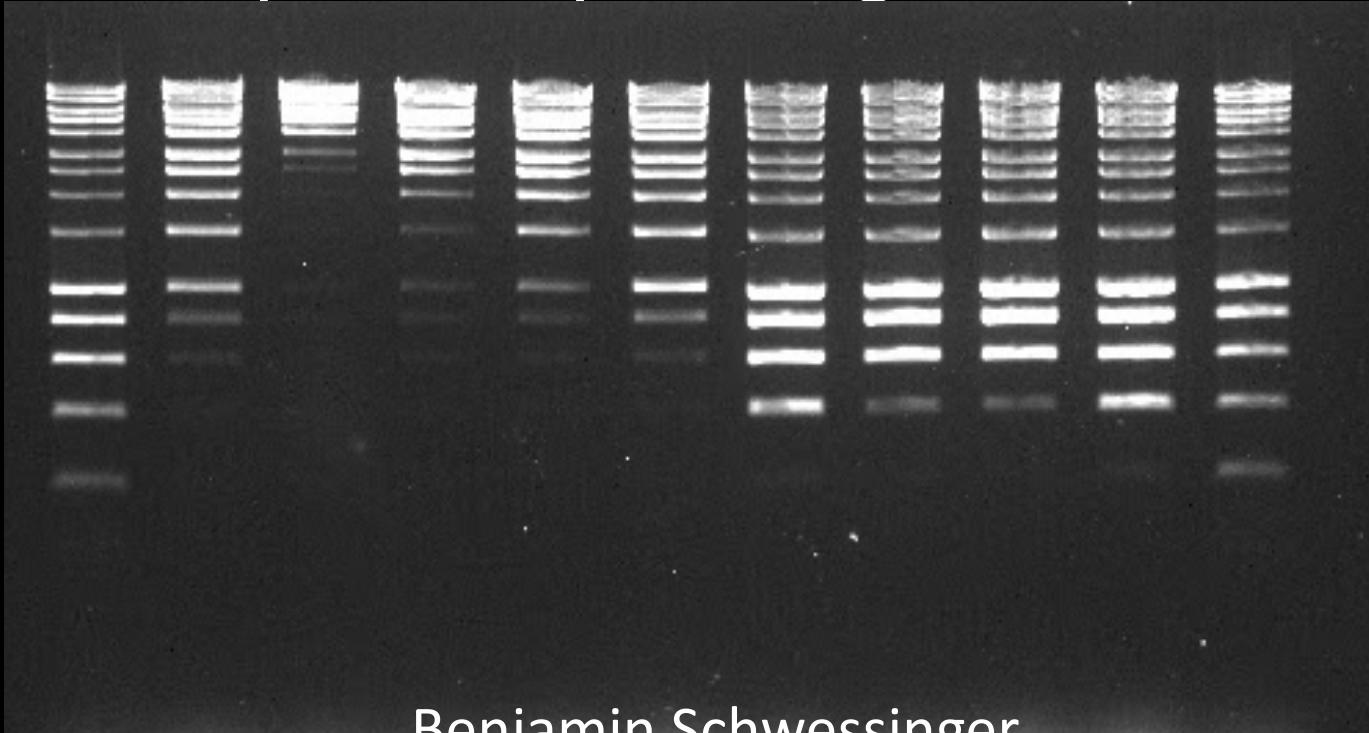


Insights into DNA extraction and library preparation for Nanopore sequencing on the MinION



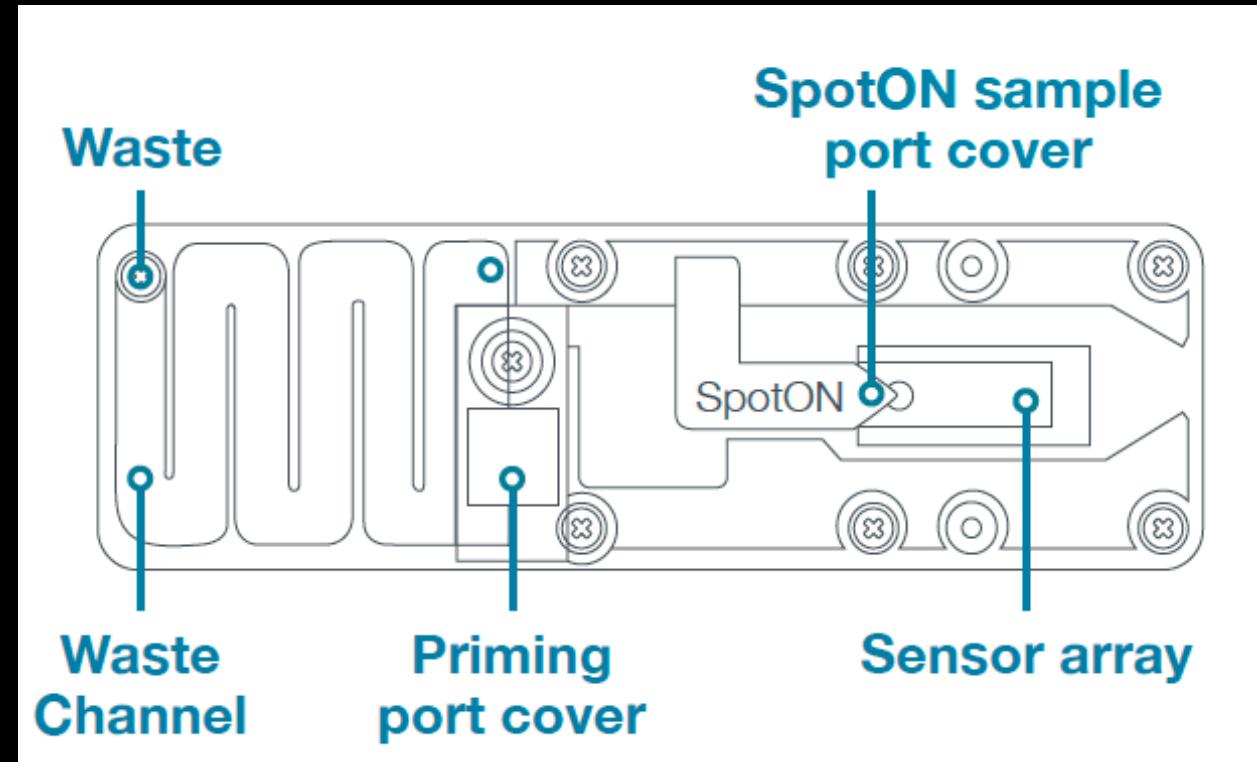
Benjamin Schwessinger
in collaboration with

