Sep 23, 2020

SMRT-OTS

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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 SMRT sequencing[1]. The protocol is a modified version described by Tsai et al (2017). 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. Tsai, Y-C., et al. (2017). "Amplification-free, CRISPR-Cas9 Targeted Enrichment and SMRT Sequencing of Repeat-Expansion Disease Causative Genomic Regions". bioRxiv: 203919.

THIS PROTOCOL ACCOMPANIES THE FOLLOWING PUBLICATION

https://www.biorxiv.org/content/10.1101/2020.02.09.940486v1

PROTOCOL CITATION

Ida Hoijer, Josefin Johansson, Sanna Gudmundsson, Chen-Shan Chin, Ignas Bunikis, Susana Häggqvist, Anastasia Emmanouilidou, Maria Wilbe, Marcel den Hoed, Marie-Louise Bondeson, Lars Feuk, Ulf Gyllensten, Adam Ameur 2020. SMRT-OTS. **protocols.io** https://protocols.io/view/smrt-ots-bjugkntw

MANUSCRIPT CITATION please remember to cite the following publication along with this protocol

https://www.biorxiv.org/content/10.1101/2020.02.09.940486v1

CREATED

Aug 17, 2020

LAST MODIFIED

Sep 23, 2020

PROTOCOL INTEGER ID

40552

MATERIALS

NAME	CATALOG #	VENDOR

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T4 DNA Ligase Reaction Buffer - 6.0 ml	B0202S	New England Biolabs
Lambda Exonuclease - 1,000 units	M0262S	New England Biolabs
Cas9 Nuclease, S. pyogenes - 400 pmol	M0386T	New England Biolabs
UltraPure 0.5M EDTA, pH 8.0	15575-038	Thermo Fisher Scientific
Exonuclease III (E. coli)	M0206	New England Biolabs
Exonuclease VII	M0379	New England Biolabs
Alt-R® CRISPR-Cas9 tracrRNA	1072532	IDT Technologies
Alt-R® CRISPR-Cas9 crRNA		IDT Technologies
0.75% Agarose High Pass DNA Size Selection Gel casettes	PAC-20KB	sage science
T4 DNA ligase HC	EL0013	Thermo Fisher Scientific
MagBead Kit v2	100-676-500	Pacific Biosciences
Heparin 20 µg/µl		
SMRTbell Template Prep Kit 1.0-SPv3	100-991-900	Pacific Biosciences
Ampure PB 5 mLs	100-265-900	Pacific Biosciences
Exonuclease I	M0293S	New England Biolabs
Sequencing Primer v4		Pacific Biosciences

MATERIALS TEXT

Capture adapter:
Phosp-ATC TCT CTC TTA AAA AAA AAA AAA AAA AAA AAA ATT GAG AGA GAT

Annealing of Capture adapter

Prepare 10x annealing buffer:
100 mM Tris-HCl, ph 7.5
1 M NaCl

Resuspend the adapter to 20 µM in 1x annealing buffer. Anneal as follows:
Incubate at 80°C for 2 min, then ramp temperature down to 25°C at a rate of 0.1°C/sec. Hold at 4°C.
Store the annealed adapter at -20°C.

tracr buffer:
40 mM HEPES, pH 7.5
40 mM KCl
10% Glycerol

Reducing agent:
100 mM TCEP in water

5X CRISPR buffer:
100 mM HEPES, pH 7.5
500 mM KCl
25% Glycerol
25 mM $MgCl_2$

Shearing of genomic DNA

1 Shear 10-20 µg of HMW genomic DNA to 8 kb using Megaruptor 2.

2 Add 0.45x AMPure PB 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 40 µl Elution 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 or Tape station.

SMRT bell library construction

4 The following protocol is optimized for 5 µg of sheared and concentrated DNA. Scale the reaction if needed.

5 ExoVII treatment

Add the following compontents to a new 1.5 ml eppendorf tube:

38 µl sheared DNA
5 µl DNA damage repair buffer
0.5 µl NAD+
5 µl ATP high
0.5 µl dNTP
1 µl ExoVII

Mix by gently flicking the tube, spin down. Incubate at 37ºC for 15 min. Place the tube on ice.

