

nanoCAGE – DAY 1

RNA samples:

Before start checklist:

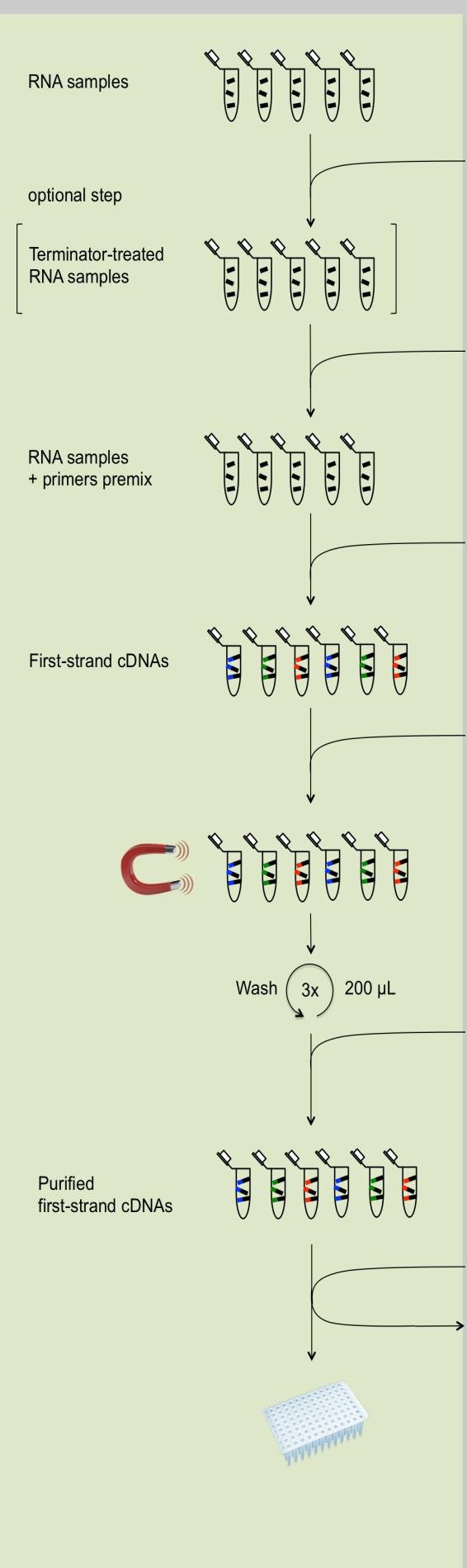
- Pipettes 1000 µL, 200 µL, 10 µL, 2 µL
- Filtered Tips Low-bind
- 0.2 mL PCR tubes low-bind + caps
- 1.5 mL microcentrifuge tubes low-bind
- 96-well PCR plate low-bind
- 96-well Real-Time PCR plate
- Ultra-Pure Water
- Magnet for beads separation
- AMPure XP beads, resuspended at room temperature
- 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 (0.66 M/3.3 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

	<ul style="list-style-type: none"> <input type="checkbox"/> ≥ 50 ng/µL of total RNA per sample or <input type="checkbox"/> ≥ 500 ng/µL of total RNA per sample, if performing the Terminator treatment 	<ul style="list-style-type: none"> - Use in preference high quality total RNA to optimize nanoCAGE library preparation for best sequencing results 	
	<p>(optional step)</p> <p>Terminator-treated RNA samples</p> <ul style="list-style-type: none"> <input type="checkbox"/> 1.3 µL of SuperScript III First-Strand Buffer (5x) <input type="checkbox"/> 0.2 µL of Water <input type="checkbox"/> 0.5 µL of Terminator Exonuclease (1 U/µL) <p>Mix by pipetting + spin down Incubate at 30°C for 1 h</p>		
	<p>For each total RNA sample, add:</p> <ul style="list-style-type: none"> <input type="checkbox"/> 1 µL of a primers premix stock solution <input type="checkbox"/> 1 µL of total RNA <p>Mix by pipetting 10 times carefully + spin down Incubate at 65°C for 10 min Transfer on ice for at least 2 min</p> <p>Add per sample:</p> <ul style="list-style-type: none"> <input type="checkbox"/> 2 µL of SuperScript III First-Strand Buffer (5x) <input type="checkbox"/> 1 µL of DTT (0.1 M) <input type="checkbox"/> 2.5 µL of dNTPs (2.5 mM) <input type="checkbox"/> 1.5 µL of Betaine (5 M) <input type="checkbox"/> 1 µL of SuperScript III (200 U/µL) <p>Incubate at 22°C for 10 min, 50°C for 30 min, 75°C for 15 min, and hold at 4°C</p>	<ul style="list-style-type: none"> - 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 (0.66 M/3.3 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 	
	<ul style="list-style-type: none"> <input type="checkbox"/> Add 18 µL of AMPure XP beads at RT <input type="checkbox"/> Mix by pipetting 10x slowly and incubate for 5 min <input type="checkbox"/> Pellet on magnet and pipette off the supernatant <input type="checkbox"/> Keep on magnet, wash 3x with 200 µL fresh 70% EtOH, do not disturb the pellet <input type="checkbox"/> Resuspend pellet in 30 µL water, incubate at RT for 5 min <input type="checkbox"/> Pellet beads on magnet, elute purified first-strand cDNAs and transfer to new PCR tubes low-bind 	<ul style="list-style-type: none"> - Aspirate supernatant carefully - In order to avoid losing the sample, be sure not to aspirate beads in the pipette tip together with the solution 	
	<p>Mix per qPCR replicate:</p> <ul style="list-style-type: none"> <input type="checkbox"/> 5 µL of Takara SYBR Premix Ex Taq (2x) <input type="checkbox"/> 0.1 µL of cDNA PCR primer, Forward (10 µM) <input type="checkbox"/> 0.1 µL of cDNA PCR primer, Reverse (10 µM) <input type="checkbox"/> 0.2 µL of Rox Reference Dye II (50x) <input type="checkbox"/> 3.1 µL of Water <input type="checkbox"/> 1.5 µL of purified first-strand cDNA <p>Analyze qPCR results Calculate the average Ct value for each cDNA sample Determine the optimum number (N) of cDNA PCR cycles to be performed for each sample</p>	<ul style="list-style-type: none"> - 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 	