Miriam Schalamun, Ramawatar Nagar, Robert Lanfear, John Rathjen, et al.,
DECRA Fellow, Australian National University



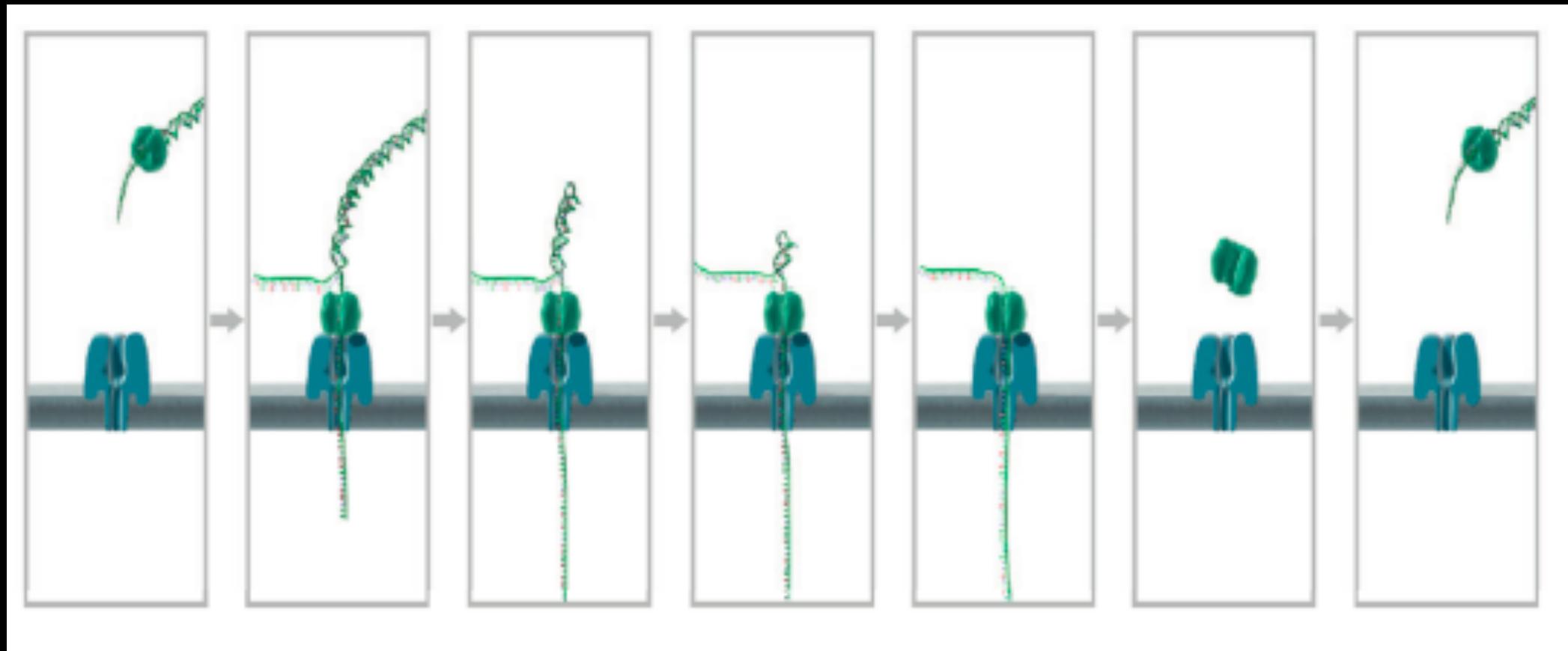
19-20/03/2018
UTS, Sydney

