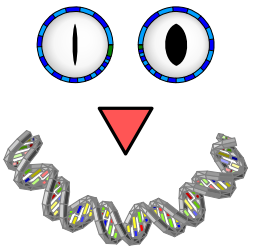
Sample Preparation - Rapid Barcoding

GrinGene Bioinformatics



Version: 2.2.3
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Whakapapa / Tohutoro (References)

- Based on the ONT Community Protocols for the V14 Rapid Barcoding Sequencing Kit (SQK-RBK114.24) [MinION / GridION / Flongle (https://community.nanoporetech.com/docs/prepare/library_prep_protocols/rapid-sequencing-gdna-barcoding-sqk-rbk114/v/rbk_9176_v114_revj_27nov2022)] and the Input DNA/RNA QC Protocol (https://community.nanoporetech.com/protocols/input-dna-rna-qc/v/idi_s1006_v1_revb_18apr2016)
- Additional explanations by David Eccles (knowledge from ONT talks, and practical experience)

Quick Summary

1. Add 1.5 µl barcoded fragmentation mix (RBXX) to 8.5 µl genomic DNA (25 ng/µl) into a 1.5 ml tube.
2. Heat 120s at 30°C, then 120s at 80°C.
3. Pool samples together, then take 11µl into a new tube.
4. Add 1 µl of diluted Rapid Adapter (RA); wait 10 minutes at RT.
5. 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
6. 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
7. 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 library for sequencing on a ONT MinION or Flongle flow cell, where a quick sample preparation is desired, and variable fragment length is acceptable. 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.

Note: this workflow is identical to that of the Rapid Sequencing Kit (SQK-RAD114). The only difference between the kits is that the fragmentation mix incorporates a fixed sequence for the Rapid Sequencing Kit, i.e.

```
5' - GCTTGGGTGTTTAACC - <sequence unknown> - GTTTTCGCATTTATCGTGAAACGCTT
TCGCGTTTTTCGTGCGCCGCTTCA - 3'
```

whereas this sequence is variable for the Rapid Barcoding Kit, i.e.

```
5' - GCTTGGGTGTTTAACC - barcode - GTTTTCGCATTTATCGTGAAACGCTTTCGCGTTTTTC
GTGCGCCGCTTCA - 3'
```

[See barcode-sequences (barcode-sequences.html) for the barcode sequences]

Potential Hazards

The most dangerous component of the Rapid Barcoding 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 Rapid Barcoding Kit Safety Data Sheet (docs/MSDS-SQK-RBK114_Rapid_Barcoding_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

Before You Begin

Make sure the input DNA is long enough to be cut with the fragmentation enzyme at least once. Our experimentation suggests that this is achievable with fragment lengths of at least 100bp - it can cut the shortest band of the TrackIt 1kb+ DNA ladder - although yield will be improved is the input is within the range of 2-50kb. Long template DNA may knot up and block pores (reducing the immediate sequencing capacity of the flow cell), whereas short template DNA leaves relatively longer gaps between successive DNA translocation events, during which smaller molecules can flow through the pores (increasing the flow cell depletion rate over time).

Materials (ONT)

- Rapid Barcoding Kit (SQK-RBK114.24)
    - 1.5µl of the appropriate index (RLBXX) for each sample (e.g. 30µl for 12 samples); mix **by flicking**, spin down, and store on ice
    - 1µl diluted Rapid Adapter (RA); mix **by flicking**, spin down, and store on ice
    - 34µl Sequencing Buffer (SB), thawed at RT, then stored on ice [15 µl for Flongle]
    - 25.5µl Loading Beads (LIB), thawed at RT, then stored on ice [10 µl for Flongle]

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)

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

Ampure XP Bead Purification

The official ONT protocol recommends carrying out this step after pooling samples together. It is necessary as a preparation step prior to fragmentation for direct PCR products that include unincorporated primer sequences, and can also be used when input DNA quality is low.

