

# nanoCAGE – DAY 1

RNA samples:

## Before start checklist:

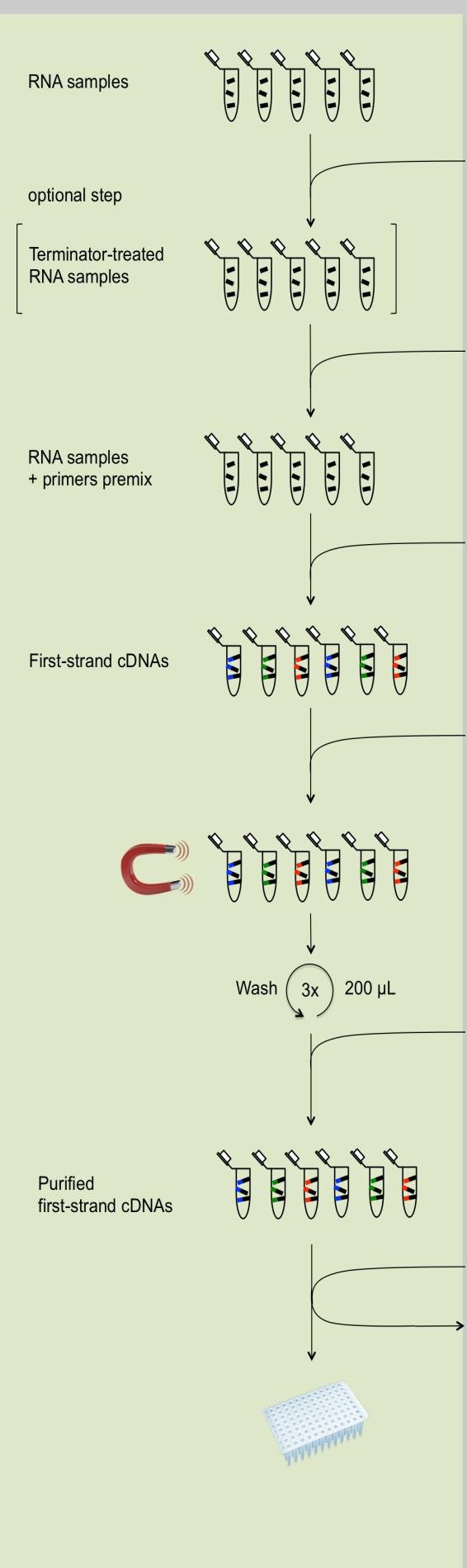
- Pipettes 1000 µL, 200 µL, 10 µL, 2 µL
- Filtered Tips Low-bind
- 0.2 mL PCR tubes low-bind + caps
- 1.5 mL microcentrifuge tubes low-bind
- 96-well PCR plate low-bind
- 96-well Real-Time PCR plate
- Ultra-Pure Water
- Magnet for beads separation
- AMPure XP beads, resuspended at room temperature
- 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"> <li><input type="checkbox"/> ≥ 50 ng/µL of total RNA per sample or</li> <li><input type="checkbox"/> ≥ 500 ng/µL of total RNA per sample, if performing the Terminator treatment</li> </ul>	<ul style="list-style-type: none"> <li>- Use in preference high quality total RNA to optimize nanoCAGE library preparation for best sequencing results</li> </ul>	
	<p>(optional step)</p> <p>Terminator-treated RNA samples</p> <ul style="list-style-type: none"> <li><input type="checkbox"/> 1.3 µL of SuperScript III First-Strand Buffer (5x)</li> <li><input type="checkbox"/> 0.2 µL of Water</li> <li><input type="checkbox"/> 0.5 µL of Terminator Exonuclease (1 U/µL)</li> </ul> <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"> <li><input type="checkbox"/> 1 µL of a primers premix stock solution</li> <li><input type="checkbox"/> 1 µL of total RNA</li> </ul> <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"> <li><input type="checkbox"/> 2 µL of SuperScript III First-Strand Buffer (5x)</li> <li><input type="checkbox"/> 1 µL of DTT (0.1 M)</li> <li><input type="checkbox"/> 2.5 µL of dNTPs (2.5 mM)</li> <li><input type="checkbox"/> 1.5 µL of Betaine (5 M)</li> <li><input type="checkbox"/> 1 µL of SuperScript III (200 U/µL)</li> </ul> <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"> <li>- Use a different barcode (primers premix solution) for each RNA sample and for each sample replicate</li> <li>- 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]</li> <li>- Trehalose/Sorbitol