nanoCAGE-XL Protocol

All reagents were from Sigma-Aldrich (https://www.sigmaaldrich.com) and mixtures were prepared on ice using nuclease-free plastics and reagents, unless otherwise noted.

Primer Sequences Used

Experiment 1

Template switching (TS): 5'-TAG TCG AAC TGA AGG TCT CCA GCA rGrGrG-3'

Reverse transcription (RT): 5'-TAG TCG AAC TGA AGG TCT CCG AAC CGC TCT TCC GAT CTN NNN NN-3'

Forward second-strand: 5'-TAG TCG AAC TGA AGG TCT CCA GC-3'

Reverse second-strand: 5'-TGA CGT CGT CTA GTC GAA CTG AAG GTC TCC GAA CC-3'

Forward library PCR: 5'-AAT GAT ACG GCG ACC ACC GAG ATC TAC ACT AGT CGA ACT GAA GG-3'

Reverse library PCR: 5'-CAA GCA GAA GAC GGC ATA CGA GAT CGG TCT CGG CAT TCC TGC TGA ACC GCT CTT CCG ATC T-3'

Sequencing primer: 5'-TAG TCG AAC TGA AGG TCT CCA GCA-3'

Experiment 2

Template switching-1 (TS-1): 5'-TAG TCG AAC TGA AGG TCT CCA GCA ATC GTG GCT ATA rGrGrG-3'

Template switching-2 (TS-2): 5'-TAG TCG AAC TGA AGG TCT CCA GCA GAT CGA GCT ATA rGrGrG-3'

Template switching-3 (TS): 5'-TAG TCG AAC TGA AGG TCT CCA GCA TCG AGC GCT ATA rGrGrG-3'

Reverse transcription (RT): same as Exp.1

Forward second-strand: same as Exp. 1

Reverse second-strand: same as Exp. 1

Forward library PCR: same as Exp. 1

Reverse library PCR: 5'-CAA GCA GAA GAC GGC ATA CGA GAT GTG ACT GGA GTT CAG ACG TGT GCT CTT CCG ATC T-3'

Sequencing primer: same as Exp. 1

Experiment-3

Template switching-1 (TS-1): 5'-TAG TCG AAC TGA AGG TCT CCA GCA CGA TGT rGrGrG-3'

Template switching-2 (TS-2): 5'-TAG TCG AAC TGA AGG TCT CCA GCA TGA CCA rGrGrG-3'

Template switching-3 (TS-3): 5'-TAG TCG AAC TGA AGG TCT CCA GCA GCC AAT rGrGrG-3'

Template switching-4 (TS-4): 5'-TAG TCG AAC TGA AGG TCT CCA GCA CAG ATC rGrGrG-3'

Template switching-5 (TS-5): 5'-TAG TCG AAC TGA AGG TCT CCA GCA CTT GTA rGrGrG-3'

Template switching-6 (TS-6): 5'-TAG TCG AAC TGA AGG TCT CCA GCA CCG TCC rGrGrG-3'

Reverse transcription (RT): same as Exp. 1, Exp. 2

Forward second-strand: same as Exp. 1, Exp. 2

Reverse second-strand: same as Exp. 1, Exp. 2

Forward library PCR: same as Exp. 1, Exp. 2

Reverse library PCR: same as Exp. 2

Sequencing primer: same as Exp. 1, Exp. 2

First Strand cDNA synthesis

Prepare Mix-1 by combining:
4 µl of primer/sorbitol//trehalose solution prepared as in (Salimullah et al., 2011)
x µl (~200 ng) of rRNA-depleted RNA
x µl of H_2O to a final volume of 8 µl.

Incubate at 65^{o}C for 10 min in a thermocycler, and cooled in ice water bath for 5 min.

