Version 2

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Nano-OTS V.2

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1 *Works for me* This protocol is published without a DOI.

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ABSTRACT

This is a protocol that detects Cas9 off-target sites *in vitro* using nanopore sequencing[1]. The protocol is a modified version published by Gilpatrick et al (2020)[2]. Guide RNAs have been designed using CHOPCHOP (https://chopchop.cbu.uib.no/).

1. Höijer, I., et al. (2020). "Amplification-free long read sequencing reveals unforeseen CRISPR-Cas9 off-target activity." bioRxiv: 2020.2002.2009.940486.

2. Gilpatrick, T., Lee, I., Graham, J.E.*et al.*Targeted nanopore sequencing with Cas9-guided adapter ligation.*Nat Biotechnol***38,**433–438 (2020). https://doi.org/10.1038/s41587-020-0407-5

EXTERNAL LINK

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Nano-OTS, Cas9, off-target sequencing, OTS, CRISPR-Cas9, ONT, nanopore sequencing, MinION

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MATERIALS TEXT

MATERIALS

NEBNext Quick Ligation Module - 20 rxns **New England Biolabs Catalog #E6056S**

dATP Solution - 25 umol **New England Biolabs Catalog #N0440S**

Agencourt AMPure XP magnetic beads **Beckman Coulter Catalog #A63880**

Alt-R® S.p. HiFi Cas9 Nuclease V3 **IDT Catalog #1081060**

Ligation sequencing kit 1D **Oxford Nanopore Technologies Catalog #SQK-LSK109**

Alt-R® CRISPR-Cas9 tracrRNA **IDT Technologies Catalog #1072532**

Alt-R® CRISPR-Cas9 crRNA **IDT Technologies**

TE pH 8.0 (1X TE Solution) **IDT Technologies Catalog #11-01-02-05**

Nuclease Free Duplex Buffer **IDT Technologies Catalog #11-01-03-01**

Quick CIP **New England Biolabs Catalog #M0525S**

Taq DNA Polymerase **New England Biolabs Catalog #M0273S**

TE pH 7.5 **IDT Technologies Catalog #11-01-02-02**

CutSmart® Buffer **New England Biolabs Catalog #B7204S**

High Pass Plus Gel Cassettes **sage science Catalog #BPLUS10**

ABSTRACT

This is a protocol that detects Cas9 off-target sites *in vitro* using nanopore sequencing[1]. The protocol is a modified version published by Gilpatrick et al (2020)[2]. Guide RNAs have been designed using CHOPCHOP (https://chopchop.cbu.uib.no/).

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Shearing of genomic DNA

1 Shear 5-10 µg of HMW genomic DNA to 20 kb using Megaruptor 2.

2 Add 0.45x AMPure XP beads to the sample and incubate at 2000 rpm using a vortex mixer for 10 min.

2.1 Place the tube on a magnetic rack until the beads collect on the side of the tube and the solution appears to be clear. Slowly pipette off the supernatant and discard. Be careful not to disturb the beads.

2.2 Wash the beads using 70% freshly prepared ethanol.

- Fill the tube with the ethanol
- Do not disurb the bead pellet
- Wait 30 seconds and pipette off the ethanol and discard

2.3 Repeat step 2.2

2.4 Remove any residual ethanol.

- Spin the tube briefly and put back on the magnetic rack
- Pipette off any remaining ethanol using a P10 pipette

2.5 Add 32 µl Low TE buffer and incubate at 2000 rpm using a vortex mixer for 5 min to elute the sample.

2.6 Place the tube on the magnetic rack until the beads collect on the side of the tube and the solution appears clear. Slowly pipette off the supernatant and transfer to a new eppendorf tube.

Quality control of fragmented DNA

3 Measure concentration of the sample using Qubit and fragment size using Bioanalyzer, Tape station or FEMTO Pulse.

Size selection of sheared DNA

4 Perform size selection with a 10 kb cut-off using the BluePippin and the High pass plus gel casette with the "15 kb High Pass Plus Marker U1" casette definition (the 10 kb cut-off is not supported by Sage Science).

5 Add 0.45x AMPure XP beads to the sample and incubate at 2000 rpm using a vortex mixer for 10 min.

5.1 Place the tube on a magnetic rack until the beads collect on the side of the tube and the solution appears to be clear. Slowly pipette off the supernatant and discard. Be careful not to disturb the beads.

5.2 Wash the beads using 70% freshly prepared ethanol.
- Fill the tube with the ethanol

- Do not disurb the bead pellet
- Wait 30 seconds and pipette off the ethanol and discard

5.3 Repeat step 5.2

5.4 Remove any residual ethanol.

- Spin the tube briefly and put back on the magnetic rack
- Pipette off any remaining ethanol using a P10 pipette

5.5 Add 24 µl Low TE buffer and incubate at 2000 rpm using a vortex mixer for 5 min to elute the sample.

5.6 Place the tube on the magnetic rack until the beads collect on the side of the tube and the solution appears clear. Slowly pipette off the supernatant and transfer to a new eppendorf tube.

Preparing the Cas9 ribonucleoprotein complexes (RNPs)

6 Resuspend crRNAs and tracrRNA to a final concentration of 100 mM in TE pH 7.5.

7 Pool all crRNAs used in the same experiment equimolarly by combining 1 µl of each crRNA in a new eppendorf tube. Unused crRNa mix can be stored at -70ºC.