Intro into MinION sequencing



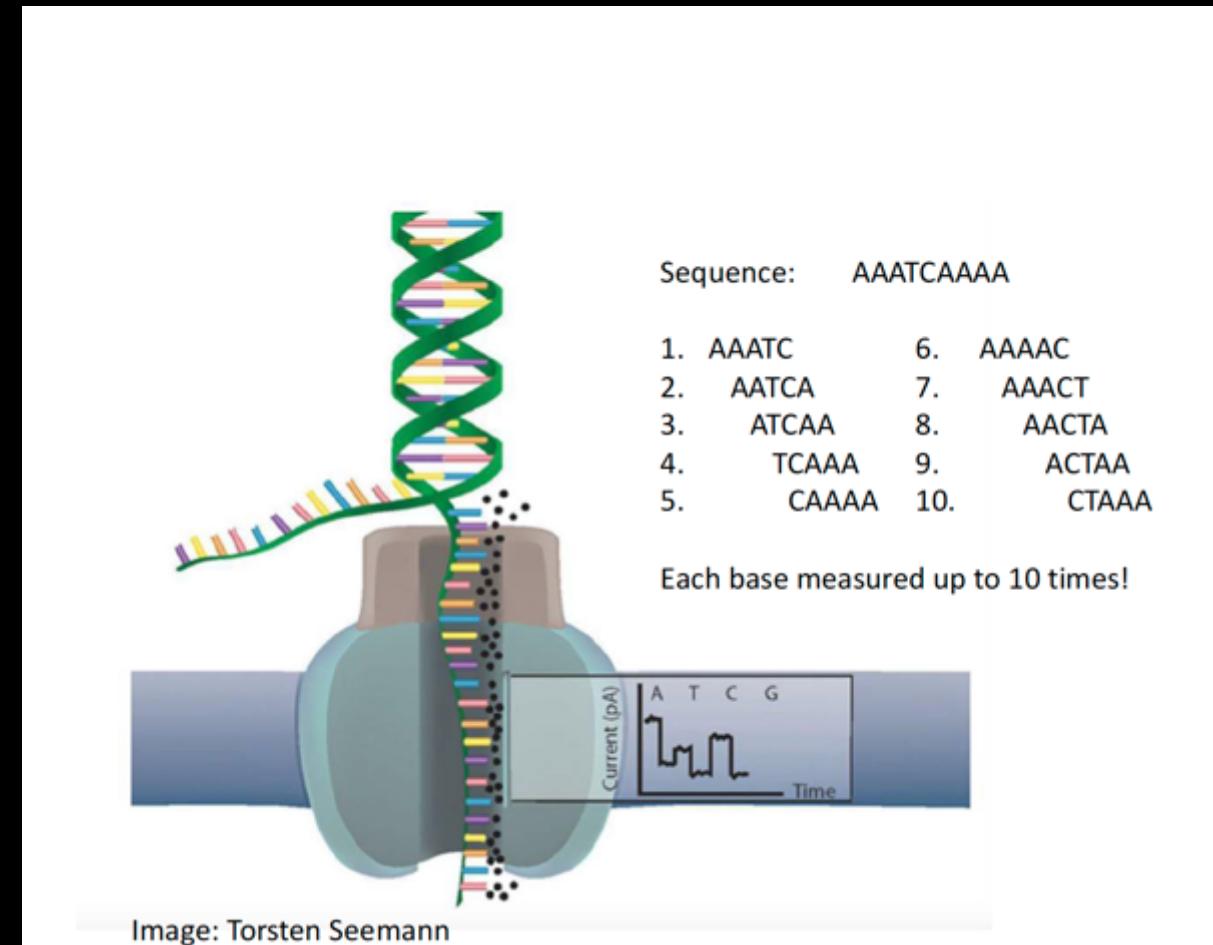
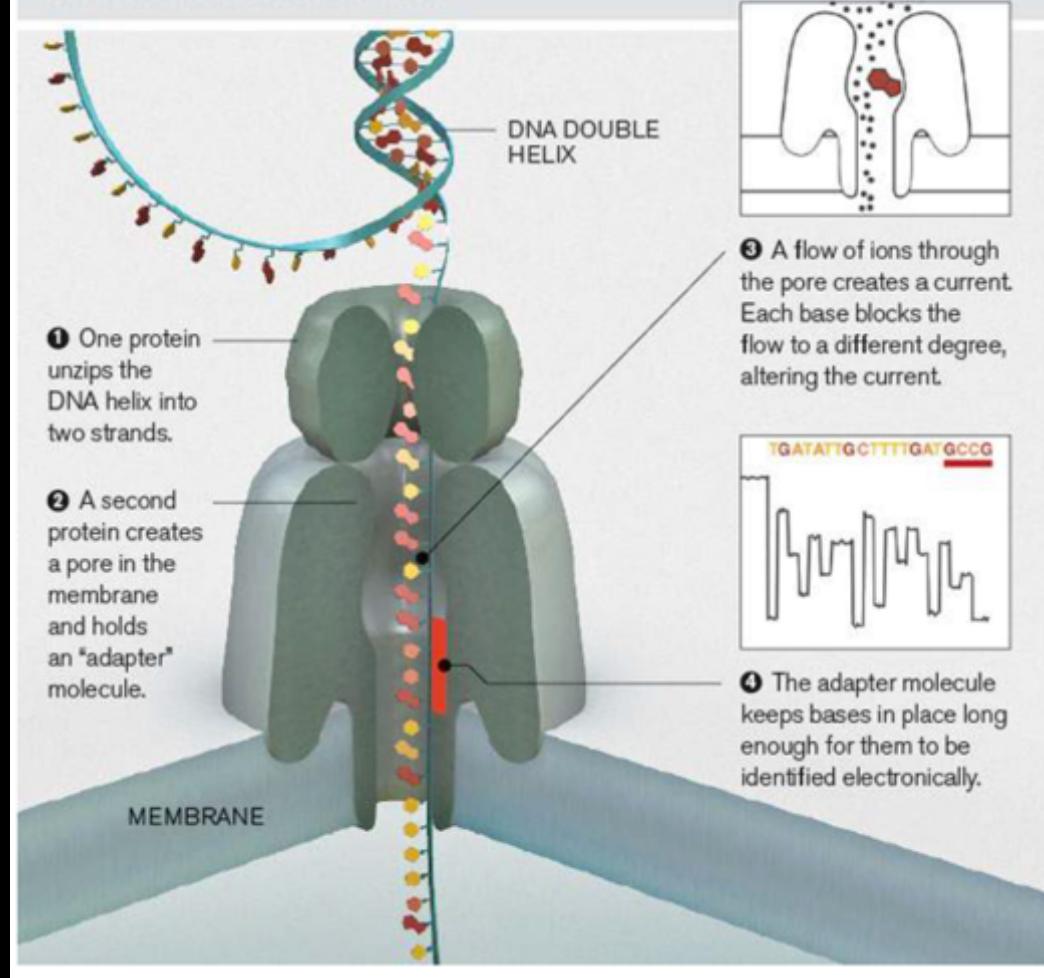
<https://community.nanoporetech.com/posts/getting-started-knowledge>

