<td>5ul</td>
<td>Xul</td>
<td>Yul</td>
<td>50ul</td>
<td></td>
</tr>
</tbody>
</table>

4. Incubate 2 hours each, 37C.
5. Run 1ul on ~1% gel to check. Make sure to run some non-linearized plasmid to compare for supercoiled and relaxed plasmid.
6. Assuming complete digestion, clean up reactions using PCR clean up kit. Make sure to use one miniprep column for each spike-in. This means pool the two reactions onto a single column. Quantify using nanodrop to have an idea for concentration. Concentration should be in the range of 100-200 ng/ul, when resuspend in 30ul.

For n reactions, add n+1 ul of rATP, rCTP, rGTP, and rUTP (10mM stocks) into clean tube to create "rNTP" (2.5mM each) as referenced below.

1. Add 6.25ul of linearized plasmid DNA. For a kit positive control, dilute 1ul of the provided standard with 5.25ul of water.
2. Set up master mix:

<table>
<thead>
<tr>
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<th>1x</th>
<th>10x</th>
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<tbody>
<tr>
<td>Txn optimized 5x buffer</td>
<td>4ul</td>
<td>40ul</td>
</tr>
<tr>
<td>DTT (100mM)</td>
<td>2ul</td>
<td>20ul</td>
</tr>
<tr>
<td>Recombinant RNasin</td>
<td>0.75ul</td>
<td>7.5ul</td>
</tr>
<tr>
<td>rNTP (as above)</td>
<td>4ul</td>
<td>40ul</td>
</tr>
<tr>
<td>4sUTP (10mM)</td>
<td>2ul</td>
<td>20ul</td>
</tr>
<tr>
<td>SP6 RNA Polymerase</td>
<td>1ul</td>
<td>10ul</td>
</tr>
<tr>
<td>TOTAL</td>
<td>13.75ul</td>
<td></td>
</tr>
</tbody>
</table>

3. Add 13.75ul of master mix to each tube of linearized DNA. Incubate 1hr in 30/37C water bath. Use 30C if you get multiple txn products, 37C if you need more product. (Darach- 2 hours at 30C works well for non-thiolated spikeins)
4. Remove 2ul into PCR tube for later gel.
5. Add 1ul of RQ1 RNase-Free DNase.
6. Incubate 15min at 37C.
8. Vortex vigorously and centrifuge at 12k RCF for 2min.
9. Transfer top aqueous layer to new eppendorf.
10. Add 1/10 volume (~1.8ul) 3M NaAcetate and <15ug of glycogen for a carrier.
11. Add 2.5 volumes (45ul) of absolute EtOH.
12. Chill 30min in -80C, or -20C overnight.
13. Spin down at full speed in cold room for ~25 min. Remove supernatant.
14. Wash with 1ml 70% EtOH. Spin down 5min. Remove and repeat.
15. Resuspend in 10ul PCR-grade H2O.

Denaturing Gel

Take your samples and run them on a gel. You can do either Denaturing gel (formaldehyde) or Non-denaturing RNA gel, non-denaturing gel isn't going to be accurate sizing but the spikeins don't appear to form secondary structures that alter their mobility.

Run both the before and after DNAse treatment samples. If they look good, proceed with the extraction and you've got your spike-ins.
Quantify on qubit, dilute. Benjy suggests using 4ng spikein (each) : 100ug total RNA, so 0.1ng/ul is a good working mix.

**ALTERNATIVES IN CLEAN UP**

Darach has fiddled around, and found that it's easier and works good to do an Ampure bead cleanup. Simply add 2x beads, incubate bench 5min, collect, wash beads twice with 80% EtOH, dry, resuspend in 20ul hyclone H₂O.

Also, you can just run a 1% agarose gel, non-denaturing. Works fine, I think.