

# 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

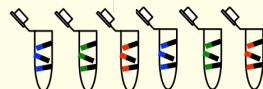
## WORKFLOW

## INSTRUCTIONS

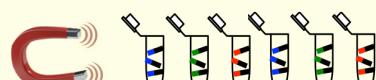
## NOTES/OBSERVATIONS

## 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