solutions are viscous; mix very carefully by pipetting up and down slowly for at least 10 times</li> <li>- Prepare a master mix for the RT reaction</li> <li>- Prepare a positive control of the RT reaction by using 1 µL of a control RNA sample</li> <li>- Prepare a negative control of the RT reaction by using 1 µL of water</li> </ul>	
	<ul style="list-style-type: none"> <li><input type="checkbox"/> Add 18 µL of AMPure XP beads at RT</li> <li><input type="checkbox"/> Mix by pipetting 10x slowly and incubate for 5 min</li> <li><input type="checkbox"/> Pellet on magnet and pipette off the supernatant</li> <li><input type="checkbox"/> Keep on magnet, wash 3x with 200 µL fresh 70% EtOH, do not disturb the pellet</li> <li><input type="checkbox"/> Resuspend pellet in 30 µL water, incubate at RT for 5 min</li> <li><input type="checkbox"/> Pellet beads on magnet, elute purified first-strand cDNAs and transfer to new PCR tubes low-bind</li> </ul>	<ul style="list-style-type: none"> <li>- Aspirate supernatant carefully</li> <li>- In order to avoid losing the sample, be sure not to aspirate beads in the pipette tip together with the solution</li> </ul>	
	<p>Mix per qPCR replicate:</p> <ul style="list-style-type: none"> <li><input type="checkbox"/> 5 µL of Takara SYBR Premix Ex Taq (2x)</li> <li><input type="checkbox"/> 0.1 µL of cDNA PCR primer, Forward (10 µM)</li> <li><input type="checkbox"/> 0.1 µL of cDNA PCR primer, Reverse (10 µM)</li> <li><input type="checkbox"/> 0.2 µL of Rox Reference Dye II (50x)</li> <li><input type="checkbox"/> 3.1 µL of Water</li> <li><input type="checkbox"/> 1.5 µL of purified first-strand cDNA</li> </ul> <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"> <li>- Analyze each first-strand cDNA and control samples in triplicates</li> <li>- Prepare a master mix for the real-time qPCR reaction</li> <li>- Prepare a negative control of the real-time qPCR reaction by adding 1.5 µL of water</li> </ul>	