Intro into MinION sequencing

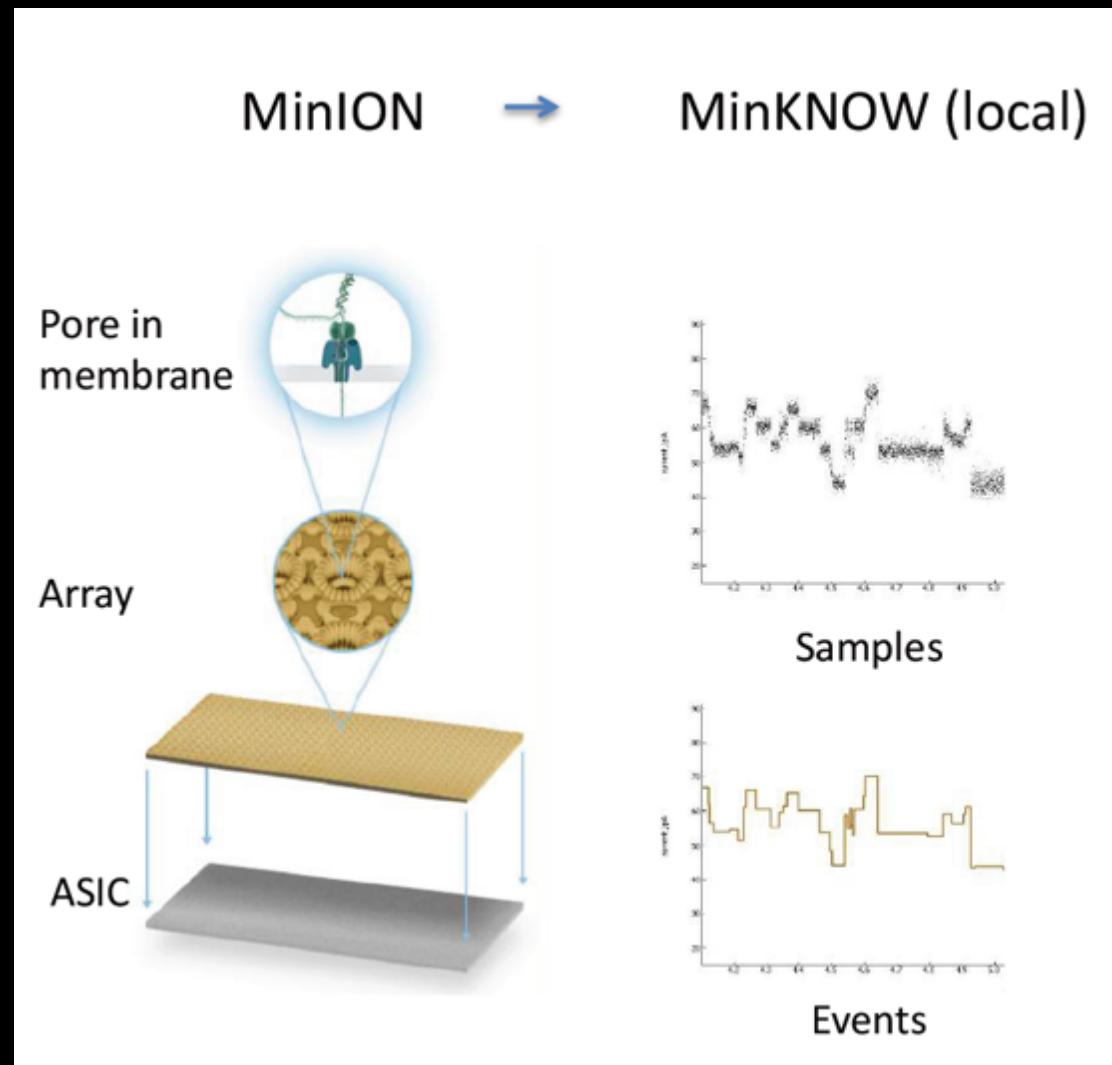


Intro into MinION sequencing

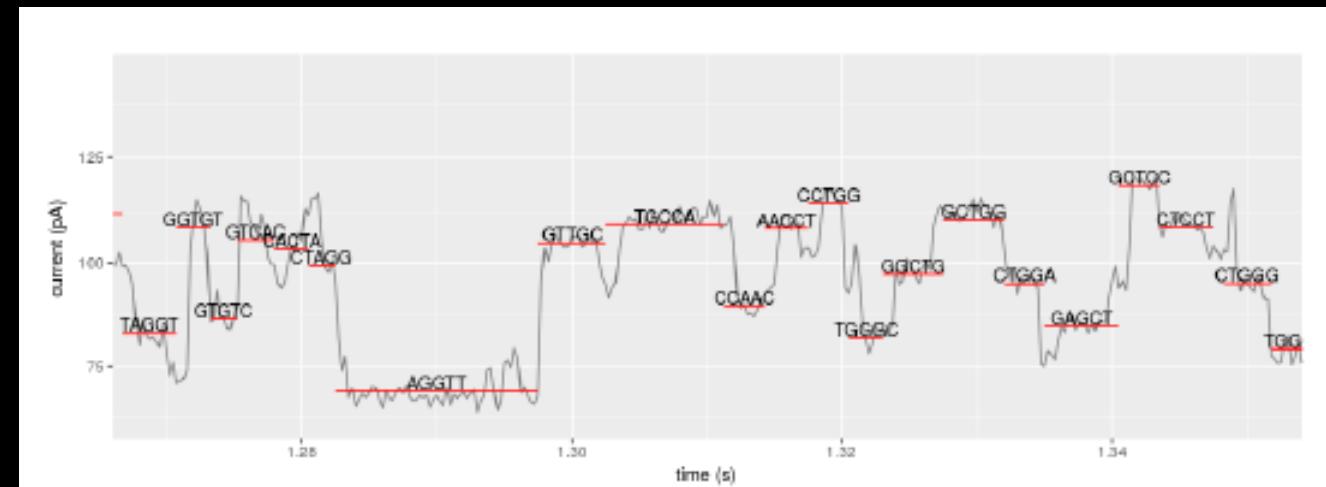
DNA can be sequenced by threading it through a microscopic pore in a membrane. Bases are identified by the way they affect ions flowing through the pore from one side of the membrane to the other.