8 Anneal crRNA and tracrRNA. In a 0.2 ml tube (preferably low-bind) combine:

8 µl Duplex buffer
1 µl crRNA pool (100 µM)
1 µl tracrRNA (100 µM)

Inclubate at 95ºC for 5 min in a thermocycler. Remove the tube from the thermocycler and let it cool down to room temperature (~5 min). Place the tube on ice.

9 Assemble the RNPs. Add the following components to a new 1.5 ml eppendorf tube:

5 µl annealed crRNA-tracrRNA
5 µl 10x NEB CutSmart buffer
39.6 µl nuclease-free water
0.4 µl HiFi Cas9 (62 µM)

Incubate at room temperature for 30 min, then place on ice.

While the RNPs are incubating, proceed to the next step.

Dephosphorylation of sheared and size selected DNA

10 3 µg of sheared and size selected DNA is needed for this step. Bring the sample to 24 µl using nuclease-free water if needed.

11 Add the following components in a 0.2 ml thin-walled PCR tube:

3 µl 10x NEB Cut Smart buffer
24 µl sheared and size selected DNA (3 µg)

Mix gently by flicking the tube, spin down.

Add 3 µl CIP to the tube.

Incubate at 37ºC for 10 min, 80ºC for 2 min and then hold at 20ºC on a thermocycler.

Cas9 digestion and a-tailing

12 Make a 10 mM dATP solution by combining 1 µl 100mM ATP and 9 µl nuclease-free water. Do not keep the solution for future experiments.

13 Make a 1x NEB Cut Smart solution by combining 1 µl 10x NEB Cut Smart Buffer and 9 µl nuclease-free water. Do not keep the solution for future experiments.

14 Add the following components to the PCR tube containing the dephosphorylated DNA sample:

30 µl dephosyphorylated DNA
7 µl 1x NEB Cut smart buffer
3 µl RNPs
1 µl 10 mM dATP
1 µl NEB Taq polymerase

Mix carefully by inverting the tube, spin down.

Incubate at 37ºC for 15 min, 72ºC for 5 min and hold at 4ºC. Then place the tube on ice.

Adapter ligation

15 Carefully transfer the contents of the 0.2 ml PCR-tube to a new 1.5 ml eppendorf tube.

16 Add the following compontents to a separate 1.5 ml eppendorf tube. It is important to add the adapter mix (AMX) last.

20 µl ligation buffer (LNB)
3 µl nuclease-free water
10 µl NEBNext Quick T4 DNA ligase
5 µl adapter mix (AMX)

Mix thoroughly by pipetting.

17 Add 20 µl of the adapter ligation mix to the Cas9 digested and dA-tailed DNA sample. Mix gently by flicking the tube. Do not spin down at this stage.

18 Immediately after mixing, add the remaining adapter ligation mix. Mix gently by flicking the tube, spin down.

Inclubate at room temperature for 10 min.

AMPure XP bead purification

19 Add 1 volume (80 µl) of TE pH 8.0 to the adapter ligated sample. Mix gently by flicking the tube.

20 Add 0.3x (48 µl) AMPure XP beads to the sample. Mix by gentle inversion.

Incubate at room temperature for 10 min. Do not agitate the sample.

21 Spin down the sample and place on a magnetic rack until the beads collect on the side of the tube and the solution appears to be clear. Pipette off the supernatant and discard. Be careful not to disturb the pellet.

22 Wash the beads by adding 250 µl long fragment buffer (LFB). Resuspend the beads by flicking the tube and place back on the magnetic rack.

When the solution appears clear, pipette off the supernatant and discard.

23 Repeat step 22.

24 Remove any residual buffer.

- Spin the tube briefly and put back on the magnetic rack
- Pipette off any remaining buffer using a P10 pipette

25 Remove the tube from the magnetic rack and add 13 µl elution buffer (EB) to elute the sample.

Incubate at room temperature for 10 min. Do not agitate the sample.

26 Place the tube on the magnetic rack until the beads collect of the side of the tube and the solution appears clear. Pipette off 12 µl of the supernantant and transfer to a new 1.5 ml eppendorf tube. Place on ice.

Sequencing on the MinION

27 Open the priming port on the MinION flow cell. Draw back a small volume to remove any bubbles by:

- Set a P1000 to 200 µl
- Insert the tip into the priming port
- Turn the wheel of the pipette until the dial shows 220-230 µl, or until you can see a small volume of buffer in the pipette tip

28 Add 30 µl of flush tether (FLT) directly to the flush buffer (FLB). Mix by pipetting.

Load 800 µl of the priming mix to the priming port. Avoid the introduction of bubbles. Wait 5 min.

29 Add the following components to a new 1.5 ml eppendorf tube:

25 µl sequencing buffer (SQB)
13 µl loading beads (LB)
12 µl DNA library

30 Complete the flow cell binding by:

- Gently open the SponON sample port cover
- Load 200 µl of the priming mix to the priming port. Avoid the introdution of bubbles.

31 Mix the prepared library gently by pipetting just prior to loading.

Add 50 µl of the library SpotON sample port in a dropwise fashion. Ensure each drop flows into the port before adding

the next one.

32 Start the sequncing run through the MinKNOW software.

Data analysis

33 Align the reads using minimap2. Use the mapped reads for detection of off-target sites using our custom tool Insider:

https://github.com/UppsalaGenomeCenter/InSiDeR