Prepare Mix-2 by combining:
8 µl of 5 X Prime Script buffer
2.5 µl of 10 mM dNTPs
4 µl of 0.1 M DTT
6 µl of 5 M Betaine
4 µl of Prime Script Enzyme
x µl of H_2O to a final volume of 32 µl

Combine Mix-1 and Mix-2, and incubate 10 min at 22^{o}C, 30 min at 40^{o}C, and 15 min at 75^{o}C; snap cool in ice water bath for 5 min. Purify Agencourt RNA Clean XP magnetic beads (Beckman Coulter; https://www.beckmancoulter.com) following the manufacturer's instructions. Elute with 80 µl H_2O.

Quantitative Real-Time PCR

Using SYBR Premix Ex Taq kit (TaKaRa; http://www.clontech.com/takara), for each sample, prepare in triplicate 8.5 µl mix by combining:

5.0 µl of 2X *SYBER* Premix *Ex Taq*
0.1 µl of 10 µM forward second-strand PCR primer
0.1 µl of 10 µM reverse second-strand PCR primer
0.2 µl of 50X ROX Reference Dye
3.1 µl of H2O

Add 1.5 µl of purified cDNA or water (as negative control). Run PCR for 1 cycle at 95^{o}C for 1 min; 30 cycles of 95^{o}C for 15 sec, 65^{o}C for 10 sec, 68^{o}C for 2 min; hold at 12^{o}C. Determine the cycle threshold for each sample (usually 13-16 cycles) and negative control (usually no amplification), and calculate the optimal number of cycles by adding 4 (usually 17-20 cycles).

Second-Strand cDNA Synthesis

Prepare 400 µl of PCR mix by combining:

40 µl of X10 *Ex Taq* buffer
8 µl of 10 mM dNTPs
4 µl of 10 µM forward second-strand PCR primer
4 µl of 10 µM reverse second-strand PCR primer
2 µl of *Ex Taq* polymerase
282 µl of H_2O
60 µl of cDNA

Amplify as in real-time PCR using the determined number of cycles. Purify with Agencourt AMPure XP beads (Beckman Coulter; https://www.beckmancoulter.com) following the manufacturer's instructions. Elute with 120 µl of H_2O. Determine concentration by NanoDrop spectrophotometer and by Qubit dsDNA HS Assay Kit (Molecular Probes; http://www.lifetechnologies.com) following the manufacturer's instructions. Concentration should be ≥10 ng per µl by Qubit.

Dilute samples to 10 ng per µl by the Qubit values

Note: High rRNA content may result in the generation of 'spiky' libraries and a loss of sequencing information (see Supplementary Figure 1). To verify the library profile, second-strand cDNA can be prepared in a 10% format (40 µl) and analyzed by Agilent Bioanalyzer before performing a full-scale format.

Addition of Sequencing Adapters

For each library, prepare 700 µl of PCR mix:

140 µl of HF buffer
14 µl of 10 mM dNTPs
14 µl of forward library primer
14 µl of reverse library primer
7 µl of Phusion polymerase (Thermo Scientific; http://www.thermoscientificbio.com)
399 µl of H_2O
112 µl of diluted second-strand cDNA

Run PCR for 1 cycle at 98°C for 1 min; 1 cycle at 98 °C for 15 sec, 55 °C for 10 sec, 68 °C for 2 min; 8 cycles of 98°C for 15 sec, 65°C for 10 sec, 68°C for 2 min; hold at 12°C.

Exo I Nuclease Digestion

Combine samples, and add 4 µl of Exo I, mix, incubate at 37°C for 30 min.

To purify the resulting product, mix with 5 volumes (3.5 ml) of PB buffer (Qiagen; http://www.qiagen.com) and purify through a single column using a PCR-purification kit (Qiagen; http://www.qiagen.com) following the manufacturer's instructions. Elute with 25 µl of water.

Library quantification

Determine concentration by NanoDrop spectrophotometer (usually ~50-100 ng per µl). Dilute

1 µl to 15 µl with water, and analyze library size by Agilent Bioanalyzer (usually ~1,400-1,700 bp). Libraries are used for qPCR analysis without further dilution, and library molarity calculated based on the obtained values (usually ~2-4 nM). Libraries were sequenced at concentrations of 1.3 to 2.3 nM.