Quadram Institute Science Health Food Innovation

How to obtain good quality nucleic acid for MinION sequencing

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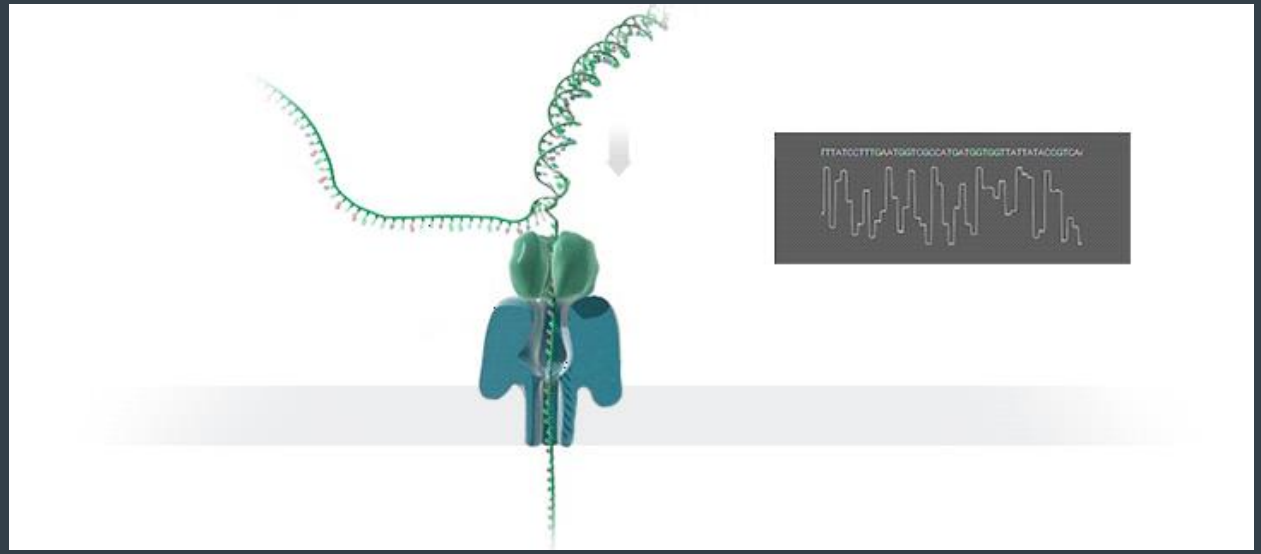
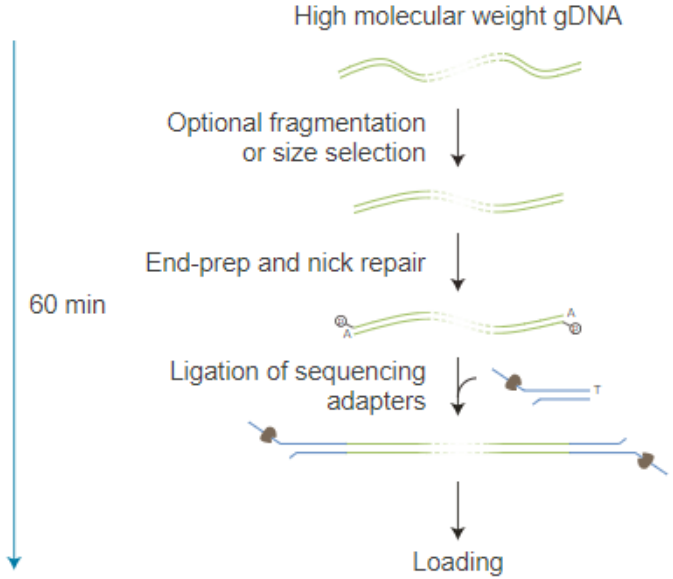
Sequencing chemistry



Types of kits

Broadly speaking, DNA:

1) Ligation Kits – adaptor is ligated to the end of DNA with ligase



PCR, multiplex, and 1D^2 options

Pros:

