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Title of resource
Cas9 Targeted Sequencing Protocol for *Simulium sp* and *Onchocerca volvulus*
Authored by
When using this protocol, the following should be referenced: Samuel Armoo[1], Romain Derelle[2], Daniel Boakye[3], Mike Y. Osei-Atweneboana[1], Charles Brockhouse[4], John Colbourne[2.]. Adapted from Gilpatrick *et al.* (2020) [1]Council for Scientific and Industrial Research – Water Research Institute, Accra, Ghana. [2]University of Birmingham, Birmingham, UK [3]Noguchi Memorial Institute for Medical Research, Accra, Ghana [4]Creighton University, Omaha, NE, USA
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Description
This protocol is for the preparation of Cas9 guide RNA targeted MinION sequencing library from DNA extracted from *Simulium* sp and *Onchocerca volvulus* worms. This protocol also describes DNA library loading procedures. This protocol is adapted from Gilpatrick *et al.* (2020)
Intended use
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Resource history

GN 23 Cas9 targeted Sequencing Protocol for *Simulium sp* and *Onchocerca volvulus*

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1.0 Introduction

This GNATWORK project employed a Cas9 guide RNA system to ensure targeted sequencing of the of the mitochondrial genomes of *Onchocerca volvulus* and *Simulium sp*. This Cas9 guide RNA system used here (Figure 1) is similar to that used by Gilpatrick and colleagues (Gilpatrick et al., 2020). This protocol is a revised and personalized version of the Cas9 targeted sequencing protocol from the Oxford Nanopore Community protocol repository (https://nanoporetech.com/community).

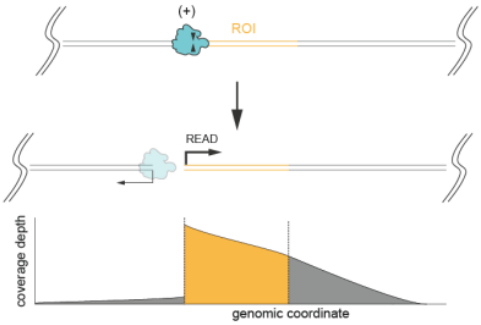

Figure 1: Using a single Cas9 guide RNA to cut the circular mitochondrial genome to linearize it to expose ends sequencing adapter ligation. In this approach, an initial dephosphorylation of the genomic DNA, prior to Cas9 guide RNA cleavage, blocks external sticky phosphate ends available for sequencing adapter ligation. A guided Cas9 RNA cleavage leaves sticky phosphate ends for sequencing adapter ligation at the targeted region. Therefore, sequencing reads will be made up of targeted region of the genome, significantly reducing background reads.

2.0 Forming Cas9 Ribonucleoprotein Complexes (RNPs)

Cas9 is loaded with crRNA and tracrRNA to form ribonucleoprotein complexes (RNPs) in preparation for the cleavage reaction.

2.1 Consumables

- Alt-R® S. pyogenes HiFi Cas9 nuclease V3, 100 µg or 500 µg (IDT, Cat # 1081060 or # 1081061)
- CutSmart Buffer (NEB Cat # B7204)
- S. pyogenes Cas9 Alt-R™ crRNAs (resuspended at 100 µM crRNA in TE pH 7.5). Sequence for *O. volvulus* Cas9 guide RNA used is: 5'-AAA TTT TTA GAC CAC ATA ATG-3'; and sequence of for *Simulium sp.* Cas9 guide RNA is 5'-TTA GAC ATT CTT TTT TTA AG-3'.
- S. pyogenes Cas9 tracrRNA (e.g., IDT Alt-R™, Cat # 1072532, 1072533 or 1072534) resuspended at 100 µM in TE pH 7.5
- Nuclease-free TE, pH 7.5 (10 mM Tris (pH 7.5), 0.1 mM EDTA; e.g., IDT Cat # 11-01-02-02)

- 0.2 ml thin-walled PCR tubes
- 1.5 ml Eppendorf DNA LoBind tubes
- Nuclease-free water (e.g. ThermoFisher, cat # AM9937)
- Nuclease-free duplex buffer (IDT Cat # 11-01-03-01)

2.2 Equipment

- Thermal cycler
- Ice bucket with wet ice
- P200 pipette and tips
- P10 pipette and tips
- P2 pipette and tips

2.3 Methods to Follow

- Pre-heat a thermal cycler to 95º C.
- Thaw an aliquot of NEB CutSmart Buffer, mix by vortexing, and place on ice.
- In a 1.5 mL Eppendorf DNA LoBind tube, mix *crRNA* (guide RNA) and *tracrRNA* using volumes below. Mix by pipetting and spin down.