6 DNA damage repair

Add the following component to the EvoVII treated sample:

2 µl DNA damage repair mix

Mix by flicking the tube gently, spin down. Incubate at 37ºC for 20 min. Place the tube on ice for 1-5 min.

7 End repair

Add the following component to the DNA damaged repaired treated sample:

2.5 µl End repair mix

Mix by gently flicking the tube, spin down. Incubate at 25ºC for 5 min. Place the tube on ice.

8 Purify the DNA with AMPure PB beads

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

8.1 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.

8.2 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

8.3 Repeat step 8.2

8.4 Remove any residual ethanol.

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

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

8.6 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.

9 Adapter ligation

Add the following reagents to the end repaired and purfied DNA sample:

1 µl Blunt adapter
Mix by the tube by gently flicking the tube, spin down.
4 µl Template prep buffer
2 µl ATP low
Mix by the tube by gently flicking the tube, spin down.
1 µl Ligase

Mix by gently flicking the tube, spin down. Incubate at 25ºC overnight. Incubate at 65ºC for 10 min to inactivate the ligase. Place the tube on ice.

10 Exonuclease III and VII treatment

Add the following compontents to the adapter ligated sample:

1 µl ExoIII
1 µl ExoVII

Mix by gently flicking the tube, spin down. Incubate at 37ºC for 1 hour, then place the tube on ice. It is important to immediately proceed to purification after this step.

11 Purify the DNA with AMPure PB beads

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

11.1 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.

11.2 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

11.3 Repeat step 11.2

11.4 Remove any residual ethanol.

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

11.5 Add 40 µl Elution buffer and incubate at 2000 rpm using a vortex mixer for 5 min to elute the sample.

11.6 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 0.2 ml PCR tube.

12 Lambda exonuclease and exonuclease I treatment

Add the following components to the Exo III and VII treated sample:

5 µl Exonuclease I reaction buffer (10X)
4 µl Lambda exonuclease (5U/µl)
1 µl Exonuclease I (20U/µl)

Mix by gently flicking the tube, spin down. Incubate at 37ºC for 1 hour, 75ºC for 10 min and then hold at 4ºC using a thermocycler. Carefully tranfer the sample to a new 1.5 ml eppendorf tube.

13 Purify the DNA with AMPure PB beads

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

13.1 Place the tube on a magnetic rack until the beads collect on the side of the tube and the solution appears to be clear

13.2 Slowly pipette off the supernatant and discard. Be careful not to disturb the beads.

13.3 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

13.4 Repeat step 13.3

13.5 Remove any residual ethanol.

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

13.6 Add 32 µl Elution buffer and incubate at 2000 rpm using a vortex mixer for 5 min to elute the sample

13.7 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 1.5 ml eppendorf tube.

Quality control of SMRT bell library

14 Measure concentration of the sample using Qubit and fragment size using Bioanalyzer or Tape station.

Size selction of SMRT bell library

15 Perform size selection of the SMRT bell library using a 1.5-4 kb cut-off (depending on how the fragment profile of the SMRT bell library) using the "0.75% DF marker S1 high pass 6-10 kb vs3" casette.

16 Purify the DNA with AMPure PB beads

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

16.1 Place the tube on a magnetic rack until the beads collect on the side of the tube and the solution appears to be clear

16.2 Slowly pipette off the supernatant and discard. Be careful not to disturb the beads.

16.3 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

16.4 Repeat step 16.3

16.5 Remove any residual ethanol.

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

16.6 Add 22 µl Elution buffer and incubate at 2000 rpm using a vortex mixer for 5 min to elute the sample

16.7 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 1.5 ml eppendorf tube.