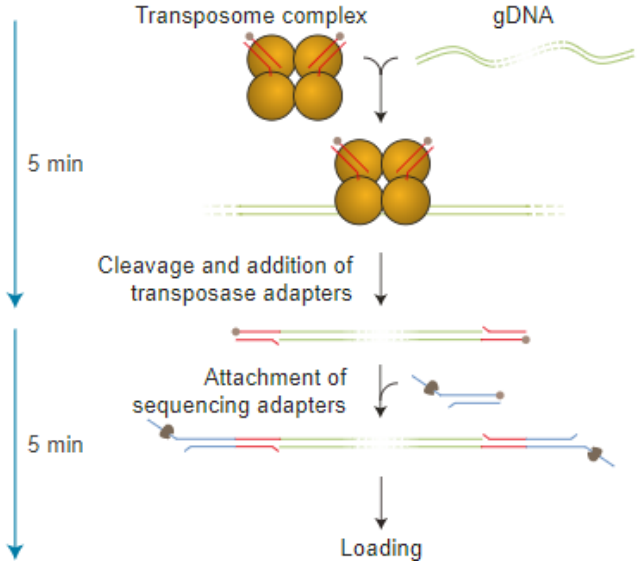
- Most control over size
- Highest yield

Cons:

- Longer
- More room for error
- More expensive (additional reagents)

Types of kits

2) Rapid Kits – transposase cuts DNA and allows adaptor to attach



PCR and multiplex options

Pros:

- Very fast
- Simple

Cons:

- Less control over size
- Lower yield

Types of kits

RNA:

1) Direct native RNA sequencing (Ligation based)

Pros:
- No reverse transcription or PCR bias

Cons:
- Much lower yield
- Early technology

2) cDNA sequencing (Ligation based)

Pros:
- Higher yield
- PCR option (lower input)

Cons:
- Takes much longer
- Requires reverse transcription

Extraction – up to you

- Extraction method will depend on what you are trying to extract and the purpose.
- **Generally**: Fresh cells and fresh extractions are best
- **Lysis**: Chemical, mechanical (bead-beating), enzymatic
- **Isolation**: phenol/chloroform, spin-column, SPRI beads
- **Consider**: Depletion or enrichment pre-extraction
- **Consider**: DNase or RNase step
- **Options**: Manual, automated

How much do you need? (DNA input)

- **Nanopore sequencing is not so much about mass (e.g. nanograms) but about molecules (e.g. femtomoles).**
- **If size and mass is known, calculate fmol. However, if size is difficult to (HMW) determine. Use input guidelines per kit.**

DNA Ligation kits

✓ **1000ng**: use as is *(per sample)*

➢ **100-500ng**: shear DNA

➢ **100ng**: use PCR (PSK004 kit)

➢ Short: calculate fmol, use **200 fmol**

DNA Rapid kits

✓ **400ng** (*per sample)*

➢ If you want longer reads: **1600+ ng**

➢ If low DNA, use PCR (RPB004 kit)

– input **1-10ng**

How much do you need? (RNA input)

Direct RNA sequencing

✓ **500ng** of poly-A RNA

cDNA sequencing

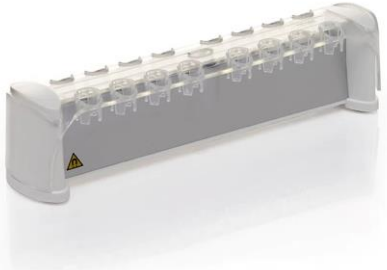
✓ **100ng** of input poly-A RNA

➢ If low, use PCR cDNA kit

- input **1ng** poly-A RNA or **50ng** total RNA

How much do you load?

- Follow all protocol-specified DNA washes. Purity is important for PCRs and sequencing.
  Biggest source of DNA loss



- If you end up with a surplus, do not be tempted to load all of it.
- Overloading will mean sequencing fuel is used up faster = lower yield
- If possible (e.g. for short DNA/Rapid Kits/PCR kits)
  measure size and quantity (mass), calculate to load ~50-100 fmol
- If still HMW after processing e.g. for Ligation kits, load ~400-600 ng

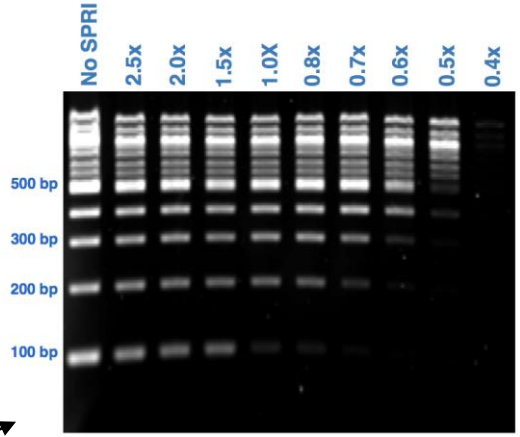
How long should it be? Up to you

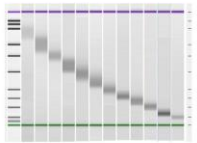
- No fragmentation or size selection required.
- Record read length: 2,200,000+ basepairs
- Longest reads achieved with Rapid Kit. More input = longer reads (but poor yield)
- Longer reads = better assemblies.
- More reliable sequencing with ligation kit if DNA is sheared (more ‘ligatable’ ends)
- Not degraded or too short - (Rapid Kit input recommendation >30kb)

Selecting the size

Size will depend on extraction but you can tweak average size after.

