Hybridization-capture for nanopore sequencing

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ABSTRACT

Large-scale genomic anomalies – structural variations (SVs) – are pervasive in cancer. Due to the scale of the SVs and the repetitive nature of the sequences usually flanking them, they are difficult to measure with conventional short-read sequencing. The long reads possible with nanopore sequencing provide an alternative to advance the understanding of SVs.
In this application note, we applied SureSelectXT to nanopore long read sequencing, enriching for CDKN2A and SMAD4 tumor suppressor genes, to improve the depth and variant calling accuracy of nanopore sequencing.
This application note focuses on optimizing the SureSelectXT protocol to long-read sequencing and using open-source softwares nanopolish and sniffles to improve the base calling accuracy and detect single nucleotide variants (SNVs) and structural variants (SVs), demonstrating the utility of SureSelect system on third-generation long-read sequencing platforms.

nanopore capture Sure Select App Note.pdf

GUIDELINES

Protocols were adopted from the original protocol provided by Agilent's SureSelect protocol.

The probe design was optimized by Josh Zhiyong Wang at Agilent Technologies, using Agilent's probe design algorithm, and validated experimentally to increase the on-target percentage.
Optimizations to the probe design include strategic placement of probes with appropriate, i.e. larger, probe spacing to enrich for larger regions, utilization of stringent probes to decrease non-specific binding, and increased number of probes around regions previously determined to contain SVs.
For this specific case, probes were designed with no tiling and with an average of 400 bp between each probe, targeting for ~ 2 Mbps region in human genome.

MATERIALS

NAME	CATALOG #	VENDOR
NEBNext End Repair Module - 20 rxns	E6050S	New England Biolabs
LongAmp Taq 2X Master Mix - 500 rxns	M0287L	New England Biolabs
Agencourt AmPure XP beads	A63880	
NEBNext Ultra II End Repair/dA-Tailing Module - 24 rxns	E7546S	New England Biolabs
SureSelectXT Reagent Kit	G9611A	Agilent Technologies
0.75% Agarose, 1-10 kb size selections or 4-20kb High Pass	BLF7510	sage science
Dynabeads MyOne Streptavidin T1	65601	Thermo Fisher Scientific
NEB Blunt/TA Ligase Master Mix	M0367	

BEFORE STARTING

We recommend users to consult with their local Agilent representative to aid in probe design to fit their purpose.

Shearing with Bioruptor Pico

Shearing with Bioruptor Pico

1 Shear 3-5 µg DNA in 100 µL to ~2kb using the following settings on Diagenode bioruptor pico : 5 cycles, 4 sec on / 30 sec off

End repair and dA-tailing

2 Split into two reactions to maximize the efficacy of the reactions.

3 Mix the following components in a sterile, nuclease-free tube:

Reagent	Volume
Fragmented DNA	50 µL
Nuclease-free water	5.5 µL
NEBNext End Repair Reaction Buffer (10X)	6.5 µL
NEBNext End Prep Enzyme Mix	3 µL
Total	65 µL

4 Incubate in thermocycler :
30 minutes at 20°C
30 minutes at 65°C
hold at 4°C
01:00:00

5 Combine two reactions together in preparation for AMPure XP cleanup.

AMPure XP Cleanup

6 Add 65 µL of AMPure beads (1X v/v ratio) at room temperature.

7 Incubate for 5 minutes at room temperature.
00:05:00
20 °C

8 Spin down briefly on a minifuge and place on a magnetic rack until beads separate out of the solution.

9 Carefully remove the supernatant, making sure not to remove the beads.

10 Keeping the tube on the magnetic rack, add 200 µL of 70 % ethanol in nuclease free water, being careful not to disturb the bead pellet.

11 Incubate for at least 30 seconds at room temperature
00:00:30

12 Repeat the wash steps (steps 9-11) for a total of two washes, and remove the supernatant (step 9).

13 Spin down on a minifuge to pellet the residual ethanol, place on the magnetic rack, and remove the residual ethanol using a P10 pipet.

14 Resuspend in 27 µL of nuclease-free water (25 + 2 to account for transfer loss.)

15 Incubate for 10 minutes at room temperature
00:10:00
20 °C

16 Spin down briefly on a minifuge and place on a magnetic rack until beads separate out of the solution.

17 Retain the supernatant, which now contains the eluted DNA.

Adaptor Ligation

18 Add all reagents together as follows:

Reagent	Volume
Nuclease-free water	31 µL
T4 DNA ligase buffer	20 µL
Adapter Oligo mix	6 µL
T4 DNA ligase	3 µL
Template DNA	25 µL
Total	85 µL

19 Carefully mix the reagents by pipetting up and down.

20 Briefly spin down in a microfuge.

21 Incubate in thermocycler, 20C for 15 minutes, then hold at 4C.
00:15:00
20 °C

22 You can pause at this step and store the library at -20C

23 Purify the adapted DNA with 1.8X Ampure XP (v/v), and elute in 32 µL nuclease-free water.
Refer to Ampure XP Cleanup step.

