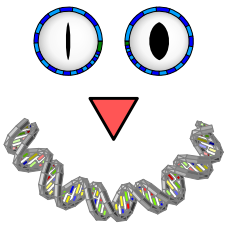
Sample Preparation - Ligation

GrinGene Bioinformatics



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Whakapapa / Tohutoro (References)

- Based on the ONT Community Protocols for the V14 Ligation Kit (SQK-LSK114) [MinION / GridION / Flongle (https://community.nanoporetech.com/docs/prepare/library_prep_protocols/genomic-dna-by-ligation-sqk-lsk114/v/gde_9161_v114_revt_29jun2022)] and the Input DNA/RNA QC Protocol (https://community.nanoporetech.com/protocols/input-dna-rna-qc/v/idi_s1006_v1_revb_18apr2016)
- End repair details from here (https://www.seqanswers.com/forum/applications-forums/sample-prep-library-generation/48241-end-repair-a-tailing-how-do-they-do-it)
- Additional explanations by David Eccles (knowledge from ONT talks, and practical experience)

Quick Summary

1. Dilute 200 fmol input DNA in TBS to a 53.5 µl total volume in a 1.5 ml tube.
2. Add 3.5 µl Ultra II End-prep Reaction Buffer and 3µl Ultra II End-prep Enzyme mix.
3. Heat 5 minutes at RT, then 5 minutes at 65°C.
4. Purify on a magnetic rack using 60 µl AXP (1X) and 2 × 150 µl 80% Ethanol.
5. Elute in 60 µl TBS (without changing the tube).
6. Add 25 µl of LNB, 10 µl Quick T4 DNA Ligase, and 5 µl of LA; incubate 10 minutes at RT.
7. Purify on a magnetic rack using 60 µl AXP (0.6X) and 2 × 250 µl LFB (or SFB).
8. elute in 13 µl EB (without changing the tube); incubate 10 minutes at 37°C.
9. Dilute 20-50 fmol into 12 µl EB (in a new tube).
10. Prepare flow cell:
    - MinION: 30 µl FCT + 1170 µl FCF (priming buffer); prime with 800 µl
    - Flongle: 3 µl FCT + 117 µl FCF (priming buffer); drop 30 µl
    - PromethION: 30 µl FCT + 1170 µl FCF (priming buffer); prime with 500 µl
11. Mix with sequencing buffers:
    - MinION: 37.5 µl SB; 25.5 µl LIB; 12 µl sequencing library
    - Flongle: 14 µl SB; 10 µl LIB; 6 µl sequencing library
    - PromethION: 100 µl SB; 68 µl LIB; 20 µl NFW; 12 µl sequencing library
12. Load onto flow cell:
    - MinION: prime with 200 µl priming buffer, then load 75 µl via SpotON port
    - Flongle: drop 30 µl priming buffer, then drop 30 µl library
    - PromethION: prime with 500 µl priming buffer, then load 200 µl library via priming port

Scope and Purpose

This procedure is for fragmenting and preparing a barcode-free library for sequencing on a ONT MinION or Flongle flow cell, where maximum yield from a single flow cell is desired, or it's necessary to produce full-length sequences that correspond directly with the input DNA. It is recommended that a Flow Cell QC (flow-cell-QC.html) is carried out just prior to doing sample preparation, to make sure that a working Flow Cell will be available for a freshly-prepared sample, and to ensure that the protective environment used for shipping the flow cells is maintained for as long as possible.

Potential Hazards

The most dangerous component of the Ligation Sequencing Kit is glycerol (CAS#: 56-81-5; EC#: 200-289-5), which is not classified as hazardous. All kit components are in small quantities and are unlikely to be considered a significant hazard, as long as standard laboratory practises are observed. For more details, please refer to the Ligation Kit Safety Data Sheet (docs/MSDS-SQK-LSK114_Ligation_Sequencing_Kit.pdf).

Safety / Procedural Precautions

- Ultra-long DNA is sensitive to breakage. Even if length may not be an issue for the particular experiment being carried out, it is good practise to get into the habit of carefully handling samples containing *any* DNA. In particular, take extra care to mix only via the prescribed method (i.e. by pipetting, vortexing, or flicking).