- **Shearing: Covaris g-Tubes**
- **Small fragment removal: SPRI beads (limited size range) 0.4x absolute minimum.**
- **Size fractionation device such as the SageELF**

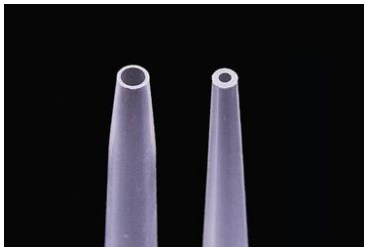




Handling DNA

To preserve long fragments (if a priority)

- **Avoid unnecessary pipetting or vortexing of DNA**
- **Avoid freeze-thawing DNA**
- **Use wide-bore pipette tips**



- **For 45kb+ DNA, drying step not recommended using SPRI beads**
- **Incubate DNA for longer and at higher temperatures during elution steps (e.g. at 37 C)**

Number of samples / yield

- Maximum MinION yield ~30 gigabases (normally lower)
- Don't think about how many samples you CAN multiplex, but how many samples you SHOULD multiplex
- Consider using other devices if yield is redundant or too low

PromethION max yield: ~148gb

Flongle max yield: ~1.8gb

Example:
- MinION run of stool sample yields **15gb**
- Detecting Campylobacter (1.6mb genome)
- Usually low proportion of reads (e.g. 1%)
- 1 Sample run = **93x coverage**
- 12 Sample run = **8x coverage**

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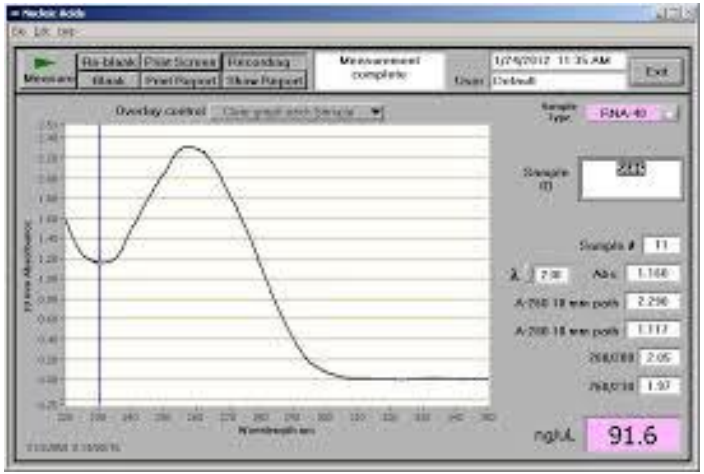
Checking your DNA/RNA

Quantity

- Check quantity with Qubit
- Use higher volumes (if possible to spare) for more accurate readings
- New standards each time you create the working buffer
- **Avoid Nanodrop** for quantification – overrepresents quantity

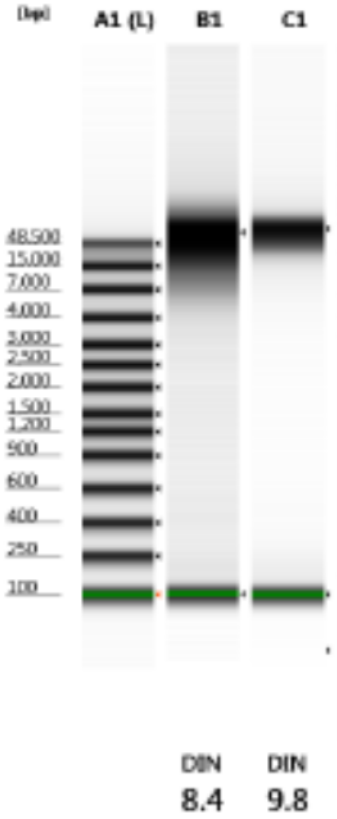
Quality

- Use Nanodrop to only check for potential contaminants
- 260/280 ratio of 1.8 = ‘pure DNA’
- 260/280 ratio of 2.0 = ‘pure RNA’
- 260/230 ratio of 2.0-2.2 = ‘clean’
  Lower = impure
- Carryover Ethanol not easily detected on Nanodrop
- Ratios unreliable at low concentrations

