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Cas9-targeted Nanopore sequencing (CANS)

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Pavel Merkulov^{1,2}, Ilya Kirov^{1,2}

¹Moscow Institute of Physics and Technology, 141701 Dolgoprudny, Russia;

²All-Russia Research Institute of Agricultural Biotechnology, Timiryazevskaya Str. 42, 127550 Moscow, Russia

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Pavel Merkulov

Moscow Institute of Physics and Technology, 141701 Dolgoprud...

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We use this protocol and it's working

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Abstract

Here we provide a protocol for Cas9-targeted Nanopore sequencing.

We successfully applied this method for targeted sequencing and DNA methylation profiling of genes in cereal genomes, as well as for insertions of transposable elements (inherited and somatic) in Arabidopsis.



Materials

Materials:

- PCR kit
- PCR and gel extraction kit
- T7 in vitro transcription kit
- Total RNA and miRNA isolation kit
- 5 µg high molecular weight genomic DNA (recommended); 1–10 µg (or 0.1–2 pmol) can be used accordingly.
- Quick Calf Intestinal Phosphatase (NEB cat #M0525)
- 1.5 ml Eppendorf DNA LoBind tubes
- 0.2 ml thin-walled PCR tubes
- Nuclease-free water (e.g. ThermoFisher, cat # AM9937)
- Taq polymerase (NEB Cat # M0273)
- dATP solution (100 mM) (NEB Cat # N0440S)
- LSK109 components
- Ampure XP beads (Beckman Coulter A63881)
- Flow Cell Wash Kit (EXP-WSH003 or EXP-WSH004)

Equipment:

- Thermal cycler
- P100 pipette and tips
- P10 pipette and tips
- P20 pipette and tips
- Vortex mixer

- Water bath
- Ice bucket with wet ice



In vitro transcription of sgRNAs

2d

- 1 Design a specific oligonucleotide for synthesizing a single guide RNA (sgRNA) template according to desired cut site in your target sequence (~20 nucleotides length and must be followed by a protospacer adjacent motif (PAM) sequence of NGG).

1d

	A	B
	Specific oligo	GGATCCTAATACGACTCACTATAGG[target sequence]GTTTTAGAGCTAGAA.
	CRISPR R	AAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTAACTTGCTATTTCTAGCTCTAAAAC
	T7 F	GGATCCTAATACGACTCACTATAG
	T7 R	AAAAAAGCACCGACTCGG

- 2 Combine following components for sgRNA template synthesis:

10m

A	B
Component	Volume, μ L
Specific oligo, 1 μ M	2
CRISPR R, 1 μ M	2
T7 F, 100 μ M	2
T7 R, 100 μ M	2
dNTP mix, 10 mM of each	2
10x buffer	10
High fidelity polymerase	1
Nuclease-free water	79
Total	100

- 3 Set the reaction with following program:

2h

1. 95°C- 2 min
2. 30 cycles:
 - 98°C - 30 sec
 - 60°C - 30 sec



- 72°C - 30 sec

3. 72°C - 1 min

4 Check the structure of synthesized templates with agarose gel electrophoresis (single band for best results, but note that T7-sequences can lead to dimers forming).

1h

5 Purify your sgRNA template with your system of choice. We use a column-based kit for gel extraction (*in the case of dimers or non-specific products*) and PCR purification (*in the case of a single band*).

30m

6 Combine following components for T7 in vitro transcription of your sgRNA:

10m

A	B
Component	Volume, μL
5x buffer	10
25x DTT	2
rNPT mix, 25 mM of each	2
sgRNA template (500 ng)	X
T7 RNA (150U/ μL)	1 μL
Nuclease-free water	to get 50 μL total volume
Total	50

7 Incubate your reaction at 37 °C for 02:00:00 . The incubation time can also be extended up to 16:00:00 (overnight) to obtain a higher sgRNA yield.

2h

8 Purify your sgRNA template with your system of choice. We use a kit for the isolation of total RNA and microRNA.

30m

9 Check the structure of synthesized sgRNA with agarose gel electrophoresis (the number of bands depends on the secondary sgRNA structure).

