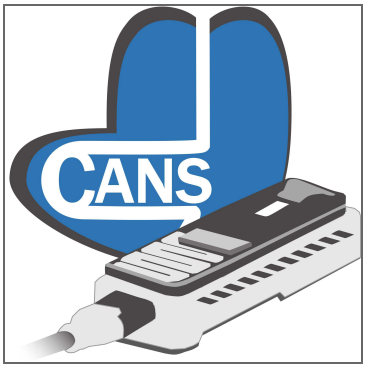

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Protocol status: Working
We use this protocol and it's working

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Cas9-targeted Nanopore sequencing (CANS)

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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.

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PROTOCOL integer ID: 50606

Keywords: Cas9, Nanopore, CANS

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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	AAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTT ATTTTAACTTGCTATTTCTAGCTCTAAAAC
	T7 F	GGATCCTAATACGACTCACTATAG
	T7 R	AAAAAAGCACCGACTCGG

2 Combine following components for sgRNA template synthesis: 10m

	A	B
	Component	Volume, µL
	Specific oligo, 1 µM	2
	CRISPR R, 1 µM	2
	T7 F, 100 µM	2
	T7 R, 100 µM	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 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.
LINK
https://doi.org/10.1186/s13059-015-0846-3

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

	A	B
	gRNA (50ng/µl ~ 1pmol/ul) 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… 32m

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

CITATION

Boas Pucker. Plant DNA extraction and preparation for ONT sequencing. protocols.io.
LINK
https://protocols.io/view/plant-dna-extraction-and-preparation-for-ont-seque-bcvyiw7w

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 i 30s
is at Room temperature before use

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 32m
at Room temperature

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 [M] 10 millimolar (mM) . In a 0.2 ml thin-walled PCR tube, make a [M] 10 millimolar (mM) dATP solution by adding 1 µL of the [M] 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
	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 therma cycler 30s

31 Using the thermal cycler, incubate at 37 °C for 15-60 (00:15:00 are recommended) minutes, th 25m 72 °C for 00:10:00 and hold at 4 °C or return to the tube to ice

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 (pH 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 supernatar 2m

41 Wash the beads by adding either 250 µL Long Fragment Buffer (LFB) or 250 µL Short Fragment 2m 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

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 µL 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 µL 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