Quality control of size selected SMRT bell library

17 Measure concentration of the sample using Qubit and fragment size using Bioanalyzer or Tape station.

Preparation of guide RNAs

18 Resuspend crRNA to 10 nM in nuclease-free water and tracrRNA to 10 nM in tracr buffer.

19 Guide RNA preparation

Add the following components in a new 0.2 ml PCR tube:

5 µl crRNA (10 nM)
5 µl tracrRNA (10 nM)

Incubate at 50ºC for 5 min, ramp down to 25ºC at 0.1ºC/s, 25ºC for 5 min and hold at 4ºC.

20 For a multiplexed approach; combine equal volumes of gRNAs in a new eppendorf tube. Do not keep left over gRNA for future experiments.

Cas9 digestion

21 Reconstitute 5X CRISPR buffer by combining 95 µl 5X CRISPR buffer with 5 µl Reducing agent in a new 1.5 ml eppendorf tube. Do not keep the remaining buffer for future experiments.

22 1 µg of size selected SMRT bell library is needed in this step. Calculate the volume (X) for 1 µg of the sample. In step 23, the volume of nuclease-free water is 30-X µl.

23 Add the following compontents to a 0.2 ml PCR tube:

10 µl reconstituted 5X CRISPR buffer
30-X µl nuclease-free water
2 µl guide RNA
0.5 µl Cas9 nuclease

Mix by gently flicking the tube, spin down. Incubate at 37ºC for 10 min. Immediately place the tube on ice.

24 Add the following component to the same tube:

7.5 µl heparin (20 µg/µl)

Mix gently by flicking the tube, spin down. Incubate at 37ºC for 3 min. Immediately place the tube on ice.

25 Add 1 µg (X µl) of size selected SMRT bell library to the PCR tube. Mix gently by flicking the tube, spin down.

Incubate at 37ºC for 1 hour. Immediately place the tube on ice.

26 Add 10 µl of 0.5 M EDTA to stop the reaction. Mix gently by flicking the tube, spin down. Place the tube on ice.

27 Carefully transfer the sample to a new 0.5 ml eppendorf tube.

AMPure PB purification of Cas9 digested sample

28 Add 0.5x AMPure PB beads to the sample and incubate at 2000 rpm using a vortex mixer for 10 min.

29 Place the tube on a magnetic rack and allow the beads to collect for at least 5 min or until the solution appears clear. Slowly pipette off the supernatant and discard. Be careful not to disturb the beads.

30 Wash the beads using 80% 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

31 Repeat step 30 twice.

32 Remove any residual ethanol.

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

33 Add 39.5 µl Elution buffer to the beads. Mix until the solution is homogenous by flicking the tube.

Incubate at room temperature for 5 min. Incubate at 2000 rpm for 2 min using a vortex mixer to elute the sample.

34 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 0.5 ml eppendorf tube.

Quality control of the Cas9 digested sample

35 Use 1 µl of sample to measure DNA concentration using Qubit dsDNA HS assay kit. The typical DNA yield is between approximately 80-90% of the input amount into the Cas9 digestion.

Capture adapter ligation

36 Add the following reagents to the Cas9 digested DNA sample:

1 µl Capture adapter (annealed)
Mix by the tube by gently flicking the tube, spin down.
5 µl T4 DNA ligase reaction buffer (10x)
Mix by the tube by gently flicking the tube, spin down.
1.5 µl T4 DNA ligase

Mix gently by flicking the tube, spin down. Incubate at 16ºC overnight.

If no 0.5 ml heat block or thermocycler is available, place a 1.5 ml eppendorf tube in the heat block and add 1 ml nuclease-free water to the tube. Place the 0.5 ml tube with the ligation reaction in the water-filled tube. The same applies to step 37, 39 and 85.

37 Incubate at 65ºC for 10 min to inactivate the ligase.

38 Centrifuge the sample at 14 000 rcf for 5 min in a micro centrifuge. Transfer the supernatant to a new 0.5 ml eppendorf tube. Proceed to the next section.

Exonuclease treatment

39 Add the following components to the capture adapter ligated DNA sample:

1 µl ExoIII
1 µl ExoVII

Incubate at 37ºC for 1 hour, the place the tube on ice. Proceed immediately to the next step.