Bluepippin (Sage Science) size selection

24 Use 0.75% cassette high-pass for size selection protocol. (estimated time 45 minutes) 00:45:00

25 Let it sit ~45 min after elution for best recovery.
00:45:00

Post adapter-ligation PCR

26 Split 40 ul eluate to 2 x 20 µL

27 Add all reagents together as follows for two reactions:

Nuclease-free water	2.5 µL
NEB LongAmp Master Mix	25 µL
SureSelect Primer (brown cap)	1.25 µL
SureSelect R Primer (clear cap in box 2)	1.25. µL
DNA library	20 µL
Total	50 µL

28 Carefully mix the reagents by inverting a few times.

29 Briefly spin the reagents down on a minifuge.

30 Amplify using the following cycling conditions:

03:00:00

Segment Number	Number of cycles	Temperature	Time
1	1	94	30 sec
2	12	94	20 sec
		55	30 sec
		65	12:30 min (50 sec/1 kb)
3	1	65	10 min
4	1	4	Hold

31 Purify the DNA with 1X Ampure XP (v/v), and elute in 15 µL nuclease-free water.
Refer to Ampure XP Cleanup step.

Hybridization

32 Mix together these reagents to make Hyb buffer, volumes given are for one reaction:

Reagent	Volume
SureSelect Hyb 1	6.63 µL
SS Hyb 2	0.27 µL
SS Hyb 3	2.65 µL
SS Hyb 4	3.45 µL
Total	13 µL/reaction

33 Make SureSelect Block Mix.

Reagent	Volume
Indexing Block #1	2. 5 µL
Block #2	2.5 µL
Indexing Blcok #3	0.6 µL
Total	5.6 µL/reaction

34 Prepare RNAse block solution by diluting the RNAse block 1:10 with nuclease free water. (7 µL needed per reaction)

35 In a strip tube, add 3.4 µL gDNA to 5.6 ul SureSelect block mix

36 Mix by pipetting, and denature the DNA on a thermocycler by incubating at 95C for 5 minutes, followed by incubationg at 65C for at least 5 minutes

00:10:00

37 Meanwhile, make the capture library mix :

Reagent	Volume
Hyb buffer mix	13 µL
RNAse block dilution	5 µL
Capture library	2 µL
Total	20 µL/reaction

38 Keep at room temperature briefly, and add to the denatured DNA while the DNA is still on the thermocycler

39 Incubate for 16 or 24 hours at 65C with heated lid at 105C.

16:00:00

65 °C

Streptavidin beads preparation

40 Prewarm SureSelectXT Wash buffer #2 at 65C.

41 Vigorously resuspend MyOne Streptavidin T1 beads on a vortex mixer.

42 Add 50 µL beads (per rxn) to a 1.5 mL tube.

43 Add 200 µL of SureSelect Binding buffer.

44 Mix the beads on a vortex mixer for 5 seconds.

45 Put the tubes into a magnetic rack

46 Remove and discard the supernatant.

47 Repeat above for a total of 3 washes.

48 Resuspend the beads in 200 µL of SureSelectXT Binding buffer

Capture

49 Take out hybridization mixture from thermal cycler, add directly to bead solution, invert 3-5X to mix.
*If less than 20 µL hyb mixture remains after incubation, be wary of suboptimal capture performance.

50 Incubate hybrid-bait/bead solution on tube rotator at 10 rpm top-to-bottom for 30 minutes at room temperature. If sample is not properly mixing, mix with heat using a thermomixer at 30 C 800 rpm.

00:30:00

20 °C

51 Briefly spin down on a minifuge.

52 Place on magnetic rack, remove and discard supernatant.

53 Resuspend in 500 µL wash buffer I by vortexing for 5 seconds

54 Incubate for 15 minutes at room temperature, occasionally mixing.

00:15:00

20 °C

55 Spin down briefly on a minifuge, place on magnetic rack, and remove supernatant.

56 Resuspend beads in 200 µL 65C prewarmed wash buffer, mix on a vortex mixer for 5 seconds to resuspend beads.

57 Incubate 10 minutes at 65C

00:10:00

65 °C

58 Briefly spin down on a minifuge, place on magnetic rack, and discard supernatant. Repeat the wash steps for a total of 3 washes.

59 Remove all wash buffer

60 Add 30 µL nuclease free water to each sample, pipet to resuspend. Keep samples on ice until next use.

Capture Library Amplification

61 Assemble PCR reaction, 2 for each capture reaction :

Reagent	Volume
Nuclease-free water	5 µL
NEB Long AMP MM	25 µL
SureSelect ILM post-capture forward PCR primer	1 µL
SS indexed primer	5 µL

Bead-bound DNA	14 µL
Total	50 µL

62 Amplify using the following cycling conditions:

02:30:00

Segment Number	Number of cycles	Temperature	Time
1	1	94	30 sec
2	14	94	15 sec
		60	30 sec
		65	8:20 min
3	1	65	10 min
4	1	4	Hold

63 Purify the DNA with 1X Ampure XP (v/v), and elute in 30 µL nuclease-free water.
Refer to Ampure XP Cleanup step.

Oxford Nanopore sequencing library preparation (Protocol for SQK-LSK108 Kit)

64 Perform NEBNext Ultra II End-Repiar/dA-tailing in 60 µL.

65 Purify the DNA with 1X Ampure XP (v/v), and elute n 30 µL nuclease-free water.

66 Ligate on adaptors using NEB Blunt/TA Ligase Master Mix.

67 Purify the adptor-ligated DNA following the protocol's specifications.

68 Load on ONT MinION following the protocol's specifications and sequence for up to 48 hours.