Intro into MinION sequencing



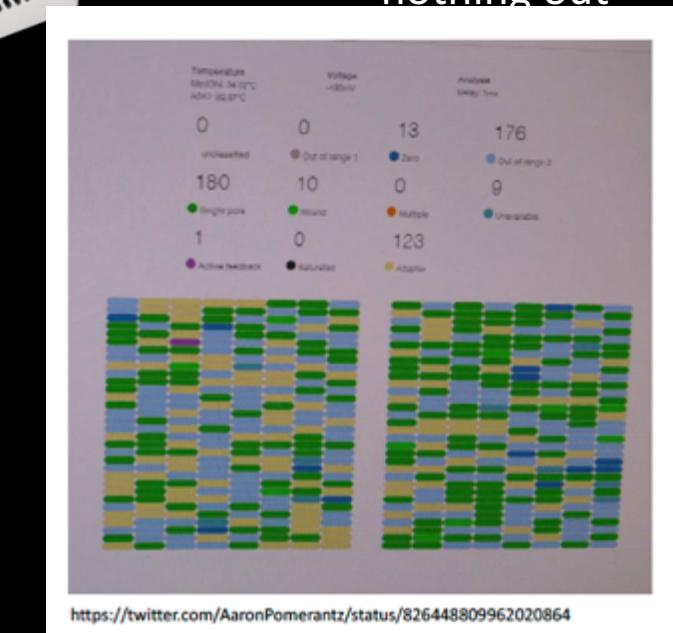
Basecalling within MinNOW, or on server e.g. albacore (nanonet, scrappie, guppy)



fast5 w/ basecalls and/or fastq

<https://github.com/rrwick/Basecalling-comparison>

Intro library prep and DNA quality!!!



Intro library prep and DNA quality!!!

DNA quality!!! is king

Recommended input DNA quality:

- OD 260:280 >1.8 (value of 2.0 suggests presence of RNA; consider another RNase step)
- OD 260:230 between 2.0-2.2 (lower value suggests contamination with carbohydrates/phenol/guanidine/glycogen; consider re-purifying your sample)
- The input DNA should be used based on a Qubit concentration measurement
- Genomic DNA > 30 kb as assessed by gel analysis with size markers or Bioanalyser trace shows intact DNA
- If the DNA could be nicked a DNA repair step is recommended (it is an optional step in the genomic DNA protocols)
- If there is any concerned about the quality of the library please consider following the Damaged Library Improvement protocol

Your concentration measured by nanodrop should be similar to Qubit max 3:1.

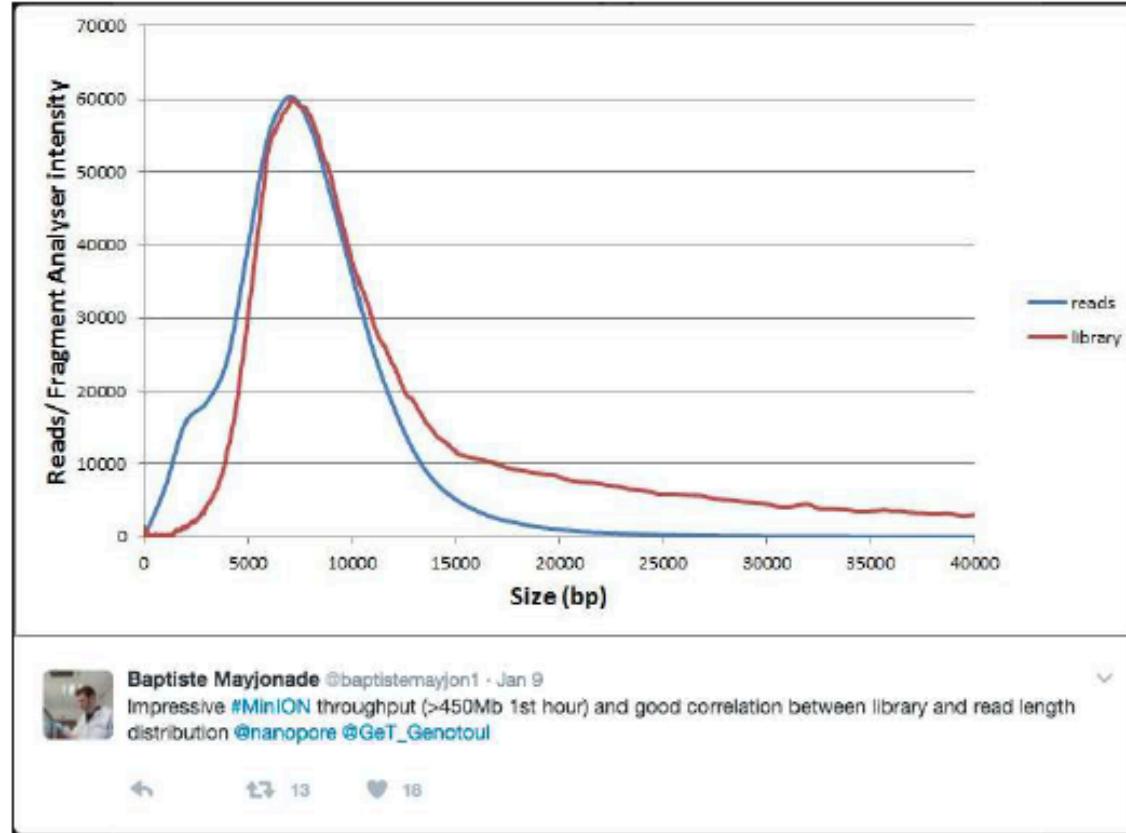
Can be EXTREMELY DIFFICULT AND THE MOST TIME CONSUMING STEP