2x AMPure PB purification of exonuclease treated sample

40 Perform two consecutive AMPure PB bead purifications. Elute in 50 µl Elution buffer after the first purification and 21 µl Elution buffer after the second purification.

41 Add 0.5x AMPure PB beads to the sample and incubate at 2000 rpm using a vortex mixer for 10 min.

42 Place the tube on a magnetic rack and allow the beads to collect for at least 5 min or until the solution appears clear. Slowly pipette off the supernatant and discard. Be careful not to disturb the beads.

43 Wash the beads using 80% 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

44 Repeat step 43 twice.

45 Remove any residual ethanol.

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

46 Add 50/22 µl Elution buffer to the beads. Mix until the solution is homogenous by flicking the tube.

Incubate at room temperature for 5 min. Incubate at 2000 rpm for 2 min using a vortex mixer to elute the sample.

47 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 0.5 ml eppendorf tube.

The purified DNA can be stored at 4ºC overnight or at -20ºC for longer duration before proceeding to the next step.

Quality control of exonuclease treated sample

48 Use 1 µl of DNA sample to measure the DNA concentration using the Qubit dsDNA HS assay kit. Typical DNA yield is approximately 50-70% of the input amount going into Cas9 digestion.

Enrichment of assymmetric SMRTbell templates

49 **Tips for success:**

- Bring MagBeads to room temperature before use.
- Mix the MagBeads well, until homogeneous, before dispensing. Pipette the bead solution slowly as the bead mixture is viscous. Precise volume are critical to the purification process.
- Minimize bubble formation during the mixing of the beads and buffer by gently tapping the finger on the tube and/or magnet pulling.
- Do not pellet the beads when spinning to collect liquid.
- Bring the buffers to room temperature before use in the MagBead washing step (Step 60-67).

MagBead preparation

50 Add 50 µl of MagBeads to a 1.5 ml eppendorf tube.

51 Collect the beads to the side of the tube in a magnetic rack. Remove the supernatant and discard.

52 Add 50 µl of MagBead Wash Buffer v2 to the tube and mix. Spin briefly.

53 Collect the beads to the side of the tube in a magnetic rack. Remove the supernatant and discard.

54 Add 50 µl of MagBead Binding Buffer v2 to the tube and mix. Spin briefly.

55 Collect the beads to the side of the tube in a magnetic rack. Remove the supernatant and discard.

56 Add 7 µl of MagBead Wash Buffer v2 to the tube and mix. Spin briefly. Proceed to the next step.

MagBead binding

57 Add 21 µl of the CRISPR-Cas9 generated SMRTbell library to 7 µl of the prepared MagBeads. Gently mix, ensure the beads are completely resuspended. Spin briefly.

58 Incubate at 4ºC for 2 hours with gentle rotation in a tube rotator.

59 Spin briefly and place the tube on ice. Proceed to the next step.

MagBead washing

60 *Note: If multiple samples, process one sample at a time starting at Step 60. Keep addtional samples on ice until ready to process.*

Collect the beads to the side of the tube in a magnetic rack. Remove the supernatant and discard.

61 Add 100 µl of room temperature MagBead bindning buffer v2 to the tube. Mix gently and spin briefly.

62 Collect the beads to the side of the tube in a magnetic rack. Remove the supernatant and discard.

63 Add 100 µl of room temperature MagBead bindning buffer v2 to the tube. Mix gently and spin briefly. Transfer the soltion to a new 1.5 ml eppendorf tube.

64 Collect the beads to the side of the tube in a magnetic rack. Remove the supernatant and discard.

65 Add 100 µl of room temperature MagBead bindning buffer v2 to the tube. Mix gently and spin briefly.

66 Collect the beads to the side of the tube in a magnetic rack. Remove the supernatant and discard.

67 Add 7 µl Elution buffer to the tube to elute the captured SMRTbell library. Mix gently and spin briefly.

Note: Keep samples in Elution buffer at room temperature if processing additional samples.