Table 2.1 Annealing crRNA and tracrRNA mix.

Reagent	**Volume (uL)**
Duplex buffer	8
crRNA (100 µM) - guide	1
tracrRNA (100 µM)	1
Total	10

- Using a thermal cycler, heat the above reaction mix at 95º C for 5 mins, then remove the tube from the thermal cycler and allow it to cool to room temperature (18-23 C), then spin down the tube to collect any liquid in the bottom of the tube.
    - *Storage and reuse of the annealed mix is not recommended*.
- To form Cas9 RNPs, assemble the components in table 2.2 (below) in a 1.5 ml Eppendorf DNA LoBind tube in the stated order.

Table 2.2: Forming the Cas9 RNP complex

Number of reactions	**3**	**5**	**10**
Reagent	**Volume (uL)**		
Annealed crRNA•tracrRNA pool (10 µM)- from table 2.1	3	5	10
10x NEB CutSmart buffer	3	5	10
Nuclease-free water	23.7	39.6	79.2
HiFi Cas9 (62 µM)	0.3	0.4	0.8
Total	30	50	100

- Form the RNPs by incubating at room temperature (18-23) for 30 mins, then return the RNPs on ice until required. *Tip*: *proceed to the next step (gDNA dephosphorylation) during the 30 min RNP incubation step*.

3.0 Dephosphorylating Genomic DNA

This step reduces background reads by removing 5' phosphates from non-target DNA ends

3.1 Materials

- 5,000 ng of high molecular weight genomic DNA (recommended)

3.2 Consumables

- Quick Calf Intestinal Phosphatase (NEB Cat #M0525)
- 1.5 ml Eppendorf DNA LoBind tubes
- Nuclease-free water (e.g. ThermoFisher, cat # AM9937)
- 0.2 ml thin-walled PCR tubes

3.3 Equipment

- Thermal cycler
- P100 pipette and tips
- P10 pipette and tips

3.4 Steps to Fellow

- Transfer 5,000 ng (total volume of 24 µl) genomic DNA into a 0.2 ml thin-walled PCR tubes (Table 3.1).
- Mix thoroughly by flicking the tube avoiding unwanted shearing.
- Spin down briefly in a microcentrifuge.
- Mix the Quick calf intestinal phosphatase (CIP) in the tube by pipetting up and down. Ensure that it is at room temperature before use.

Table 3.1: Genomic DNA dephosphorylation mix

Reagent	**Volume (uL)**
NEB CutSmart Buffer (10x)	3
HMW genomic DNA (at ≥ 210 ng/µl)	24
CIP**	3**
Total	30

- Mix gently by flicking the tube, and spin down.
- ***Add 3 µl of CIP to the tube.
- Using a thermal cycler, incubate at 37º C for 10 minutes, 80º C for 2 minutes then hold at 20º C.

4.0 Cleaving and dA-tailing Target DNA

In this step, Cas9 RNPs (section 2.0) and Taq polymerase are added to the dephosphorylated genomic DNA sample (section 3.0). This process cleaves target DNA, and dA-tails all available DNA ends in one step- activating the Cas9 cut sites for ligation

4.1 Materials

- crRNA-tracrRNA-Cas9 ribonucleoprotein complexes (section 2)
- 5,000 ng of high molecular weight dephosphorylated genomic DNA (section 3).

4.2 Consumables

- Nuclease-free TE, pH 8.0 (10 mM Tris (pH 8.0), 0.1 mM EDTA; e.g., IDT Cat # 11-01-02-05)
- Alt-R® S. pyogenes HiFi Cas9 nuclease V3, 100 µg or 500 µg (IDT, Cat # 1081060 or # 1081061)
- CutSmart Buffer (NEB Cat # B7204)
- Taq polymerase (NEB Cat # M0273)
- dATP solution (100 mM) (NEB Cat # N0440S)
- 1.5 ml Eppendorf DNA LoBind tubes
- 0.2 ml thin-walled PCR tubes
-

4.3 Steps to Follow

- Thaw the dATP tube, vortex to mix thoroughly and place on ice.
- Spin down and place the tube of Taq polymerase on ice.
- In a fresh 1.5 ml DNA Eppendorf LoBind tube, make a 10 mM dATP solution by adding 1 µl of the 100 mM dATP stock to 9 µl of nuclease-free water. Vortex to mix, then spin down.
- To the PCR tube containing 30 µl dephosphorylated DNA sample (section 3.0), add the following (table 4.1):