Please share your protocols openly like Josh Quick, us, and others on



Intro library prep and DNA quality!!!

- Read length = fragment size
- All other technologies limited by chemistry in some way



<https://twitter.com/baptistemayon1/status/818484473780203520>

Things to consider when extracting DNA

- Be gently
- Don't vortex
- Use wide pore tips (or cut them)
- Avoid or get ride of small fragments
- think molarity

Factor influencing your DNA length:

- lysis method of tissue and cells
- purification method
- precipitation method (e.g. ETOH vs. CTAB)
- clean up (SPRI vs. propanol)

Typical size fragmentation:

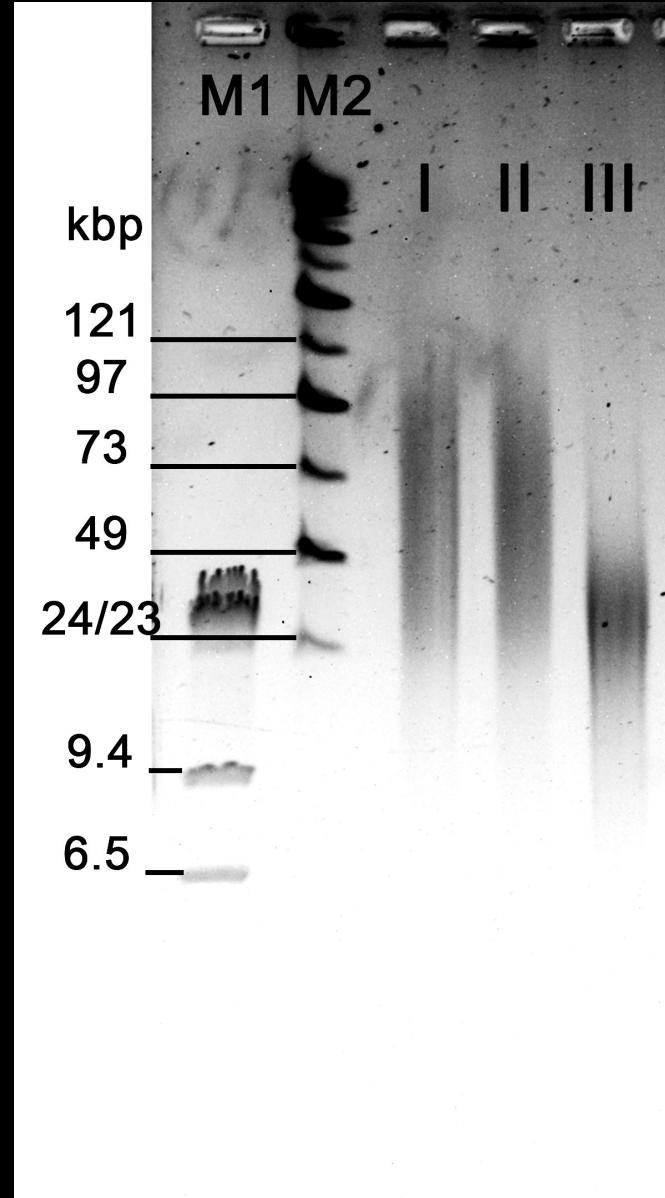
- Spin column 20-100kb depending on brand
- Precipitation 20-300kb
- Dialysis >200kb
- Agarose plug digestion

Things to consider when extracting DNA

- Be gentle
- Don't vortex
- Use wide pore tips (or cut them)
- Avoid or get rid of small fragments
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Factor influencing your DNA length:

- lysis method of tissue and cells
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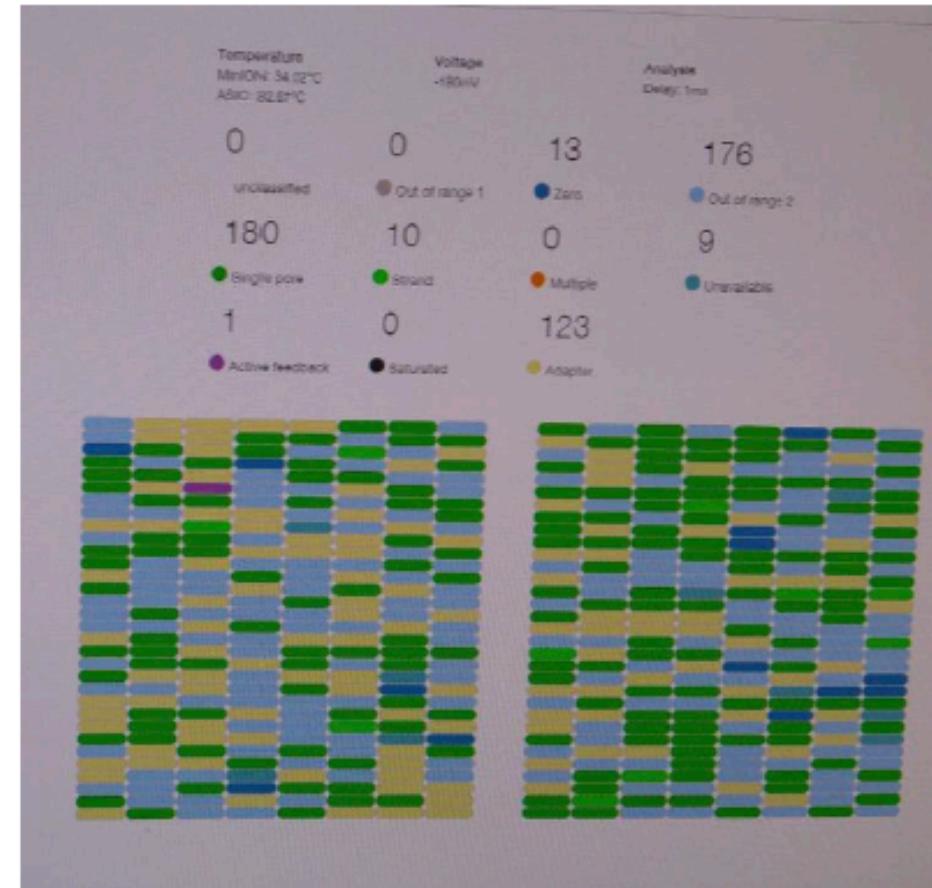


