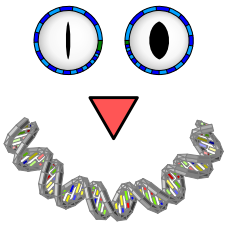
Sample Preparation – Rapid PCR Barcoding

GrinGene Bioinformatics



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Whakapapa / Tohutoro (References)

- Based on the ONT Community Protocols for the Rapid PCR Barcoding Kit (SQK-RPB114.24) [MinION / GridION / Flongle (https://nanoporetech.com/document/rapid-sequencing-dna-v14-pcr-barcoding-sqk-rpb114-24)] 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)
- Additional modifications from MARC River Water Sequencing project [paper (https://academic.oup.com/gigascience/article/9/6/giaa053/5855463#204848914), co-authored by David Eccles]

Scope and Purpose

This procedure is for preparing a shotgun PCR library for sequencing on a ONT MinION or Flongle flow cell, where nothing is known in advance about the sample, or a relatively unbiased survey of the DNA content of a sample is desired (e.g. metagenomics). 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 Rapid PCR 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 PCR Barcoding Kit Safety Data Sheet (docs/MSDS-SQK-RPB004_Rapid_PCR_Barcoding_Kit.pdf).

Safety / Procedural Precautions

- Ultra-long DNA is sensitive to breakage. While the fragmentation and PCR barcoding process used in this protocol will break DNA down to lengths where shearing is not an issue, 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

- Check to make sure that the input DNA is long enough to be cut with the fragmentation enzyme at least *twice*. This probably requires fragment lengths of at least 2kb, but ideally over 5kb. If template DNA is too small, then it won't amplify, and the total DNA amount after amplification will be *lower* than the amount before amplification.

Materials (ONT)

- Rapid PCR Barcoding Kit (SQK-RPB114.24)
    - 1µl fragmentation mix (FRM) for each sample (e.g. 12µl for 12 samples); mix **by flicking**, spin down, and store on ice
    - 1µl rapid PCR barcodes (RLB) for each sample (RLB01-24; 10µM); 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
- P1000 pipette & tips
- P200 pipette & tips
- P100 pipette & tips
- P20 pipette & tips
- P10 pipette & tips
- P2 pipette & tips
- Timer
- Thermal cycler
- Hula mixer or rotator mixer
- Magnetic rack
- 1.5 ml Eppendorf DNA LoBind tubes [at least 1 per sample]
- 0.2 ml thin-walled PCR tubes [at least 1 per sample]
- Nuclease-free water (e.g. ThermoFisher, cat # AM9937) [at least 23 µl per sample]
- Agencourt AMPure XP beads [at least 30µl per sample]
- LongAmp Taq 2X Master Mix (e.g. NEB M0287) [at least 50 µl per sample]; mix by pipetting until bits go away, store on ice
- 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]
- 1-5 ng high molecular weight genomic DNA per sample

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

Input QC

To obtain good results from Nanopore sequencing, use high quality, pure genomic DNA (gDNA). Good results can be obtained from DNA which meets the following criteria:

- Purity as measured using Nanodrop – OD 260/280 of 1.8 and OD 260/230 of 2.0–2.2
- Average fragment size, as measured by pulsed-field gel analysis (>30 kb)

For long-term storage of high molecular weight (HMW) gDNA we recommend the use of TE buffer.

There are a number of known common contaminants that can influence the results of QC checks, as well as nanopore sequencing: ethanol, isopropanol, EDTA, NaCl, Guanidinium chloride, Guanidinium isothiocyanate, and Phenol. Where possible, try to reduce the input of these contaminants into the sample preparation process process.

The majority of RNA should be removed by RNase digestion, e.g. by using the RNase Cocktail Enzyme Mix (ThermoFisher, AM2286). DNA should be quantified during the sample preparation process by a labelled fluorescence-based method (e.g. Quantus or Qubit), as RNA bases can still remain as a contaminant after digestion and will influence Nanodrop results.

For more details, see the DNA QC protocol (DNA-QC.html).