Table 4.1: Cleaving and dA-tailing mix

Reagent	**Volume (uL)**
Dephosphorylated genomic DNA sample (from section 3.0)	30
Cas9 RNPs (from section 2.0)	10
10 mM dATP	1
NEB Taq polymerase	1
Total	42

- Carefully mix the contents of the tube by gentle inversion, then spin down and place the tube in the thermal cycler.
- Using the thermal cycler, incubate at 37º C for 45 min, then 72º C for 5 min and hold at 4º C or return to tube on ice.

5. 0 Adapter Ligation

AMX adapters from the Ligation Sequencing Kit (SQK-LSK109), are ligated to the ends generated by Cas9 cleavage

5.1 Materials

- Ligation Sequencing Kit (SQK-LSK109)

5.2 Consumables

- 1.5 ml Eppendorf DNA LoBind tubes
- T4 DNA ligase, from NEBNext Quick Ligation Module (E6056)
- Agencourt AMPure XP beads

5.3 Equipment

- Ice bucket with wet ice
- Vortex mixer
- P1000 pipette and tips
- P100 pipette
- P20 pipette and tips

5.4 Steps to Follow

- Thaw Ligation Buffer (LNB) at room temperature, spin down and mix by pipetting. Due to viscosity, vortexing this buffer is ineffective. Place on ice immediately after thawing and mixing.
- Carefully transfer the contents of the 0.2 ml thin-walled PCR tube (table 4.1) to a fresh 1.5 ml Eppendorf DNA LoTube using a wide-bore pipette tip.
- Thaw an aliquot of Adapter Mix (AMX), mix by flicking the tube, pulse-spin to collect the liquid in the bottom of the vial, then return the vial to ice.
- Place the AMPure XP beads at room temperature.
- Assemble the following (table 5.1) 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.

Table 5.1 Adapter ligation mix

Reagent	**Volume (uL)**
Ligation Buffer (LNB)	20
Nuclease-free water	3
NEBNext Quick T4 DNA Ligase	10
Adapter Mix (AMX)*	5
Total	38

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

- Mix by pipetting the above ligation mix thoroughly. Ligation Buffer (LNB) is very viscous, so the adapter ligation mix needs to be well-mixed.
- **IMPORTANT**: Add 20 µl of the adapter ligation mix (table 5.1) to the cleaved and dA-tailed sample in the tube (table 4.1). Mix gently by flicking the tube. Do not centrifuge the sample at this stage.

Immediately after mixing, add the remainder of the adapter ligation mix (table 5.1) to the cleaved and dA-tailed sample (table 4.1), to yield an 80 µl ligation mix.

- Mix gently by flicking the tube, and spin down.
- Incubate the reaction for 10 minutes at room temperature.
- **IMPORTANT:** A white precipitate may form upon addition of the adapter ligation mix (table 5.1) to the dA-tailed DNA (table 4.1). 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.

6.0 AMPure XP Bead Purification

This step removes excess un-ligated adapters and other short DNA fragments, and concentrates and buffer-exchanges the library in preparation for sequencing

6.1 Materials

- Long Fragment Buffer (LFB)
- Short Fragment Buffer (SFB)
- Elution Buffer (EB)

6.2 Consumables

- Agencourt AMPure XP beads
- 1.5 ml Eppendorf DNA LoBind tubes
- Nuclease-free TE, pH 8.0 (10 mM Tris (pH 8.0), 0.1 mM EDTA; e.g., IDT Cat # 11-01-02-05)

6.3 Equipment

- P1000 pipette and tips
- P200 pipette and tips
- P20 pipette and tips
- Magnetic separator, suitable for 1.5 ml Eppendorf tubes
- Eppendorf 5424 centrifuge (or equivalent)

6.4 Steps to Follow

- Resuspend the AMPure XP beads by vortexing and ensure they are at room temperature before use. Thaw an aliquot of SFB (Short Fragment Buffer) from SQK-LSK109, as required, and an aliquot of EB (Elution Buffer).
- Add 1 volume (80 µl) of TE (pH 8.0) to the ligation mix (section 5). Mix gently by flicking the tube.
- Add 0.3x volume (48 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 liquid.
- Incubate the sample for 10 minutes at room temperature. Do not agitate or pipette the sample.
- Spin down the sample and pellet on a magnet. Keep the tube on the magnet, and pipette off the supernatant.