Procedure

Materials (ONT)

- Ligation Sequencing Kit (SQK-LSK114)
    - 60 + XX µl Ampure XP beads (AXP); thawed at room temperature, then resuspended **by vortexing**
    - 25 µl Ligation Buffer (LNB); spun down briefly, mixed **by pipetting**, spun down again, then placed on ice
    - 5 µl Ligation Adapter (LA); spun down briefly, then placed on ice
    - 25 µl Elution Buffer (EB); tawed at room temperature, then resuspended **by vortexing**

Materials (Other Suppliers)

- NEBNext Companion Module for Oxford Nanopore Technologies Ligation Sequencing (NEB E7180S or E7180L)
    - 3.5 µl Ultra II End-prep Reaction Buffer
    - 3 µl Ultra II End-prep Enzyme Mix
    - 10 µl NEBNext Quick T4 DNA Ligase
- 400 µl 80% ethanol

Despite the expense of this NEB reagent (about $1,300 for 24 runs), it works out currently as the cheapest per-run commercial kit for ligation sequencing, even if only the dA-tailing and ligation buffers are used.

Equipment & Consumables (Other Suppliers)

- Microfuge
- P200 pipette & tips
- P20 pipette & tips
- P10 pipette & tips
- Timer
- Gentle agitator (e.g. hula mixer or rotator mixer)
- Magnetic rack
- high molecular weight genomic DNA; 10 µl per sample at a concentration of 20-100 ng/µl
- 1.5 ml tubes [at least 1 per sample, and 1 for the pooled mixture]
- 4.5 µl Nuclease-free water (e.g. ThermoFisher, cat # AM9937) [MinION Only]

If bead purification is needed:

- 1.5 ml tubes [at least 1 per sample]
- Agencourt AMPure XP beads [at least 2.5X input DNA volume, per sample]
- Freshly prepared 70% ethanol in nuclease-free water [at least 500µl per sample]
- Tris-buffered saline (TBS), at least 12µl per sample; 10 mM Tris-HCl pH 8.0 with 50 mM NaCl [see preparation steps below]

Magnetic Rack

If you don't have access to a magnetic rack, see here for a magnetic rack frame that is printable without supports on a filament 3D printer:

https://www.printables.com/model/532085-open-walled-magnetic-rack (https://www.printables.com/model/532085-open-walled-magnetic-rack)

The 20x10x5mm N52 magnets to fit into these racks can be purchased from AMF Magnets:

https://amfmagnets.co.nz/products/neodymium-block-20mm-x-10mm-x-5mm-n52 (https://amfmagnets.co.nz/products/neodymium-block-20mm-x-10mm-x-5mm-n52)

Preparing TBS solution

1. Make 1M stock of NaCl by adding 2.922g of NaCl into a 50 ml Falcon tube, and make up to 50 ml with MilliPore water
2. Create 50 mM TBS stock by adding 750 µl 1M NaCl solution to a 15 ml Falcon tube, and make up to 15 ml using Qiagen Elution Buffer (EB, i.e. 10 mM Tris-HCl at pH 8.0)
3. Confirm pH to be 7.9-8.1 using a pH indicator strip [e.g. EMD Millipore MColorpHast 1.09543.0007], or pH meter

Before You Prepare Samples

- Make sure input DNA is sufficient quality; see the Sample QC protocol (sample-QC.html).
- Begin a Flow cell QC check.
- Start heating a heating block up to 65°C.

DNA Preparation

1. Transfer 100-200 fmol genomic DNA into a 1.5 ml tube. This corresponds to approximately 100-200 ng for an average input DNA length of 1.5 kbp, or 10-20 ng for an average input DNA length of 150 bp, or 1-2 µg for an average input DNA length of 15 kbp.
2. Adjust the volume to 53.5 µl with Tris-buffered saline (TBS; see above for a preparation protocol).
3. Mix gently **by flicking** the tube.
4. Spin the sample down briefly (1-3s) in a microfuge.

End Repair and dA tailing

1. Add 3.5 µl Ultra II End-prep Reaction Buffer.
2. Add 3 µl Ultra II End-prep Enzyme Mix.
3. Mix thoroughly **by pipetting**. If DNA is long (>10 kb), mix using a wide-bore pipette tip to reduce shearing.
4. Incubate the sample tube for at least 5 minutes at room temperature (RT; 18-23°C). This will repair the ends of DNA fragments (i.e. filling in 5' overhang and removing 3' overhang.
5. Incubate the sample tube for at least 5 minutes at 65°C. This will heat-kill the end repair enzyme and add a single dA to the 3' end of blunt-ended DNA.