# nanoCAGE – DAY 2

Purified First-Strand cDNA 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
- 384-well microplate for fluorescence assays
- Ultra-Pure Water
- Kapa HiFi HotStart Ready Mix (2x), on ice
- cDNA PCR primers, Forward and Reverse (10 µM), on ice
- Magnet for beads separation
- AMPure XP beads, resuspended and at room temperature
- Freshly prepared 70% EtOH, at room temperature
- Lambda DNA standard stock solution (1 ng/µL), on ice
- Quant-iT PicoGreen Reagent (200x), in fridge until use
- TE buffer (1x), at room temperature
- Bioanalyzer DNA High Sensitivity DNA kit, at room temperature 30 min before use
- Purified first-strand cDNA samples, on ice

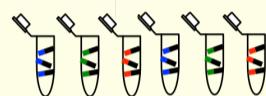
## WORKFLOW

## INSTRUCTIONS

## NOTES/OBSERVATIONS

## TIME/DATE

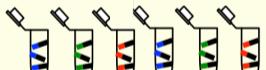
First-strand  
cDNA  
samples



cDNA PCR  
products



Purified cDNA  
PCR products



Pool of purified cDNA  
PCR products



Quantified pool of  
purified cDNA PCR  
products



- Purified first-strand cDNA samples prepared on Day 1

- Add for each sample:
- 25 µL of Kapa HiFi HotStart Ready Mix (2x)
- 0.5 µL of cDNA PCR primer, Forward (10 µM)
- 0.5 µL of cDNA PCR primer, Reverse (10 µM)
- 4 µL of Water
- 20 µL of first-strand cDNA sample
- Mix by vortexing + spin down
- Incubate at 95°C for 3 min, then perform [N] PCR cycles of 98°C for 15 s, 65°C for 10 s, 72°C for 2 min, and incubate at 72°C for 2 min, hold at 4°C

- Add 90 µL of AMPure XP beads at RT
- Mix by pipetting 10x slowly and incubate for 5 min
- Pellet on magnet and pipette off the supernatant
- Keep on magnet, wash 3x with 200 µL fresh 70% EtOH, do not disturb the pellet
- Resuspend pellet in 25 µL water, incubate at RT for 5 min
- Pellet beads on magnet, elute purified cDNA PCR products and transfer to new PCR tubes low-bind

- Use 3 µL of each purified cDNA PCR products samples for quantification by Quant-iT PicoGreen dsDNA assay

- Pool purified cDNA PCR products tagged with different barcodes together [Mix at least 2 ng of each purified cDNA PCR products sample at this step]

- Use 3 µL of the pool of cDNA PCR products for quantification of the mix by Quant-iT PicoGreen dsDNA assay

- (optional)  Use 1 µL to visualize the size profile of the pool of cDNA PCR products and confirm the final concentration of the mix on an Agilent Bioanalyzer High Sensitivity DNA chip

- Prepare a master mix of the cDNA PCR reaction, including extra volumes for the positive and negative controls of the RT reaction

- Perform [N] cDNA PCR cycles for each first-strand cDNA sample based on the Ct values obtained from the real-time qPCR experiment performed on Day 1

- Aspirate supernatant carefully

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

- It is also possible to mix purified cDNA PCR products tagged with different barcodes by group/experimental condition and further perform one fragmentation reaction per group using one Nextera N-series Index primer per reaction, and then pool the different nanoCAGE libraries obtained together, prior to library quantification and sequencing

- The concentration of the mix should be at least 0.1 ng/µL and the volume of at least 2.5 µL in order to perform the fragmentation reaction

- Prepare 5 µL of a 1 ng/µL dilution of the pool of cDNA PCR products and measure the concentration of the dilution in triplicate by applying 3 × 1 µL on the Bioanalyzer chip
- Quantify the cDNA products in the 100 bp – 9,000 bp range

# nanoCAGE – DAY 3

Quantified pool(s) of cDNA PCR products:

## 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
- 384-well microplate for fluorescence assays
- Ultra-Pure Water
- Magnet for beads separation
- AMPure XP beads, resuspended and at room temperature
- Freshly prepared 70% EtOH, at room temperature
- Lambda DNA standard stock solution (1 ng/µL), on ice
- Quant-iT PicoGreen Reagent (200x), in fridge until use
- TE buffer (1x), at room temperature
- Bioanalyzer DNA High Sensitivity DNA kit, at room temperature 30 min before use
- Tagment DNA Buffer / NT Buffer, on ice
- Amplicon Tagment Mix / Nextera PCR Mastermix, in freezer until needed
- 12 Nextera N-series Index primers / nanoCAGE custom S-series primer (10 µM), on ice
- NaOH 5 M, pH ≥ 13/ Qiagen EB buffer, at room temperature
- Illumina sequencing kit (e.g., MiSeq Reagent Kit v2) / PhiX Sequencing Control v3 / nanoCAGE Sequencing primer Read 1 (100 µM), in freezer until needed
- Quantified pool(s) of purified cDNA PCR products, on ice

