Aug 29, 2025 Version 2

TEnCATS - Transposable Element nanopore Cas9-Targeted Sequencing V.2

DOI
https://dx.doi.org/10.17504/protocols.io.kqdg3q66ev25/v2

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Protocol Citation: Torrin McDonald, Camille Mumm, Jessica Switzenberg, Alan Boyle 2025. TEnCATS - Transposable Element nanopore Cas9-Targeted Sequencing. **protocols.io** https://dx.doi.org/10.17504/protocols.io.kqdg3q66ev25/v2Version created by **Jessica Switzenberg**

Protocol status: Working
We use this protocol and it's working currently for tissue and cell line capture experiments.

Created: August 28, 2025

Last Modified: August 29, 2025

Protocol Integer ID: 225710

Keywords: Cas9, nCATS, transposable elements, TE, R10, ONT, SMaHT, transposable elements within genomic dna, using oxford nanopore technology, oxford nanopore technology, using cas9, r10 flow cell, genomic dna, cas9, dna, boyle lab, transposable element

Abstract

This is the Boyle lab's protocol for using Cas9 to target transposable elements within genomic DNA using Oxford Nanopore Technologies (ONT) R10 flow cells.

Materials

Monarch HMW DNA Extraction Kit for Tissue (T3060S, NEB)
Proteinase K (3115879001, Roche)
Thermomixer
EnGen sgRNA Synthesis kit (E3322S, NEB)
Molecular biology grade water
Trizol
Chloroform
Ethanol
3M sodium acetate
Refrigerated centrifuge
RNase-free water
Qubit with BR RNA assay kit, dsDNA BR assay kit, dsDNA HS assay kit
Alt-R S.p. Cas9 HiFi Nuclease V3 (IDT)
QuickCIP (M0525S, NEB)
Taq DNA polymerase (M0273L, NEB)
10mM dATP
T4 DNA ligase (M0202M, NEB)
Thermolabile Proteinase K (P8111S, NEB)
1X TE buffer
SQK-LSK114 kit (ONT)
MinION R10 flow cells (ONT)
MinION sequencer (ONT)
Tube rotator
1.5mL microcentrifuge tubes
PCR tubes

High Molecular Weight (HMW) genomic DNA (gDNA) extraction from tissue

45m

1 Aliquot the desired amount of tissue for gDNA extraction

2 Follow the manufacturer's guidelines from the Monarch HMW DNA Extraction Kit for Tissues (T3060S, NEB) with following changes to the lysis step:

2.1 Add 40 µL 10 mg/mL Proteinase K to 580 µL Tissue Lysis Buffer

2.2 Incubate tissue sample in Tissue Lysis Buffer 56 °C 00:15:00 in Thermomixer at 1000rpm

15m

2.3 Incubate the tissue sample an additional 00:30:00 at 56 °C without agitation. If clumps are still visible, you can incubate longer including overnight

30m

2.4 Continue with Monarch protocol

in vitro transcription of guide RNAs (sgRNAs)

1h 15m

3 Lyophilized custom DNA oligos of the desired guide RNAs were resuspended in molecular biology grade water to a final concentration of 100uM. A 1:10 dilution of the oligos was used as a working stock for *in vitro* transcription

4 For each guide, set up the below mix in a PCR tube using the EnGen sgRNA Synthesis Kit (E3322S, NEB):

- 10 µL 2x sgRNA Reaction Mix
- 0.5 µL DTT
- 2.5 µL custom DNA oligo
- 2 µL EnGen Enzyme Mix
- 5 µL molecular biology grade water

5 Incubate the reaction 37 °C 01:00:00 in a thermocycler

1h

6 Add 30 µL molecular biology grade water then 2 µL DNase I to the reaction to remove the DNA oligos

7 Incubate the reaction 37 °C 00:15:00 15m

Purification of sgRNAs 55m

8 Move the sgRNAs produced from the *in vitro* transcription reactions into a new 1.5mL microcentrifuge tube

9 Add 200 µL Trizol and 50 µL Chloroform to the samples and vortex to mix thoroughly

10 Centrifuge 20000 x g, 4°C, 00:05:00 5m

11 Pipet out the aqueous layer (should be clear) carefully to avoid the pink layer and place into a new 1.5mL microcentrifuge tube

12 Add 50 µL Chloroform and vortex to mix thoroughly

13 Centrifuge 20000 x g, 4°C, 00:05:00 5m

14 Pipet out the top layer carefully to avoid the second layer and place into a new 1.5mL microcentrifuge tube

15 Add 2 volumes of cold 100% ethanol and 3M sodium acetate solution to a final concentration of 0.3M and invert to mix 10 times

