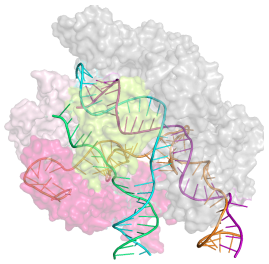
May 25, 2019 Version 1

Cas9 Enrichment for Nanopore Sequencing V.1



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Timothy Gilpatrick[1], Isac Lee[1], James E. Graham[2], Etienne Raimondeau[2], Rebecca Bowen[2], Andrew Heron[2], Fritz J. Sedlazeck[3], Winston Timp[1]

[1]Johns Hopkins University; [2]Oxford Nanopore Technologies; [3]Baylor College of Medicine

Winston Timp
Johns Hopkins University

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We use this protocol and it's working

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Abstract

This protocol is designed to help users achieve targeted enrichment for regions of interest using nanopore sequencing, providing higher coverage for myriad future analysis applications. GuideRNA components (crRNA and tracrRNA) were designed using online tools provided by IDT.

Materials

MATERIALS

- CutSmart Buffer - 5.0 ml **New England Biolabs Catalog #**B7204S
- Taq DNA Polymerase with Standard Taq Buffer - 2,000 units **New England Biolabs Catalog #**M0273L
- Quick Ligation Kit - 150 rxns **New England Biolabs Catalog #**M2200L
- Quick Dephosphorylation Kit - 500 rxns **New England Biolabs Catalog #**M0508L
- TE buffer **Thermo Fisher Scientific**
- Alt-R® S.p. HiFi Cas9 Nuclease V3 **IDT Catalog #**1081060
- Duplex Buffer **IDT Catalog #**11-01-03-01
- 100mM ATP **Thermo Scientific Catalog #**R0441
- 1D Ligation Sequencing Kit **Catalog #**SQK-LSK109
- AmpureXP beads **Beckman Coulter Catalog #**A63880

Before start

Prior to start, users should design and order their own custom crRNAs. Note that there is a strong preference for reads to start on the strand of DNA containing the PAM site (with the sequence matching the crRNA sequence and containing the PAM site)

Making Ribonucleoprotein Complex

1 Resuspend crRNA(s) and tracrRNA to final concentration of 100uM in TE, pH7.5
(2nmol crRNA in 20uL; 5nmol tracrRNA in 50uL)

2 Make equimolar mix of all crRNAs to be used by adding 0.75uL of each to new tube

3 Assemble guideRNA duplex:
8uL Nuclease Free Water (NFW)
1uL 100uM tracrRNA
1uL crRNA mix

4 Heat guideRNA duplex at +95C for 5min, allowing to cool on bench after incubation
(perform steps 5 and 6 during incubation)

5 Dilute the 10X CutSmart buffer 1:8 with Nuclease Free Water (NFW) to make 1.25X CutSmart Buffer
(14uL NFW + 2uL 10X CutSmart Buffer)

6 Dilute the HiFi Cas9 1:5 using the 1.25X CutSmart made in step 5
(1uL HiFi Cas9 + 4uL 1.25X CutSmart)

* Cas9 comes in stock of 10ug/uL or 61pM,
we dilute 1:5 to add (almost) equimolar amounts (10pmol) of the guideRNA + Cas9 enzyme in next step

7 Assemble Ribonucleoprotein Complex :
23.7uL NFW
2.9uL 10X CutSmart Buffer
2.4uL guideRNA duplex (from step 4)
1uL 1:5 dilution of Cas9 (from step 6)

Mix by gentle flicking, then keep on benchtop (room temperature) for 20min
After incubation, keep on ice or at +4C for up to a few days before use

Dephosphorylation of Free DNA Ends

8 *note: this assumes starting DNA is sufficiently concentrated that 3ug is less than 33uL.
Adjust volumes if DNA concentration is too low

Dephosphorylation Reaction:
4uL 10X CutSmart Buffer
3ug of DNA (often in TE)
NFW up to volume of 37uL