1h

Preparing the Cas9 ribonucleoprotein complexes (RNPs)

40m

10 Combine equimolar amounts of sgRNAs for a targeted fragment in a single tube.

1m

11 Add water to get  11 μL

30s

12 Heat and cool each sgRNAs to obtain pure monomers:  95 °C for  00:03:00 , then cool to  Room temperature for  00:02:00

5m



Citation

Dang Y, Jia G, Choi J, Ma H, Anaya E, Ye C, Shankar P, Wu H (2015)
 . Optimizing sgRNA structure to improve CRISPR-Cas9 knockout efficiency.
 Genome biology.

<https://doi.org/10.1186/s13059-015-0846-3>

LINK

13 To form Cas9 RNPs, assemble the components in the table in a 1.5 ml Eppendorf DNA LoBind tube in the following order:

2m

A	B
Reagent	Volume (per one cleavage reaction)
Cas9 5x buffer	3
Cas9	1
gRNA (50ng/ μL ~ 1pmol/ μL) in 11 μL water	11
Total	15

14 Mix thoroughly by *flicking the tube*

30s



15 Form the RNPs by incubating the tube at  Room temperature for  00:30:00 , then return the RNPs on ice until required (proceed to the 'Dephosphorylating genomic DNA' section during this time)

30m





Dephosphorylating genomic DNA (This step reduces background reads by removing 5' phosphates from non-target DNA ends.)

32m

- 16 Transfer 1-10 µg (with 5 µg recommended) genomic DNA into 0.2 mL tubes.

1m

Citation

Boas Pucker (2026). Plant DNA extraction and preparation for ONT sequencing.
protocols.io.

<https://protocols.io/view/plant-dna-extraction-and-preparation-for-ont-seque-bcvyi7w>
LINK

ml thin-walled PCR tubes

- 17 Adjust to  24 µL with nuclease-free water

30s

- 18 Mix thoroughly by *flicking the tube* avoiding unwanted shearing

30s



- 19 *Spin down* briefly in a microfuge

30s

- 20 Mix the Quick calf intestinal alkaline phosphatase (CIP) in the tube by pipetting up and down. Ensure that it is at  Room temperature before use

30s



- 21 Assemble the following components in a clean 0.2 ml thin-walled PCR tube:

1m

A	B
Reagent	Volume
NEB CutSmart Buffer (10x)	4 µl
HMW genomic DNA (at ≥ 210 ng/µl)*	24-30 µl
Water	to get 34 µl total volume
Total	34 µl



22 Mix gently by *flicking the tube*, and spin down

30s



23 Add  6 μL of CIP to the tube

30s

24 Mix gently by *flicking the tube*, and spin down

30s



25 Using a thermal cycler, incubate at  37 °C for  00:30:00 ,  80 °C for  00:02:00 then hold at  Room temperature

32m



Cleaving and dA-tailing target DNA

35m

26 Thaw the dATP tube, vortex to mix thoroughly, and place on ice

2m

27 Dilute dATP to concentration  10 millimolar (mM) . In a 0.2 ml thin-walled PCR tube, make a  10 millimolar (mM) dATP solution by adding  1 μL of the  100 millimolar (mM) dATP stock to  9 μL of nuclease-free water. Vortex to mix, then spin down

1m

28 *Spin down* and place the tube of Taq polymerase on ice

30s

29 To the PCR tube containing  40 μL dephosphorylated DNA sample, add:

5m

A	B
Reagent	Volume
Dephosphorylated genomic DNA sample (Section 2)	40 μl
Cas9 RNPs (Section 1)	15 μl
10 mM dATP	1.5 μl



A	B
Taq polymerase	1 µl
Total	57.5 µl

- 30 Carefully mix the contents of the tube by gentle inversion, then spin down and place the tube in the thermal cycler

30s



- 31 Using the thermal cycler, incubate at 37 °C for 15-60 (00:15:00 are recommended) minutes, then 72 °C for 00:10:00 and hold at 4 °C or return to the tube to ice