16 Centrifuge 20000 x g, 4°C, 00:30:00 30m

17 Remove supernant being careful not to disturb the white RNA pellet at the bottom

18 Wash the sgRNA by adding cold 250 µL 70% ethanol and centrifuging 20000 x g, 4°C, 00:10:00 then removing the supernant. Repeat this wash for a total of 2 washes 10m

19 Remove as much of the residual ethanol as possible and air dry on bench for 00:05:00 5m

20 Resuspend the sgRNA in 11 µL RNase-free water

21 Quantify the RNA concentration using your specific method, we used the Qubit

Cas9-targeting of transposable elements in HMW gDNA 2h 1m

22 Aliquot out 10ug of extracted gDNA using a wide-bore tip into a PCR tube, try to get this to below 30uL. If not, you can scale up the Cutsmart Buffer and water in the following reaction if needed. Usually when scaling, I try to stay at a maximum volume of 100uL total.

23 Add the following to the gDNA sample:
4 µL 10X Cutsmart Buffer
6 µL QuickCIP
X µL molecular biology grade water to a total of 40uL.
Mix by inversion then spin down briefly

24 Incubate the sample to dephosphorylate the gDNA using the below protocol in a thermocycler: 50m
37 °C 00:30:00
80 °C 00:20:00

24.1 Make a 1:5 dilution of the Cas9 nuclease as follows:
2.5 µL 1X Cutsmart Buffer
0.5 µL Alt-R S.p Cas9 HiFi nuclease

24.2 While the gDNA is dephosphorylating, make the Cas9:sgRNA (RNP) by mixing the following in a PCR tube:
- 850 ng sgRNA
- 1 µL 1:5 dilution of Cas9 nuclease
- 1.5 µL 10X Cutsmart Buffer
- X µL RNase-free water to a total of 15uL

24.3 Mix by flicking and incubate Room temperature 00:20:00

25 Cool the dephosphorylated gDNA on ice for 00:01:00 1m

26 Add the following to the gDNA sample for Cas9 cleavage and a-tailing of open ends:
- 15 µL RNP
- 1.5 µL Taq DNA polymerase
- 1 µL 10mM dATP

Mix by inversion and spin down briefly

27 Incubate the sample using the protocol below in a thermocycler: 40m
- 37 °C 00:30:00
- 72 °C 00:10:00

28 Cool the Cas9-cleaved gDNA mix for 00:01:00 on ice

29 Add 5 µL TL Proteinase K (P8111S, NEB) to the sample

Mix by inversion and spin down briefly

30 Incubate the sample using the protocol below in a thermocycler: 30m
- 37 °C 00:20:00
- 72 °C 00:10:00

ONT library preparation for R10 MinION flow cells 56m

31 Cool the Cas9-treated sample on ice for 00:01:00 1m

32 Transfer the sample to a new 1.5mL microcentrifuge tube using a wide-bore tip

33 Add the following to the sample from the ONT SQK-LSK114 kit:
25 µL LNB
5 µL LA
then add 5 µL T4 DNA ligase (M0202M, NEB) and
X µL molecular biology grade to 100uL total and mix by inversion, then spin down briefly

Note: If you scaled up your dephosphorylation reaction, you will need to scale the amount of LNB here to be 1/4 of the total reaction volume i.e. 125uL of sample (dephos gDNA reaction+RNP+Taq+dATP+LA+T4 ligase) would be 31.25uL of LNB

34 Incubate Room temperature 00:30:00 with rotation with a tube rotator 30m

35 Add 1 volume of 1X TE buffer and invert to mix

36 Add 80 µL Axygen magnetic beads to the sample and invert to mix

Note: If you scaled up your reaction, you will need to add the beads to be a final of 0.4x i.e. 250uL (Reaction volume in Step 33 + 1x TE) is 100uL of beads

37 Incubate Room temperature 00:10:00 with rotation 10m

38 Place tube on a magnetic rack and pellet the beads

39 Remove supernant

40 Wash beads twice by adding 200 µL LFB , resuspending the beads by inversion, pellet the beads on the magnetic rack and remove the supernant

41 Remove as much residual LFB as you can by spinning the tube down briefly and pelleting the bead on the magnetic rack, and pipeting out any leftover LFB

42 Add 13 µL EB to the sample and resuspend the beads in the buffer. I have also brought this up to 16uL of EB due to scaling up and used 12uL of this library for sequencing

43 Incubate 37 °C 00:10:00 10m

44 Place tube on magnetic rack and pellet the beads

45 Pipet out the eluate and place in new 1.5mL microcentrifuge tube

46 Quantify the library using your method of choice, we use the Qubit

Loading onto the R10 MinION flow cell

47 For sequencing, we load as much library as we can onto the flow cell thus the entire 12uL of library is used. Add the following to the library:
37.5 µL SB
25.5 µL LIB

48 Prime the MinION following ONT's instructions

49 Mix the library by quick inversion or flicking to resuspend the LIB and load onto the flow cell following ONT's instructions