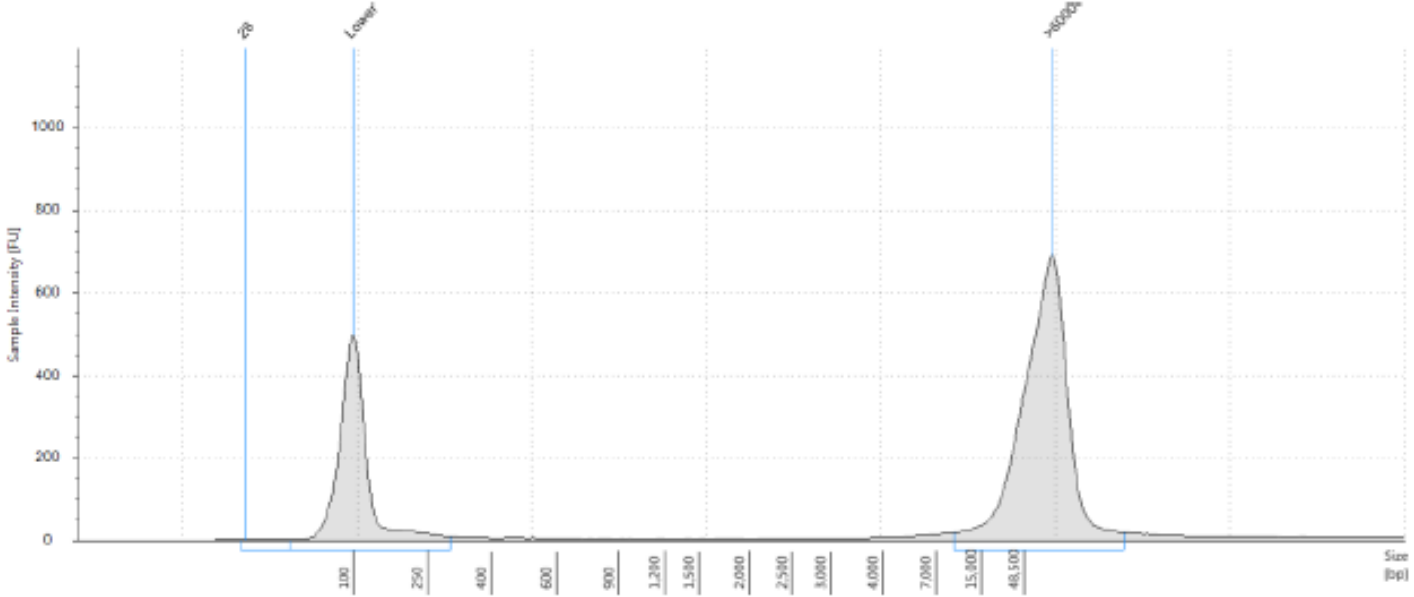


Size and integrity

- Use Tapestation for size and integrity (not degraded)
- DIN/RIN value = integrity (higher = better)
- Long fragments less reliable
  (don't assume 30kb = exactly 30kb)
- **Don't just focus on peak, focus on 100-1000bp area**



Tapestation – short fragments



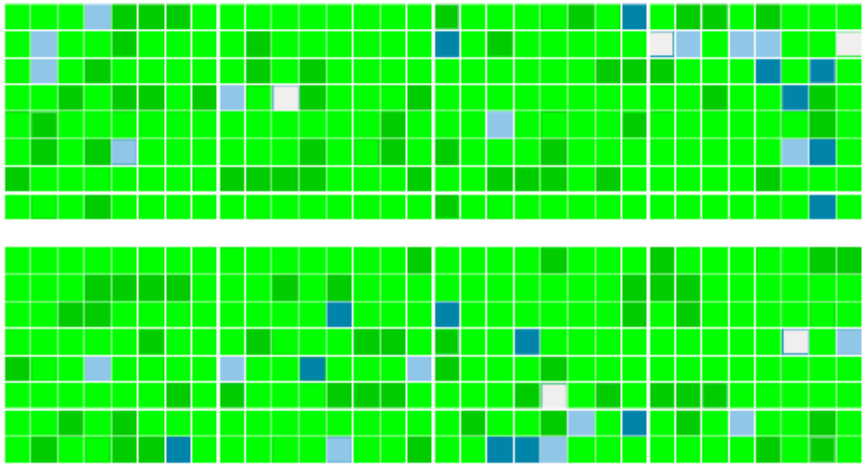
Checking quality by sequencing

- **Worth checking libraries on Flongle before expensive PromethION run**

Assessing your library in MinKNOW

Check for:

- **Available pores**
- **Pore occupancy**
- **Adaptors**



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Remember for sequencing:

GARBAGE IN = GARBAGE OUT

Questions?