inspired by Josh Quick, F1000, 2017
Schwessinger and Rathjen, 2017

DNA quality influences sequencing output



<https://twitter.com/DrT1973/status/777854022074060801>



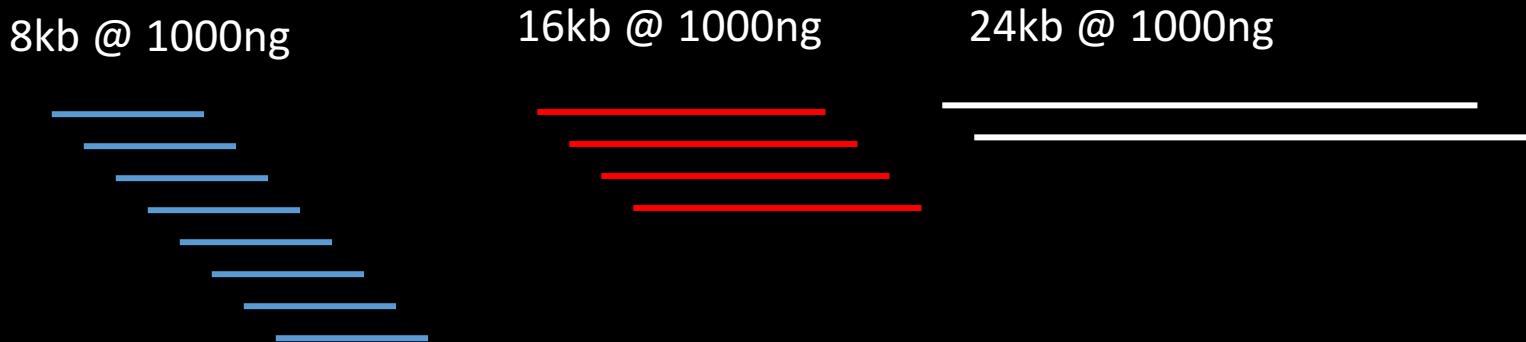
<https://twitter.com/AaronPomerantz/status/826448809962020864>

Library preps are about molarity not mass

Input requirement of 1000 ng is an oversimplification and advises at a suboptimal step in the protocol

What actually matters is the amount of free DNA ends (mols) into the adapter ligations

