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NanoSeq DNA Library Preparation Protocol

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We use this protocol and it's working

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Abstract

Nanorate sequencing (NanoSeq) is a duplex sequencing protocol with an error rate of fewer than 5 errors per billion base pairs in single DNA molecules from cell populations. It was developed by Abascal et al. (2021) to study somatic mutations in non-dividing cells across various human tissues.

The protocol described here is based on the one published by Lensing et al. (2021) and includes minor modifications reflecting the availability of chemicals in our lab, practical considerations, and differences between human and insect samples. The modified steps are 1, 3, 7, 10, 12, and 13. The entire procedure takes between 3 and 4 days, depending on the number of samples processed.

Materials

Reagents:

- Agencourt Ampure XP beads (Beckman Coulter: A63882)
- 75% Ethanol (freshly prepared)
- Qubit™ 1X dsDNA HS Assay Kit (Thermo Scientific Q33231)
- Nuclease-free water
- HpyCH4V (NEB: R0620L)
- CutSmart® Buffer (NEB: B7204S)
- dATP Solution (NEB: N0440S)
- ddNTP Set, 5 mM solutions (ddCTP, ddGTP, ddTTP) (GE Healthcare: 27204501)
- Klenow Fragment (3'→5' exo-) (M0212L)
- 10X NEBufferTM 4 (NEB: B7004S)
- ATP 100 mM (Thermo Scientific™ R0441)
- xGen CS Adapter - Tech Access (IDT: 1080799)
- T4 DNA Ligase (NEB: M0202L)
- HS D5000 reagent (Agilent Technologies 5067-5593)
- HS D5000 screen tape (Agilent Technologies 5067-5592)
- KAPA library quantification kit (KK4824)
- 10 mM TRIS-HCl buffer (pH8-8.5)
- NanoqPCR primers (only needed if qPCR is performed before attaching full-size adapters):
  - 100 µM NanoqPCR1 primer (5′ACACTCTTTCCCTACACGAC3′)
  - 100 µM NanoqPCR2 primer (5′GTGACTGGAGTTCAGACGTG3′)
- NEBNext® UltraTM II Q5® Master Mix (NEB: M0544L)
- xGen™ UDI Primers Plate 1, 8nt (IDT: 10005922)

Equipment:

- Full set of pipettes, including multichannel pipette, reservoirs, and tips
- Eppendorf twin.tec plates and plate seals
- Rocking platform (optional)
- Plate magnet
- Heat block (optional)
- Qubit instrument
- Qubit Assay Tubes
- 384 well plate and optical seal for qPCR
- Lightcycler e.g. QuantStudio 6 Flex
- TapeStation system
- Thermocycler

Troubleshooting

Procedure

1 Prepare DNA samples by taking about 50 ng gDNA in a volume of 70 µL, preferably nuclease-free water (NFW) or a buffer compatible with beads.
If skipping step 2, the working volume should be only 20 µL.

2 (OPTIONAL) Perform an Ampure bead clean-up per sample.

2.1 Add 50 µL Ampure beads to each 70 µL DNA sample. Mix well by pipetting up and down and allow DNA to bind to beads.

2.2 Wash twice with 75% EtOH (200 µL).

2.3 Re-suspend beads in 20 µL NFW.

3 Fragmentation:
Having a high quality gDNA sample and efficient fragmentation are critical for good fragment sizes and high library complexity. The fragmentation can be optimized for different types of samples by modifying the enzyme selection (step 3.1) and the incubation time (step 3.3).

3.1 Prepare a fragmentation mix with the following volumes per sample:
10X CutSmart® Buffer: 2.5 µL,
NFW: 2 µL,
HpyCH4V (5U/uL): 0.5 µL.

3.2 Add the 5 µL fragmentation mix to each 20 µL bead suspension.

3.3 Incubate at 37 °C for 2 h on a thermocycler or heat block.

4 Perform an Ampure bead clean-up:

4.1 Add 62.5 µL Ampure XP beads to each 25 µL sample. Mix well by pipetting up and down and allow DNA to bind to beads.

4.2 Wash twice with 75% EtOH (150 µL).

4.3 Elute in 12 µL NFW.

5 (OPTIONAL) Check size distribution on gel.
This step is important for optimizing the HpyCH4V incubation time and input DNA amounts. Later on, it can be skipped.

6 A-tailing:

6.1 Make up a 1 mM dATP/ddBTP mix by combining 2.5 µL 100 mM dATP, 50 µL 5 mM ddCTP, 50 µL 5 mM ddTTP, 50 µL 5 mM ddGTP and 97.5 µL NFW.
This 1 mM dATP/ddBTP mix is enough for more than 150 libraries. The step can be skipped if there is enough of the mixture already prepared.

6.2 Prepare A-tailing mix with the following volumes per sample:
10X NEBufferTM 4: 1.5 µL,
NFW: 1.85 µL,
1 mM dATP/ddBTP mix: 1.5 µL,
Klenow Fragment (3'→5' exo-): 0.15 µL.

6.3 Add the 5 µL A-tailing mix to 10 µL of the cleaned-up fragmentation product (from step 4).

6.4 Incubate at 37 °C for 30 min on a thermocycler or heat block.

7 Ligation:

7.1 Prepare ligation mix with the following volumes per sample:
10X NEBufferTM 4: 2.24 µL,
NFW: 18.9 µL,
100 mM ATP: 0.374 µL,
15 µM xGen Duplex Seq Adapters: 0.33 µL,
400 U/µL T4 DNA ligase: 0.56 µL.