1. Transfer the sample to a clean 1.5 ml Eppendorf DNA LoBind tube.
2. Resuspend the AMPure XP beads **by vortexing**.
3. Add 1X the sample volume of resuspended AMPure XP beads to the reaction and mix **by pipetting** (i.e. for a sample volume of 15µl, add 15µl Ampure XP beads).
4. Incubate on a gentle agitator (e.g. Hula mixer or rotator mixer) for at least 5 minutes at RT (18-23°C).
5. Prepare 500 µl of fresh 80% ethanol in nuclease-free water.
6. Spin the sample down briefly in a microfuge (1-3s).
7. Pellet the beads on a magnet until the eluate is clear and colourless.
8. Keeping the tube on the magnet, pipette off and discard the supernatant.
9. Keeping the tube on the magnet, slowly add freshly-prepared 80% ethanol to cover (but not disturb) the pellet (about 200µl for 1.5ml tubes).
10. Keeping the tube on the magnet, pipette off and discard the ethanol and collected contaminants, leaving the pellet behind.
11. Repeat the previous two steps (add ethanol to cover the pellet, then remove and discard the added ethanol).
12. Spin down the pellet briefly in a microfuge.
13. Place the tube back on the magnet and pipette off any residual ethanol.
14. 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.
15. Elute by adding 12 µl EB (or TBS).
16. Remove the tube from the magnet, and resuspend **by flicking**.
17. Spin the sample down briefly in a microfuge (1-3s).
18. Incubate for 2 minutes at room temperature.
19. Pellet the beads on a magnet until the eluate is clear and colourless.
20. Quantify 1 µl of the eluate using a Qubit fluorometer. The DNA concentration should be at least 5ng/µl.

The DNA has now been bead-purified. The eluate can be left as-is in a fridge (together with the beads), and transferred into another tube when needed.

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 80°C.
- Record which sample goes with which barcoded mix.

DNA Tagmentation (carried out for each sample)

1. Transfer 8.5 µl genomic DNA into a 1.5 ml tube. [Note: DNA concentration should be 25 ng/µl]
2. Add 1.5 µl barcoded fragmentation mix (RBXX).
3. Mix gently **by flicking** the tube.
4. Spin the sample down briefly (1-3s) in a microfuge.

Incubate all sample tubes in a heating block (or by hand) for 120s at 30°C to activate the fragmentation enzyme, then for 120s in a heating block (or thermal cycler) at 80°C to inactivate the fragmentation enzyme after cutting.

Barcode pooling

Pool all barcoded libraries together into a single tube, ideally in equimolar ratios.

For calculating molarity, a reasonable assumption to make for linear sequences is that the average sequence length has halved after fragmentation (i.e. 6kb template DNA will be fragmented into a mean template length of 3kb). For circular sequences less than 20kb (e.g. plasmid DNA), the most common sequence is a single cut linearised plasmid.

If the template DNA is below 2kb, then the average fragment length after fragmentation may be more than half the sequence length.

For example, imagine there are three plasmid samples with template lengths 5kb, 10kb, and 3kb. Work out the relative proportion of sample to add by dividing the adjusted template length by the length of the longest sample:

- 5 kb -> 5 / 10 -> 0.5
- 10 kb -> 10 / 10 -> 1
- 3 kb -> 3 / 10 -> 0.3

Multiply this amount by 10 µl (or 5 µl for Flongle) to determine how much to add to the pool:

- 5 kb -> 0.5 x 10 µl = 5 µl
- 10 kb -> 1 x 10 µl = 10 µl
- 3 kb -> 0.3 x 10 µl = 3 µl

If sequencing many samples with low concentration, the samples can be concentrated via XP beads after pooling (see previous XP bead purification steps), and eluted in 30 µl EB/TBS

(or 15 µl EB/TBS for Flongle).

Diluted Rapid Adapter preparation

1. Thaw the adapter buffer at RT, mix **by vortexing**, then spin down briefly (1-3s) in a microfuge, and place on ice
2. Mix the Rapid Adapter (RA) **by pipetting**, then spin down briefly (1-3s) in a microfuge, and place on ice
3. Into a 0.2 ml PCR tube, add the following as diluted Rapid Adapter:
    1. 1.5 µl Rapid Adapter (RA)
    2. 3.5 µl Adapter Buffer (ADB)

Note: The rapid adapter is diluted into a total volume of 5µl, of which only 1µl is used for sequencing. Left over diluted adapter can be stored in a -20°C freezer for use in additional rapid sequencing experiments. The lifetime of the diluted rapid adapter is not yet known (it lasts at least a month), so it is recommended to label the tube with the date of dilution to help with establishing lifetimes in the future.

Adapter addition

1. Transfer 11 µl of pooled DNA to a new tube.
2. Add 1 µl of diluted Rapid Adapter (RA).
3. Mix gently **by flicking** the tube.
4. Spin the sample down briefly in a microfuge.
5. Incubate the reaction for 10 minutes at RT. This is a great time to prepare the flow cell for sample loading with priming solution.

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. If liquid is not draining from the loading port, there is a liquid flow blockage. Place a pipette through the drop and into the loading port, then dial up about 5 µl to check for bubbles (dial up to remove bubbles if they exist).
6. If liquid is still not draining from the loading port, place a pipette into the exposed waste port (with a circular hole) and dial up 30 µl or until liquid starts dropping from the loading port.
7. If liquid is still not draining from the loading port, set the 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 the plunger and lift up the pipette.
8. If liquid is still not draining from the loading port, 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