## WORKFLOW

## INSTRUCTIONS

## NOTES/OBSERVATIONS

## TIME/DATE

Quantified pool of purified cDNA PCR products



- Quantified pool(s) of purified cDNA PCR products prepared on Day 2
- Prepare ≥ 2.5 µL of a dilution of the pool at 0.1 – 0.2 ng/µL

- Either one pool containing all cDNA PCR products samples or several pools containing cDNA PCR products samples grouped by experimental condition

nanoCAGE library



- Add in PCR tube low-bind:
  - 5 µL of Tagment DNA Buffer
  - 2.5 µL of the dilution of the pool of cDNA PCR products (0.25 – 0.5 ng)
  - 2.5 µL of Amplicon Tagment Mix
- Incubate at 50°C for 10 min, Hold at 10°C
- Add 2.5 µL of NT buffer as soon as the temperature reaches 10°C
- Mix by pipetting 10x carefully and incubate 5 min at RT
- Add:
  - 2.5 µL of a Nextera XT N-series Index primer
  - 2.5 µL of nanoCAGE custom S-series primer (10 µM)
  - 7.5 µL of Nextera PCR Mastermix
- Mix by pipetting carefully, spin down briefly
- Incubate at 72°C for 3 min, 95°C for 30 s, then perform 12 PCR cycles of 95°C for 10 s, 55°C for 30 s, 72°C for 1 min, and incubate at 72°C for 5 min, hold at 10°C

- Mix NT buffer carefully in order to avoid the formation of bubbles
- If processing several pools of cDNA PCR products, choose one Nextera N-series Index primer per pool when performing the Reduced-Cycle PCR Amplification
- If processing a single pool of cDNA PCR products, perform the PCR reaction using a single Nextera N-series Index primer and do not sequence the index.
- Alternatively, perform the PCR using 2.5 µL of an equimolar mix of the 12 Nextera N-series Index primers (i.e., mix 2 µL of each primer) and sequence the index

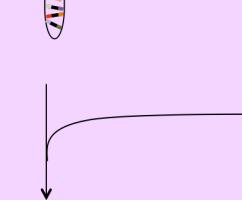
Purified nanoCAGE library



- Add 22.5 µL of AMPure XP beads at RT
- Mix by pipetting 10x slowly and incubate for 5 min
- Pellet on magnet and pipette off the supernatant
- Keep on magnet, wash 3x with 200 µL fresh 70% EtOH, do not disturb the pellet
- Resuspend pellet in 20 µL water, incubate at RT for 5 min
- Pellet beads on magnet, elute purified cDNA PCR products and transfer to a new PCR tube low-bind

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

Quantified purified nanoCAGE library



- Prepare ≥ 20 µL of 1:10 and 1:20 dilutions of the purified nanoCAGE library
- Use 3 × 1 µL of purified nanoCAGE library and 3 × 1 µL of the 1:10 and 1:20 dilutions to visualize the size profile of the nanoCAGE library and confirm the final concentration on an Agilent Bioanalyzer High Sensitivity DNA chip
- (optional)  Use 3 µL of the dilutions 1:10 and 1:20 of the purified nanoCAGE library for quantification by Quant-iT PicoGreen dsDNA assay

- If processing several nanoCAGE libraries prepared from different groups of samples, first quantify each library individually, then prepare a mix by adding equal quantities of each library and quantify dilutions of the pool as indicated
- Quantify the nanoCAGE library by selecting nanoCAGE fragments in the 150 bp – 1,500 bp range
- In general, sequence the nanoCAGE library at a concentration of 6 – 12 pM
- Add 10% of PhiX Sequencing Control library to the final nanoCAGE sequencing sample
- Output sequencing data as FASTQ files for bioinformatics analysis and demultiplexing based on barcode, index, and UMI informations

Sequence the nanoCAGE library on an Illumina sequencing system