Making spike-in transcripts for mRNA normalization

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Abstract

Citation: Darach Miller Making spike-in transcripts for mRNA normalization. **protocols.io** dx.doi.org/10.17504/protocols.io.fftbjnn
Published: 29 Jul 2016

Protocol

Preparing templates

Step 1.

Grow DH5alpha cells overnight with pSP64 poly(A) plasmid containing inserted spike-in sequence. Currently, this is DGP 104,105,106,229.

Step 2.

Miniprep plasmids, quantify. You'll need a lot of plasmid.

Step 3.

Set up the digestion reaction in 50ul 1x EcoRI compatible buffer with 3ug pSP64-NSERT-poly(A) and 5ul EcoRI. Incubate 2 hours each, 37C.

Benjy recommends you prepare at least two reactions per spike-in.

Save some plasmid for the gel below.

Step 4.

Pour a 1% agarose gel and run 100ng of digest and undigested plasmid, to check for cutting.

Step 5.

Assuming complete digestion, clean up reactions using PCR clean up kit, pooling repeats of the same plasmid digest at this stage. Quantify using nanodrop to have an idea for concentration. Concentration should be in the range of 100-200 ng/ul, when resuspended in 30ul.

in-vitro txn with SP6

Step 6.

Prepare a rNTP mastermix of rATP, rUTP, rCTP, rGTP, 2.5mM each (so 10mM rNTPs in total).

Set up SP6 reaciton master mix:

	1x	5x
Txn optimized 5x buffer	4ul	
DTT (100mM)	2ul	
Recombinant RNasin	0.75ul	
2.5mM each rNTP (as above)	4ul	
4tUTP (10mM)	2ul	
SP6 RNA Polymerase	1ul	

Step 7.

Make new tubes of 6.25ul of linarized plasmid DNA. For a kit positive control, dilute 1ul of the provided standard with 5.25ul of water.

Step 8.

Add 13.75ul of SP6 reaction 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 says 2 hours at 30C works well for non-thiolated spikeins)

Step 9.

Remove 2ul into PCR tube for later gel.

To the rest, add 1ul of RQ1 RNase-Free DNase. Incubate 15min at 37C.

Step 10.

Add 180ul water to make the next step easier. Add equal volume (200ul) saturated phenol.

Vortex vigorously and centrifuge at 10,000g for 1min.

Step 11.

Transfer top aqueous layer to new eppendorf.

Do an ethanol precipitation, that is add 1/10 volume (20ul) 3M NaAcetate, 10ug of glycogen, 2.5 volumes (500ul) of absolute etOH.

Step 12.

Put on ice for between 20min to 1 hr.

Step 13.

Spin down at full speed in cold room for 15 min.

Step 14.

Remove supernatant, wash with 1ml 80% EtOH. Spin down 5min.

Step 15.

Remove supernatant, wash with 1ml 80% EtOH. Spin down 5min.

Step 16.
Resuspend in 10ul PCR-grade H20.

Step 17.

Run 2ul on an agarose gel, alongside the 2ul that you saved before.

You can do either Denaturing gel (formaldehyde) or Non-denaturing RNA gel. A non-denaturing gel isn't going to be accurate sizing but the spikeins don't appear to form secondary structures that alter their mobility and Darach's found it to work fine.

Step 18.

Quantify on qubit, dilute. 0.1ng/ul is a good working mix.

Benjy suggests using 4ng spikein (each) : 100ug total RNA. Darach's had success with 1ng for 5e7 cells (for qPCR normalization).