Sample elution

68 *Note: If multiple samples, process one sample at a time starting at Step 69. Keep additional samples at 50ºC.*

Incubate the sample at 50ºC for 10 min in an Eppendorf ThermoMixer at full mixing speed.

69 Spin briefly to collect the liquid.

70 Collect the beads at the side of the tube in a magnetic rack. Quickly transfer the supernatant to a new tube before the solution cools down. Place the tube on ice.

71 Samples can be stored overnight at 4ºC or at -20ºC for longer duration before proceeding to the next step.

Primer annealing

72 Prepare diluted Sequencing primer v4 before reaction setup by adding 1 µl primer to 29 µl nuclease-free water.

73 Add the following components to a 0.2 ml PCR tube:

18 µl 10X Primer buffer v2
9 µl diluted Sequencing primer v4

Mix gently by flicking the tube. Spin briefly. Incubate at 80ºC for 2 minutes and hold at 4ºC using a thermocycler.

74 Add the follwing to a new 0.2 ml PCR tube:

2.7 µl conditioned sequencing primer
6.3 µl enriched SMRTbell library

Incubate at room temperatrue for 1 hr. Place the tube on ice.

Polymerase binding

75 Prepare diluted Sequel polymerase 3.0 just prior to use by adding 1 µl polymerase to 29 µl Sequel binding buffer v1 and keep on ice. Diluted polymerase must be used immediately. Discard the unused portion.

76 Add the following components to the tube containing 9 µl primer annealed SMRTbell library:

1.5 µl Sequel binding buffer v1
1.5 µl DTT
1.5 µl dNTP v3
Mix before proceeding
1.5 µl Diluted Sequel polymerase 3.0

Mix gently by flicking the tube. Spin down. Incubate at 30ºC for 4 hours and hold at 4ºC using a thermocycler.

Sequel sequencing preparation

77 Make two 100-fold serial dilutions of the Sequel DNA internal control 3.0 in MagBead binding buffer v2:

- Add 1 µl control complex to 99 µl MagBead binding buffer v2.
- Mix well by flicking the tube. Spin down.
- Add 1 µl of the diluted control complex to 99 µl MagBead binding buffer v2.
- Mix well by flicking the tube. Spin down.

78 Dispense 2.8 µl of the final control complex dilution to a sequencing sample plate for each sample. Cover the sample plate and keep on ice until ready to add the purified sample complex.

Sample complex purification

79 Carefully transfer the 15 µl of samplex complex to a new 0.5 ml eppendorf tube.

80 Add 35 µl MagBead binding buffer v2 to the tube containing 15 µl of sample complex.

81 Add 30 µl AMPure PB beads to the tube containing 50 µl diluted sample complex. Mix well by flicking the tube. Spin down.

Incubate at room temperature for 5 min.

82 Place the tube on a magnetic rack for 2 min and slowly remove the supernatant and discard.

83 Immediately resuspend the beads in 41.1 µl room temperature MagBead binding buffer v2. Mix well by flicking the tube. Spin down.

Incubate in room temperature for 15 min.

84 Place the tube on a magnetic rack for 1 min and slowly remove the supernatant and transfer to the well in the sequencing sample plate containing the control complex.

85 Add an additional 41.1 µl room temperature MagBead binding buffer v2 to the tube containing the beads. Mix well by flicking the tube. Spin down.

Incubate at 37ºC for 15 min *(same technique described in Step 36 can be used).*

86 Place the tube on a magnetic rack for 1 min and slowly remove the supernatant and transfer to the well in the sequencing sample plate containing the control complex and the first sample eluate.

87 Cover the sample plate at keep on ice until ready to sequence.

88 Sequence with diffusion loading, 4 hr immobilization time and 10 hr movie time.

Data analysis

89 Because of the assymmetric nature of the SMRTbells recalling of adapters has to be performed, using the following tool:

https://github.com/PacificBiosciences/recalladapters

90 Perform CCS analysis on the recalled data.

91 Align the CCS reads to a reference using minimap2.

92 Use the mapped reads for detection of off-target sites using our custom tool Insider:

https://github.com/UppsalaGenomeCenter/InSiDeR