25m



Adapter ligation

25m

- 32 Assemble the following at room temperature in a separate 1.5 ml Eppendorf DNA LoBind Tube, adding Adapter Mix (AMX) last, before you are ready to begin the ligation:

2m



A	B
Reagent	Volume
Ligation Buffer (LNB)	25 µl
Nuclease-free water	5 µl
NEBNext Quick T4 DNA Ligase	12.5 µl
Adapter Mix (AMX)*	5 µl
Total	47.5 µl

** The Adapter Mix (AMX) must be added last and immediately before the ligation step*

- 33 Mix by pipetting the above ligation mix thoroughly. Ligation Buffer (LNB) is very viscous, so the adapter ligation mix needs to be well-mixed

30s





34 Add  20 μL of the adapter ligation mix to the cleaved and dA-tailed sample. Mix gently by *flicking the tube*. Do not centrifuge the sample at this stage. Immediately after mixing, add the remainder ( 27.5 μL) of the adapter ligation mix to the cleaved and dA-tailed sample, to yield a  105 μL ligation mix

1m

35 Mix gently by *flicking the tube*, and *spin down*

30s



36 Incubate the reaction for  00:20:00 at  Room temperature

20m

Note

A white precipitate may form upon the addition of the adapter ligation mix to the dA-tailed DNA. Adding the ligation mixture in two parts helps to reduce precipitation. However, the presence of a precipitate does not necessarily indicate failure of ligation of the sequencing adapter to target molecule ends.

AMPure XP bead purification

1h

37 Add 1 volume ( 105 μL) of TE ( 8.0) to the ligation mix. Mix gently by *flicking the tube*

1m



38 Add 0.3x volume ( 63 μL) of AMPure XP Beads to the ligation sample. The volume of beads is calculated based on the volume after the addition of TE. Mix gently by inversion. If any sample ends up in the lid, *spin down* the tube very gently, keeping the beads suspended in a liquid

30s

39 Incubate the sample for  00:10:00 at  Room temperature

10m

Note

Do not agitate or pipette the sample to prevent long DNA fragments stick to the magnetic beads (it may decrease the elution)





40 Spin down the sample and pellet on a magnet. Keep the tube on the magnet, and pipette off the supernatant

2m



41 Wash the beads by adding either  250 μ L Long Fragment Buffer (LFB) or  250 μ L Short Fragment Buffer (SFB), depending on the size of your target molecule. Flick the beads to resuspend, then return the tube to the magnetic rack and allow the beads to pellet. Remove the supernatant using a pipette and discard

2m



42 Repeat the previous step  [go to step #41](#)

2m

43 Spin down and place the tube back on the magnet. Pipette off any residual supernatant. Allow drying for  00:00:30 , but do not dry the pellet to the point of cracking

30s



44 Remove the tube from the magnetic rack and resuspend the pellet in  13 μ L Elution Buffer (EB). Incubate for  00:10:00 at  Room temperature

10m



Note

For fragments > 30 kb, we recommend increasing the elution time to  00:30:00

45 Pellet the beads on a magnet until the eluate is clear and colorless

1m

46 Remove and retain  12 μ L of eluate which contains the DNA library in a clean 1.5 ml Eppendorf DNA LoBind tube

1m



47 Prime a MinION flow cell as specified in Nanopore protocols, and finally load the library drop-wise through the Sample port (a detailed description including video documentation can be found here: [Priming and loading the SpotON flow cell](#))

20m





Citations

Step 12

Dang Y, Jia G, Choi J, Ma H, Anaya E, Ye C, Shankar P, Wu H. Optimizing sgRNA structure to improve CRISPR-Cas9 knockout efficiency.

<https://doi.org/10.1186/s13059-015-0846-3>

Step 16

Boas Pucker. Plant DNA extraction and preparation for ONT sequencing

<https://protocols.io/view/plant-dna-extraction-and-preparation-for-ont-seque-bcvyiw7w>