

nanoCAGE – DAY 1

RNA samples:



Before start checklist:

- Pipettes 1000 µL, 200 µL, 10 µL, 2 µL
- Filtered Tips Low-bind
- 0.2 mL PCR tubes low-bind + caps
- 1.5 mL microcentrifuge tubes low-bind
- 96-well PCR plate low-bind
- 96-well Real-Time PCR plate
- Ultra-Pure Water
- Magnet for beads separation
- AMPure XP beads, resuspended and at room temperature

- Freshly prepared 70% EtOH, at room temperature
- Terminator Exonuclease (1 U/µL) / SuperScript III Reverse Transcriptase (200 U/µL) / Takara SYBR Premix Ex Taq (2x), in freezer until needed
- SuperScript III First-Strand Buffer (5x) / DTT (0.1 M) / dNTPs (2.5 mM) / Betaine (5 M), on ice
- Trehalose/Sorbitol stock solution (3.3 M/0.66 M) / Reverse Transcription Random primers (100 µM) / Template Switching Oligonucleotides (1 mM), on ice
- cDNA PCR primers Forward and Reverse (10 µM) / Rox Reference Dye II (50x), on ice
- RNA samples, in freezer until needed

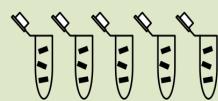
WORKFLOW

INSTRUCTIONS

NOTES/OBSERVATIONS

TIME/DATE

RNA samples

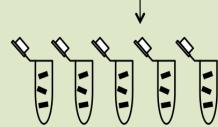


- ≥ 50 ng/µL of total RNA per sample or
- ≥ 500 ng/µL of total RNA per sample, if performing the Terminator treatment

- Use in preference high quality total RNA to optimize nanoCAGE library preparation for best sequencing results

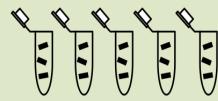
optional step

Terminator-treated RNA samples



- (optional step) Add per sample:
 - 1.3 µL of SuperScript III First-Strand Buffer (5x)
 - 0.2 µL of Water
 - 0.5 µL of Terminator Exonuclease (1 U/µL)
 Mix by pipetting + spin down
 Incubate at 30°C for 1 h

RNA samples + primers premix

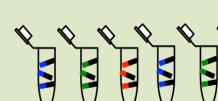


- For each total RNA sample, add:
 - 1 µL of a primers premix stock solution
 - 1 µL of total RNA
 Mix by pipetting 10 times carefully + spin down
 Incubate at 65°C for 10 min
 Transfer on ice for at least 2 min

- Add per sample:
 - 2 µL of SuperScript III First-Strand Buffer (5x)
 - 1 µL of DTT (0.1 M)
 - 2.5 µL of dNTPs (2.5 mM)
 - 1.5 µL of Betaine (5 M)
 - 1 µL of SuperScript III (200 U/µL)
 Incubate at 22°C for 10 min, 50°C for 30 min, 75°C for 15 min, and hold at 4°C

- Use a different barcode (primers premix solution) for each RNA sample and for each sample replicate
- Prepare the primers premix solutions in advance by mixing 1 µL of a Template Switching Oligonucleotide (1 mM) + 1 µL of Reverse Transcription Random primers (100 µM) + 8 µL of Trehalose/Sorbitol stock solution (3.3 M/0.66 M) – [store at -20°C]
- Trehalose/Sorbitol solutions are viscous; mix very carefully by pipetting up and down slowly for at least 10 times
- Prepare a master mix for the RT reaction
- Prepare a positive control of the RT reaction by using 1 µL of a control RNA sample
- Prepare a negative control of the RT reaction by using 1 µL of water

First-strand cDNAs



- Add 18 µL of AMPure XP beads at RT
 Mix by pipetting 10x slowly and incubate for 5 min

- Aspirate supernatant carefully

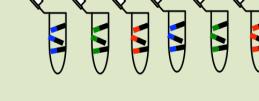
- In order to avoid losing the sample, be sure not to aspirate beads in the pipette tip together with the solution



Wash 3x 200 µL

- Keep on magnet, wash 3x with 200 µL fresh 70% EtOH, do not disturb the pellet
- Resuspend pellet in 30 µL water, incubate at RT for 5 min
- Pellet beads on magnet, elute purified first-strand cDNAs and transfer to new PCR tubes low-bind

Purified first-strand cDNAs



- Mix per qPCR replicate:
 - 5 µL of Takara SYBR Premix Ex Taq (2x)
 - 0.1 µL of cDNA PCR primer, Forward (10 µM)
 - 0.1 µL of cDNA PCR primer, Reverse (10 µM)
 - 0.2 µL of Rox Reference Dye II (50x)
 - 3.1 µL of Water
 - 1.5 µL of purified first-strand cDNA

- Analyze each first-strand cDNA and control samples in triplicates

- Prepare a master mix for the real-time qPCR reaction

- Prepare a negative control of the real-time qPCR reaction by adding 1.5 µL of water