Post-tailing Bead Cleanup

1. Resuspend the AMPure XP beads **by vortexing**.
2. Add 60µl of resuspended AMPure XP beads (i.e. 1X) to the reaction and mix **by pipetting**. If DNA is long (>10 kb), mix using a wide-bore pipette tip to reduce shearing.
3. Incubate on a gentle agitator (e.g. Hula mixer or rotator mixer) for at least 5 minutes at RT (18-23°C).
4. Prepare 400 µl of fresh 80% ethanol in nuclease-free water.
5. Spin the sample down briefly in a microfuge (1-3s).
6. Pellet the beads on a magnet until the eluate is clear and colourless.
7. Keeping the tube on the magnet, pipette off and discard the supernatant.
8. Keeping the tube on the magnet, slowly add 150 µl ethanol to cover (but not disturb) the pellet.
9. Keeping the tube on the magnet, pipette off and discard the washing solution and collected contaminants, leaving the pellet behind.
10. Repeat the previous two steps (add 150µl ethanol, then remove and discard the added ethanol).
11. Spin down the pellet briefly in a microfuge.
12. Place the tube back on the magnet and pipette off any residual ethanol.
13. Allow to air dry for at most 30 seconds, but do not dry the pellet to the point of cracking. This will happen shortly after the pellet loses shine from the liquid.
14. Elute by adding 60 µl of TBS.
15. Remove the tube from the magnet, and resuspend **by flicking**.
16. Spin the sample down briefly in a microfuge (1-3s).

Keep the sample in the same tube for the next steps; this reduces sample loss.

This is a good stopping point; the end-repaired sample can be stored on the magnetic beads at 4°C for a few days up to a few weeks without substantial degredation.

Reagent Preparation

1. Spin down the ligation adapter (LA) and Quick T4 Ligase briefly in a microfuge (1–3s).
2. Store LA and T4 ligase on ice.
3. Thaw the ligation buffer (LNB), fragment buffer (LFB for reads >3kbp, or SFB) and elution buffer (EB) at RT.
4. Spin down LNB briefly in a microfuge (1–3s), then mix **by pipetting**.
5. Mix EB and LFB/SFB **by vortexing**.
6. Spin down LNB, LFB/SFB, and EB briefly in a microfuge (1–3s), then place on ice.

Adapter Ligation

1. Add 25 µl Ligation Buffer (LNB) to the sample tube.
2. Add 10 µl NEBNext Quick T4 DNA Ligase.
3. Add 5 µl Ligation Adapter (LA).
4. Mix gently **by pipetting**. If DNA is long (>10 kb), mix using a wide-bore pipette tip to reduce shearing.
5. Spin down briefly in a microfuge (1–3s).
6. Incubate the sample tube for at least 10 minutes at RT.

Post-ligation bead cleanup

Note: after adding the adapter, keep the adapted sample away from ethanol, as it can damage the protein and prevent sequencing. ONT provides short and long fragment buffers that replace ethanol washes in bead cleanup.

1. Resuspend the AMPure XP beads **by vortexing**.
2. Add 60µl of resuspended AMPure XP beads (i.e. 1X) to the reaction and mix **by pipetting**. If DNA is long (>10 kb), mix using a wide-bore pipette tip to reduce shearing.
3. Incubate on a gentle agitator (e.g. Hula mixer or rotator mixer) for at least 5 minutes at RT (18–23°C).
4. Spin the sample down briefly in a microfuge (1–3s).
5. Pellet the beads on a magnet until the eluate is clear and colourless.
6. Keeping the tube on the magnet, pipette off and discard the supernatant.
7. Add 250 µl fragment buffer (LFB for reads >3kbp, or SFB).
8. Remove the tube from the magnet, and resuspend the beads **by flicking**.
9. Return the beads to the magnet and wait until the eluate is clear and colourless.
10. Repeat the previous 4 steps (discard supernatant; add 250 µl LFB/SFB; resuspend by flicking; pellet on magnet).
11. Spin the pellet down briefly in a microfuge (1–3s).
12. Place the tube back on the magnet and pipette off any residual buffer.
13. Allow to air dry for at most 30 seconds, but do not dry the pellet to the point of cracking. This will happen shortly after the pellet loses shine from the liquid.
14. Elute by adding 13 µl of Elution buffer (EB).
15. Remove the tube from the magnet, and resuspend **by flicking**.
16. Spin down briefly in a microfuge (1–3s).
17. Incubate for 10 minutes at 37°C.
18. Return the beads to the magnet and wait until the eluate is clear and colourless.

QC and Concentration adjustment

1. Quantify 1 µl of eluted sample using a fluorometer (e.g. Quantus or Qubit).
2. Dilute 20–50 fmol into 12 µl EB (in a new tube). Use 20 fmol if high-accuracy duplex data is desired, and 50 fmol if high-yield data is required. 50 fmol corresponds to approximately 50 ng for an average input DNA length of 1.5 kbp, or 5 ng for an average input DNA length of 150 bp, or 500 ng for an average input DNA length of 15 kbp.

The sequencing library has now been prepared for sequencing. This is an appropriate stopping point; the library can be stored on ice or at 4°C until it is ready to load.

