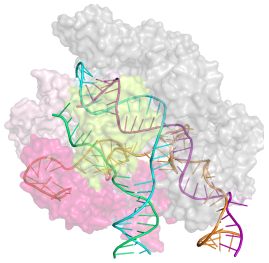
Sep 22, 2020 Version 3

Cas9 Enrichment for Nanopore Sequencing V.3



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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
- 1D Ligation Sequencing Kit **Catalog #**SQK-LSK109
- AmpureXP beads **Beckman Coulter** **Catalog #**A63880
- dATP **Zymo Research** **Catalog #**D1005

Troubleshooting

Before start

Users should design and order their own custom crRNAs. Note that there is a strong preference for reads to start on the side of the Cas9 cut containing the PAM site. The selection of guideRNAs makes a big difference in the performance of the assay, we therefore encourage using available tools to predict guideRNA on-target performace and (if applicable) off-target performance. We used the tool provided by 'Integrated DNA Technologies' to design and estimate performance of guideRNAs

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 prevent pipetting of very small volumes of the Cas9 enzyme in next step

7 Assemble Ribonucleoprotein Complex :
23uL NFW
2.8uL 10X CutSmart Buffer
3uL guideRNA duplex (from step 4) [30pmol]
1.2uL 1:5 dilution of Cas9 (from step 6) [15pmol]

Mix by gentle flicking, then keep on benchtop (room temperature) for 20min
After incubation, keep the RNP on ice. Can be stored at +4C a few days before use

Dephosphorylation of Free DNA Ends

8 *note: this assumes starting DNA is sufficiently concentrated that 3ug is less than 24uL.
If DNA concentration is too low, adjust volumes of the buffers accordingly. For volume increases up to 3X, the enzyme and RNP amounts can be kept consistent

Dephosphorylation Reaction:
3uL 10X CutSmart Buffer
3ug of DNA (often in TE)
NFW up to volume of 27uL

Mix gently by flicking before adding enzyme

Add 3uL QuickCIP enzyme (from NEB Quick dephosphorylation kit)
[total volume = 30uL]

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, vortex to mix, keep on ice after thawing,

Cleavage and A-tailing of DNA

9 Make 1:10 dilution of 100mM dATP
[5uL 100mM dATP + 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) dATP
1uL Taq polymerase
[total volume = 42uL]

Mix by gentle flicking

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

> During this incubation, get out the following components from SQK-LSK109 reagents to thaw:
Ligation Buffer (LNB) - (keep at room temp)
Adaptor Mix (AMX) (keeping on ice after thaw)

Adaptor Ligation

10 Make "Ligation mix":
20uL LNB (ligation buffer from LSK109 kit)
4.5uL NFW
10uL T4 Ligase (from NEB quick ligation kit)
3.5uL AMX* (sequencing adaptor from LSK109 kit)

* NOTE : AMX will be consumed in solution with ligase -- add right before use

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

Add 20uL of "ligation mix" to DNA*,
mix by gentle flicking

Add remaining (~18uL) "ligation mix" to DNA
mix by gentle flicking
[total volume = 80uL]

* 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 Long-Fragment Buffer (LFB) and Elution Buffer (EB) from LSK109 kit, keeping both at room temp
[or the Short Fragment Buffer (SFB), if the target region is less than 3kb]

13 After ligation, add equivolume (80uL) amount of TE (pH 8.0)
[new volume = 160uL]

14 Add 0.3X Ampure (48uL if DNA is currently in 160uL)
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
[Or use the Short Fragment Buffer (SFB), if the target region is less than 3kb]

Remove tube from magnetic stand and resuspend Ampure beads by gentle flicking until homogeneous

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 15uL of elution buffer (EB), and keep at room temperature for 10 minutes to elute
[for longer DNA fragments, it can be helpful to increase the elution time to 30minutes , or to elute with gentle agitation]

>During this elution, get out the following components from LSK-109 kit to thaw:
Flush Buffer (FLB or FB), Loading Beads (LB), Sequencing Buffer (SQB) - (all at room temp)
and Sequencing Tether (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!) ~15uL 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 15uL DNA eluate:
25uL SQB

14.5uL Loading beads (LB) *
0.5uL SQT
[Total volume of prepped Library : ~55uL]

*Loading Beads are in suspension -- vortex or flick prior to use

25 Perform second flow cell priming with 200uL of
second priming solution: (70uL SQB + 70uL NFW + 70uL FLB)

..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)