DNA Tagmentation (carried out for each sample)

1. Transfer 1-5 ng genomic DNA into a 0.2 ml PCR tube. [DNA concentration should be about 2 ng/µl]
2. Adjust the volume to 3 µl with nuclease-free water.
3. Mix gently **by flicking** to avoid unwanted shearing.
4. Add 1µl fragmentation mix (FRM).
5. Mix gently **by flicking** the tube.
6. Spin the sample down briefly (1-3s) in a microfuge.

Incubate all sample tubes in a thermal cycler for 120s at 30°C to activate the fragmentation enzyme, then for 120s at 80°C to inactivate the fragmentation enzyme after cutting.

PCR Barcoding and DNA Amplification (carried out for each sample)

Prepare solutions on a PCR tube freezer block to avoid enzyme activation prior to amplification.

1. To each tube of 4 µl of fragmented DNA, add the following reagents to a total volume of 50 µl:
    - 20 µl Nuclease-free water
    - 1 µl Rapid library barcoding primer (RLB). Use a different barcode for each sample (RLB01-RLB24)
    - 25 µl LongAmp Taq 2X Master Mix
2. Mix gently **by flicking** the tube.
3. Spin the sample down briefly in a microfuge.
4. Amplify all samples together in a thermal cycler using the following cycling conditions (20 cycles amplification):

Cycle Step	Temperature	Time
Initial denaturation	95°C	3 mins
20 cycles of...		
/ Denaturation	95°C	15 secs
Annealing	56°C	15 secs
\ Extension	65°C	6 mins
Final extension	65°C	6 mins
Hold	4°C	∞

[Advanced use: Adjust the extension time accordingly for different lengths of amplicons and the type of polymerase that is being used.]

DNA Concentration (carried out for each reaction)

1. Resuspend the AMPure XP beads **by vortexing**
2. Prepare 100 µl of fresh 80% ethanol per reaction in nuclease-free water (e.g. 1200 µl for 12 reactions)
3. To each PCR tube add 40 µl of resuspended AMPure XP beads (i.e. 0.8X) to the reaction and mix **by pipetting**
4. Incubate on a gentle agitator (e.g Hula mixer or rotator mixer, or thermomixer) for at least 5 minutes at RT (18-23°C).
5. Spin the tubes down briefly (1-3s) in a microfuge
6. Pellet the beads in a magnetic rack 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 50 µl of 80% ethanol to cover (but not disturb) the pellet
9. Keeping the tube on the magnet, pipette off and discard the ethanol and collected contaminants, leaving the pellet behind
10. Keeping the tube on the magnet, slowly add another 50 µl of 80% ethanol to cover (but not disturb) the pellet
11. Keeping the tube on the magnet, pipette off and discard the ethanol and collected contaminants,

leaving the pellet behind
12. Spin the tubes down briefly (1–3s) in a microfuge
13. Place the tubes 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. Add 12 µl of Elution Buffer (EB)
16. Remove the tube from the magnetic rack, and resuspend **by flicking**
17. Spin the tubes down briefly in a microfuge
18. Incubate on a gentle agitator (e.g Hula mixer or rotator mixer, or thermomixer) for at least 10 minutes at RT (18–23°C)
19. Pellet the beads in a magnetic rack until the eluate is clear and colourless
20. Quantify 1 µl of eluted sample using a fluorometer. The DNA concentration should be at least 20ng/µl

This is a good stopping point; concentrated DNA can be stored on beads in a non-cycling fridge at 4°C. Consider running 1µl of each sample on a gel (or a TapeStation / Bioanalyzer) to determine the average DNA fragment length

Barcode pooling

Pool all barcoded libraries in equimolar ratios into a single tube, to a total of 50–100 fmoles (i.e. 100–200ng for 3kb fragments) in 11 µl of TBS.

Where this requires less than 1µl added per sample, add at least 1µl for the most concentrated sample (with other sample amount scaled accordingly), then take 50–100 fmoles from the pooled libraries and make up to 11 µl using TBS.

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. Add 1µl of diluted rapid adapter to the amplified DNA library
2. Mix gently **by flicking** to avoid unwanted shearing
3. Spin the library down briefly (1–3s) in a microfuge
4. Incubate for at least 5 minutes at RT (18–23°C)

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