Mix gently by flicking before adding enzyme

Add 3uL QuickCIP enzyme (from Quick dephosphorylation kit)
[total volume = 40uL]

Mix by gentle flicking

Incubate at:
+37C for 10min (dephosphorylation)
+80C for 2min (enzyme inactivation)
hold at +20C

during incubation:
>> get out AmpureXP to allow to come to room temperature,
>> thaw 100mM ATP, keep on ice after thawing,

Cleavage and A-tailing of DNA

9 Make 1:10 dilution of 100mM ATP
[5uL 100mM ATP + 45uL NFW]

After allowing dephosphorylated DNA (from step 8) to cool,
Add the following components:
10uL Assembled RNP complex (from step 7)
1uL 1:10 (10mM) ATP
1uL Taq polymerase
[total volume = 52uL]

Incubate at:
+37C for 15min (Cas9 cleavage)
+72C for 5min (Mono adenylation of 3' ends by Taq/ATP)
hold at +12C

> During this incubation, get out the following components from SQK-LSK109 reagents to thaw:

LNB (keep at room temp)
AMX (keeping on ice after thaw)

Adaptor Ligation

10 Make "Ligation mix":
25uL LNB (ligation buffer from LSK109 kit)
12.5uL T4 Ligase (from NEB quick ligation kit)
5.5uL NFW
5uL AMX* (sequencing adaptor from LSK109 kit)

* NOTE : AMX will degrade in this solution, add right before use

11 Using pipette, mix ligation mix gently until homogenous (LNB is very viscous)

Add half (24uL) of "ligation mix" to DNA*,
mix by gentle flicking

Add remaining (~24uL) "ligation mix" to DNA
mix by gentle flicking
[total volume = 100uL]

* A DNA precipitate may from at this step, adding "ligation mix" in 2-steps helps to reduce this.
Formation of DNA precipitate does not appear to interfere with protocol efficiency

12 Rotate ligation for 10min at Room Temperature

>During incubation, get out LFB and EB from LSK109 kit, keeping both at room temp

13 After ligation, add equivolume (100uL) amount of TE (pH 8.0)
[new final volume = 200uL]

14 Add 0.3X Ampure (60uL if DNA is currently in 200uL)
and mix by gentle flicking until homogeneous

15 Rotate for 5 minutes,
then keep on benchtop for 5 minutes to allow Ampure beads to bind DNA

16 Place on magnetic tube rack,
allow 2.5 minutes for beads to collect on back of tube

17 Remove supernatant with a pipette, taking care not to disturb the beads

Add 200uL of LFB to beads

Remove tube from magnetic stand and resuspend by gentle flicking

18 Return sample to magnetic tube rack ,
allowing 2.5 minutes for beads to collect

19 Repeat steps 17 and 18 for a second bead wash w/ 200uL LFB

20 Remove supernatant,
briefly spin tube to collect any excess LFB and carefully remove remaining solution from above the beads

21 Add 16.6uL of elution buffer (EB), and keep at room temperature for 10 minutes to elute

>During this elution, get out the following components from LSK-109 kit to thaw:
FLB, LB, SQB (all at room temp)
and SQT (on ice after thaw)

Library Prep and Sequencing

22 Return tube with sample to magnetic rack , and allow 2min for beads to collect,

Transfer (and keep!) ~15.8uL supernatant with DNA to a new tube

23 Perform initial priming of flow cell by loading 800uL FLB into MinION priming port

24 Finish library prep by adding the following components to the DNA eluate:
26uL SQB
9.5uL Loading beads (LB)
0.5uL SQT

25 Perform second flow cell priming with 200uL of a 1:2 dilution of sequencing buffer (100uL SQB + 100uL NFW)

then immediately afterwards load sample dropwise into MinION sample loading port

26 Start sequencing run using MinKNOW software

(We commonly get low pore occupancy with 20-30 pores performing active sequencing by this method.
and runs often stop producing data after 24h)