Method - Flow Cell Loading

The sequencing library has now been prepared for sequencing. Proceed with the 12µl of library as input for ONT R10.4.1 flow cell loading, as per the RBK114.24 kit:

- Flongle Flow Cell (https://community.nanoporetech.com/docs/prepare/library_prep_protocols/rapid-sequencing-gdna-barcoding-sqk-rbk114/v/rbk_9176_v114_revj_27nov2022/loading-the-flongle-flow-cell?devices=flongle)
- MinION Flow Cell (https://community.nanoporetech.com/docs/prepare/library_prep_protocols/rapid-sequencing-gdna-barcoding-sqk-rbk114/v/rbk_9176_v114_revj_27nov2022/priming-and-loading-the-spoton-flow-cell?devices=minion)
- PromethION Flow Cell (https://community.nanoporetech.com/docs/prepare/library_prep_protocols/rapid-sequencing-gdna-barcoding-sqk-rbk114/v/rbk_9176_v114_revj_27nov2022/priming-and-loading-the-promethion-flow-cell?devices=promethion)

A quick summary of loading for each type of flow cell follows; please consult the protocols above for more information.

Loading - Flongle Flow Cells

This uses the gentle negative-pressure loading method from the community, with 5µl of library loaded in 30µl mix:

https://community.nanoporetech.com/posts/a-very-gentle-relatively (https://community.nanoporetech.com/posts/a-very-gentle-relatively)

1. Prepare Flow Cell Flush solution:
    - 117 µl Flow Cell Flush (FCF)
    - 3 µl Flow Cell Tether (FCT)
    - mix ***by pipetting***
2. Unseal the flow cell
3. Add tape to the rectangular waste port (see the sticker)
4. Drop 30 µl of flush solution onto the loading port to form a dome
5. Place pipette into loading port and dial up about 5 µl to check for bubbles (dial up to remove bubbles if they exist)
6. If liquid is not dropping from the loading port, place pipette into the exposed waste port and dial up 30 µl or until liquid starts dropping from the loading port
7. Set pipette to 30 µl, press down while in mid air to expel air, then place into the exposed waste port and slowly release the plunger. If liquid starts dropping from the loading port, stop releasing and lift up the pipette
8. Repeat the last step with a faster release speed until liquid starts dropping from the loading port
9. Wait 5 minutes (while preparing library for loading)
    - 15 µl Sequencing Buffer (SB)
    - 10 µl Library Beads, mixed ***by pipetting*** immediately before use
    - 5 µl DNA library
    - mix ***by pipetting***
10. Remove the tape from the waste port
11. Drop another 30 µl of flush solution onto the loading port, wait for it to drain through
12. Drop 30 µl of sequencing library onto the loading port, wait for it to drain through
13. Re-seal the flow cell by rolling a finger across the plastic adhesive cover, trying to avoid putting pressure on the flow cell matrix

Loading – MinION Flow Cells

This method uses BSA (can probably be omitted), with a library load amount of 12 µl in 75 µl:

1. Prepare Flow Cell Flush solution:
    - 1170 µl Flow Cell Flush (FCF), i.e. an entire tube
    - 12.5 µl Bovine Serum Albumin (BSA) at 20 mg/µl [recombinant BSA seems to work as well]
    - 30 µl Flow Cell Tether (FCT)
    - mix ***by pipetting***
2. Load 800µl flush solution into ***Priming port***
3. Wait 5 minutes (while preparing library for loading)
    - 37.5 µl Sequencing Buffer (SB)
    - 25.5 µl Library Beads (LIB), mixed ***by pipetting*** immediately before use
    - 12 µl DNA library
    - mix ***by pipetting***
4. Lift up SpotON port cover
5. Load 200µl flush solution into ***Priming port***
6. Add 75µl library dropwise into ***SpotON port***

Loading – PromethION Flow Cells

BSA is not used for PromethION flow cell loading, and the library load amount is 12 µl in 200µl:

1. Prepare Flow Cell Flush solution:
    - 1170 µl Flow Cell Flush (FCF), i.e. the entire tube
    - 30 µl Flow Cell Tether (FCT)
    - mix ***by pipetting***
2. Mix by pipetting
3. Load 500µl flush solution into inlet port
4. Wait 5 minutes (while preparing library for loading)
    - 100 µl Sequencing Buffer (SB)
    - 68 µl Library Beads (LIB), mixed ***by pipetting*** immediately before use
    - 12 µl DNA library
    - 20 µl Elution Buffer (EB)
    - mix ***by pipetting***
5. Lift up SpotON port cover
6. Load 500µl flush solution into inlet port
7. Load 200µl library into inlet port by slow pipetting