7.2 Add 22.4 µL ligation mix to the 15 µL A-tail product.

7.3 Incubate at 20 °C for 20 min on a thermocycler.

8 Perform an Ampure bead clean-up:

8.1 Add 37.4 µL Ampure XP beads to each 37.4 µL sample. Mix well by pipetting up and down and allow DNA to bind to beads.

8.2 Wash twice with 75% EtOH (120 µL).

8.3 Elute in 50 µL NFW

9 Measure DNA concentration on Qubit HS and/or TapeStation prior qPCR.

10 DNA Quantification by qPCR.
Check the KAPA library quantification kit technical data sheet for more details.

10.1 Add the supplied primer premix to the supplied KAPA SYBR FAST master mix and 20 µL of 100 µM NanoqPCR1 primer and 20 µL of 100 µM NanoqPCR2 primer to the KAPA SYBR FAST master mix (OPTIONAL). Alternatively, prepare a fresh qPCR mix with every experiment with the following volumes per sample:
qPCR Master Mix: 5 µL,
Primer Premix: 1 µL,
Dye ROX low: 0.2 µL,
NanoqPCR1 primer: 0.04 µL,
NanoqPCR2 primer: 0.04 µL.

10.2 Prepare the qPCR file on the qPCR machine (e.g., QuantStudio 6 Flex): State samples, PCR program, volume, dye, etc.

10.3 Dilute a fraction of each sample 1:100 and 1:10000 (or in the required amounts to be within the concentration range of the qPCR standards) using 10 mM TRIS-HCl buffer (pH8-8.5).

10.4 Set up in triplicates 10.28 µL qPCR reactions (6.28 µL master mix, 4 µL sample/standard) in a 384 well plate, for 20 cycles. One can use NFW instead of sample for the not template control (NTC).

10.5 Seal the plate safely and spin down at 4000 g for 8 minutes.

10.6 Introduce the plate and start the program.

10.7 Perform analysis Absolute quantification (2nd Derivative Maximum Method) with the high sensitivity algorithm.
For qPCR data analysis, download the calculation excel sheet from Roche with the only modification of multiplying the final concentration value by 1.5 to correct for the performance.
Make sure that the qPCR results are reliable: efficiency between 90 and 110; $R^2 \geq 0.99$; the consecutive standards ΔCq vary 3.1-3.6; no amplification in the NTC; % deviation between dilutions of the same sample < 10%.

11 Dilute each sample in 16 µL of NFW to the desired fmol input amount, aiming to achieve duplicate rates close to the optimal 81%.

The optimal DNA input to the PCR (in fmol, *fmol_opt*) depends on the number of paired-end reads to be sequenced (*N*), the number of (amplifiable) DNA fragments per fmol of library (*f*), and the optimal number of read pairs sequenced per original DNA fragment (*r_opt)*. The relationship is defined by the formula from Abascal et al. (2021):

$$\mathbf{fmol\_opt = N / (f \times r\_opt)}$$

r_opt is estimated as 5.1, a value that maximizes the number of starting molecules with two reads sequenced per strand (2+2).
f is estimated as 10^8. A more accurate estimate can be obtained by sequencing NanoSeq libraries with a range of dilution factors and determining the duplicate rate in each set of sequences.
N is determined by the amount of sequencing output planned or obtained, which is set based on the desired power to detect somatic mutations.

12 PCR amplification:
Conduct two PCR amplifications per sample: one using the diluted library (from step 11) and one using the undiluted library (from step 8). This allows for the filtering of germline SNPs.
In this protocol, the primers contain the indexes (UDI) that will be used for demultiplexing. Carefully write down which primer was used with each sample. Likewise, do not use the same primer with different samples if both will be sequenced in the same lane.

12.1 Add the following volumes per sample for a final PCR volume of 50 µL:
NEBNext Ultra II Q5 Master Mix: 25 µL,
UDI-containing PCR primers: 9 µL,
Template: 16 µL.

12.2 Mix and thermocycle:

Step 1: 98 °C 30 seconds,
Step 2: 98 °C 10 seconds,
Step 3: 65 °C 75 seconds,
Step 4: Return to Step 2, X times,
Step 5: 65 °C for 5 min,
Step 6: Hold at 4 °C.

The number of PCR cycles is dependent upon the input: 0.1 fmol = 16 cycles, 0.3 fmol = 14 cycles, 0.6 fmol = 13 cycles, 5 fmol = 10 cycles. For DNA amounts lower than 0.03 fmol, 25-30 cycles are needed. The number of PCR cycles can be calculated as:

$\log_2(5000 / fmol)$, where *fmol* is either *fmol_opt* for diluted samples or the total DNA amount (in fmol) for undiluted libraries.

13 Perform two consecutive 0.7X Ampure bead clean-ups and elute in 20 µL NFW.

14 (OPTIONAL) Check size distribution and presence of adapter dimers in a small representation of samples on TapeStation. If adapter dimers are still present, do another 0.7X Ampure bead clean-up.

15 Quantify the DNA concentration e.g. using Qubit or qPCR.

16 Pool the libraries to be sequenced on the same lane, ensuring similar DNA amounts for each library.
Adjust the number of samples pooled so that the total sequencing output per lane, distributed across all samples, meets the target number of reads per sample required to achieve the desired sequencing depth (from step 11).

17 Run pool on TapeStation or Qubit and adjust the pool concentration according to the sequencing strategy.

18 (OPTIONAL) Run the pool on qPCR to verify that full adapters are properly attached. For this qPCR step, do not include NanoqPCR1 and NanoqPCR2 primers in the qPCR mix.

19 Sequence on preferred Illumina sequencer.

Protocol references

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