- Wash the beads by adding of Short Fragment Buffer 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.
- Repeat the previous step.
- Spin down and place the tube back on the magnet. Pipette off any residual supernatant. Allow to dry for ~30 seconds, but do not dry the pellet to the point of cracking.
- Remove the tube from the magnetic rack and resuspend pellet in 13 µl Elution Buffer (EB). Incubate for 20 minutes at room temperature.
- Pellet the beads on a magnet until the eluate is clear and colourless.
- Remove and retain 12 uL of the eluate, which contains the DNA library, in a clean 1.5 ml Eppendorf DNA LoBind tube.
- Dispose of the pelleted beads.
- The prepared library will be used for loading the flow cell. Store the library on until ready to use.

7.0 Priming and Loading the SpotON Flow Cell

(Please note that the Sequencing Tether (SQT) tube will NOT be used in this protocol. It is provided in the kit for potential future product compatibility).

7.1 Materials

- Flush Tether (FLT)
- Flush Buffer (FB)
- Loading Beads (LB)
- Sequencing Buffer (SQB)

7.2 Consumables

- 1.5 ml Eppendorf DNA LoBind tubes
- Nuclease-free water (e.g. ThermoFisher, cat # AM9937)

7.3 Equipment

- MinION
- P1000 pipette and tips
- P100 pipette and tips
- P20 pipette and tips
- P10 pipette and tips

7.4 Steps to Follow

- Thaw the Sequencing Buffer (SQB), Loading Beads (LB), Flush Tether (FLT) and one tube of Flush Buffer (FB) at room temperature before placing the tubes on ice as soon as thawing is complete.
- Mix the Sequencing Buffer (SQB) and Flush Buffer (FB) tubes by vortexing, spin down and return to ice.
- Spin down the Flush Tether (FLT) tube, mix by pipetting, and return to ice.

- Open the lid of the Nanopore sequencing device and slide the flow cell's priming port cover clockwise so that the priming port is visible.
    - Priming and loading the SpotON Flow Cell
    - Priming and loading: The steps for priming and loading the SpotON Flow Cell. Written instructions are given below. The library is loaded dropwise without putting the pipette tip firmly into the port.
    - Take care to avoid introducing any air during pipetting.

- After opening the priming port, check for small bubble under the cover. Draw back a small volume to remove any bubble (a few µls):
    - Set a P1000 pipette to 200 µl
    - Insert the tip into the priming port
    - Turn the wheel until the dial shows 220-230 µl, or until you can see a small volume of buffer entering the pipette tip
    - Visually check that there is continuous buffer from the priming port across the sensor array.

- Prepare the flow cell priming mix: add 30 µl of thawed and mixed Flush Tether (FLT) directly to the tube of thawed and mixed Flush Buffer (FB), and mix by pipetting up and down.
- Load 800 µl of the priming mix into the flow cell via the priming port, avoiding the introduction of air bubbles. Wait for 5 minutes.
- Thoroughly mix the contents of the Loading Beads (LB) by pipetting.
- In a new tube, prepare the library for loading as follows (table 7.1):

Table 7.1 Preparing library for loading

Reagent	**Volume (uL)**
Sequencing Buffer (SQB)	25
Loading beads (LB), mixed immediately before use	13
DNA library	12
Total	50

- Complete the flow cell priming:
    - Gently lift the SpotON sample port cover to make the SpotON sample port accessible.
    - Load **200 µl** of the priming mix into the flow cell via the priming port (**not** the SpotON sample port), avoiding the introduction of air bubbles.
- Mix the prepared library gently by pipetting up and down just prior to loading.
- Add 50µl of sample to the flow cell via the SpotON sample port in a dropwise fashion. Ensure each drop flows into the port before adding the next. *Note: The final loading volume is 50 µl, less than a standard SQK-LSK109 library preparation.*
- Gently replace the SpotON sample port cover, making sure the bung enters the SpotON port, close the priming port and replace the MinION lid.

Reference

Gilpatrick, T., Lee, I., Graham, J.E., Raimondeau, E., Bowen, R., Heron, A., Downs, B., Sukumar, S., Sedlazeck, F.J., and Timp, W. (2020). Targeted nanopore sequencing with Cas9-guided adapter ligation. Nat Biotechnol *38*, 433-438.