For 8kb 1000 ng ~ 0.2pmol

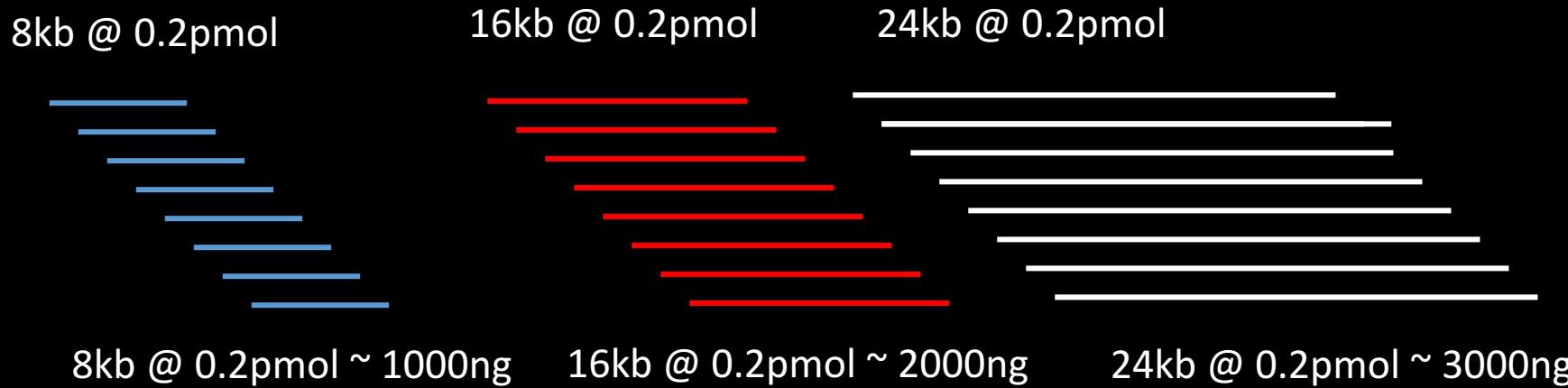


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For 8kb 1000 ng ~ 0.2pmol



DNA quality measurements

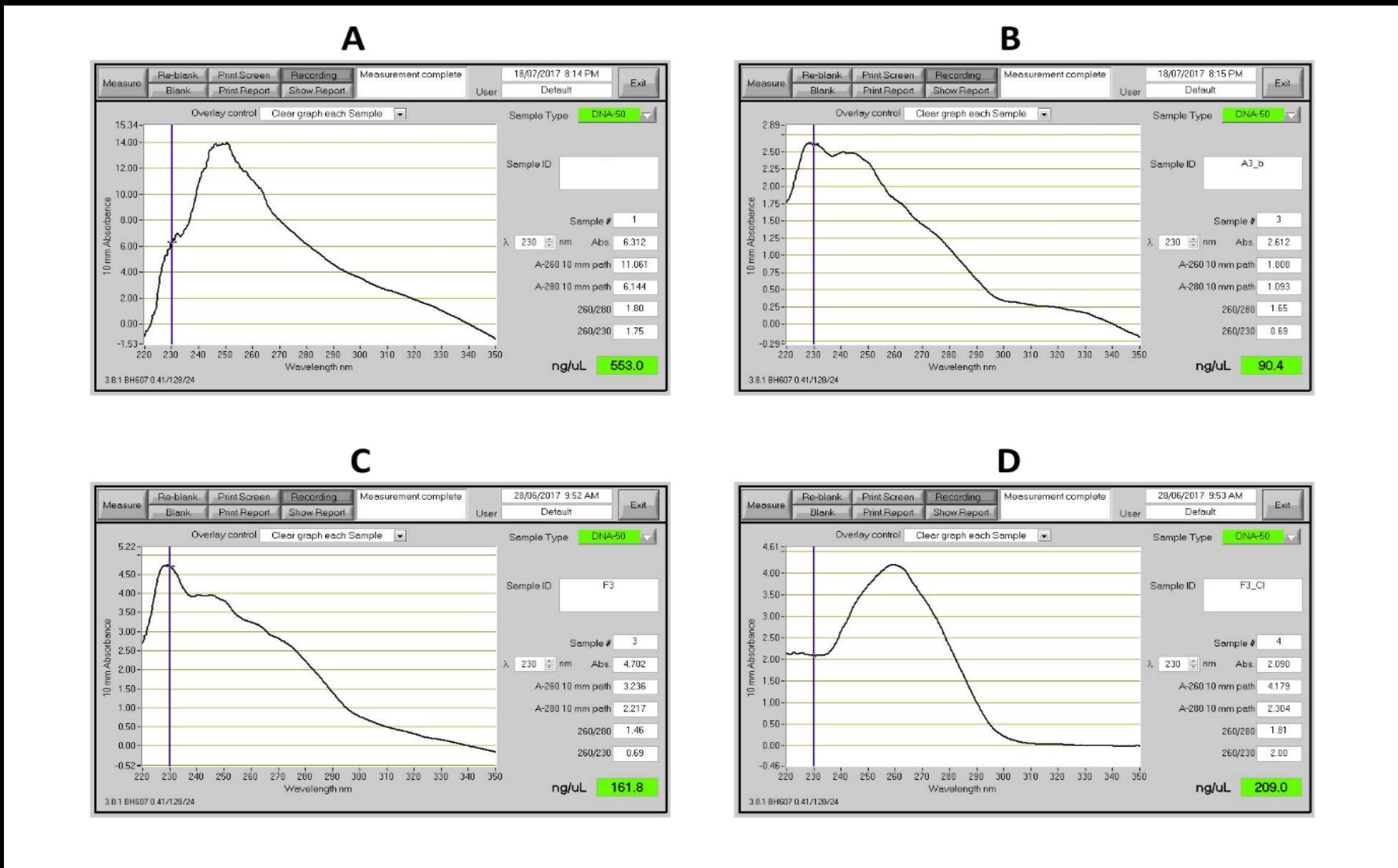
QC methods

- your eyes
- Qubit
- Nanodrop
- 0.8% agarose (up to ~25kb)
- PFGE (up to Mb)
- TapeStation

What to look for

- No color
- $c[\text{Qubit}] \sim c[\text{nanodrop}]$
- 260:230 2.0-2.2
- 260:280 > 1.80
- no smear downward especially < 2kb
- most DNA above 25kb

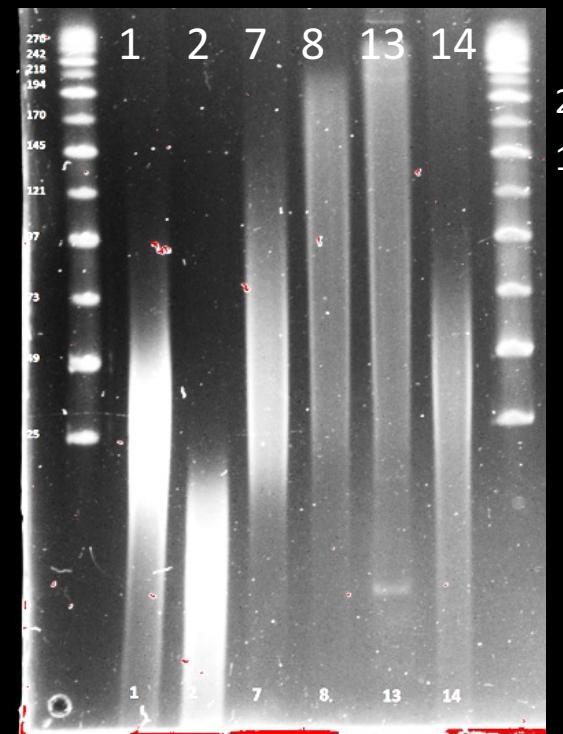
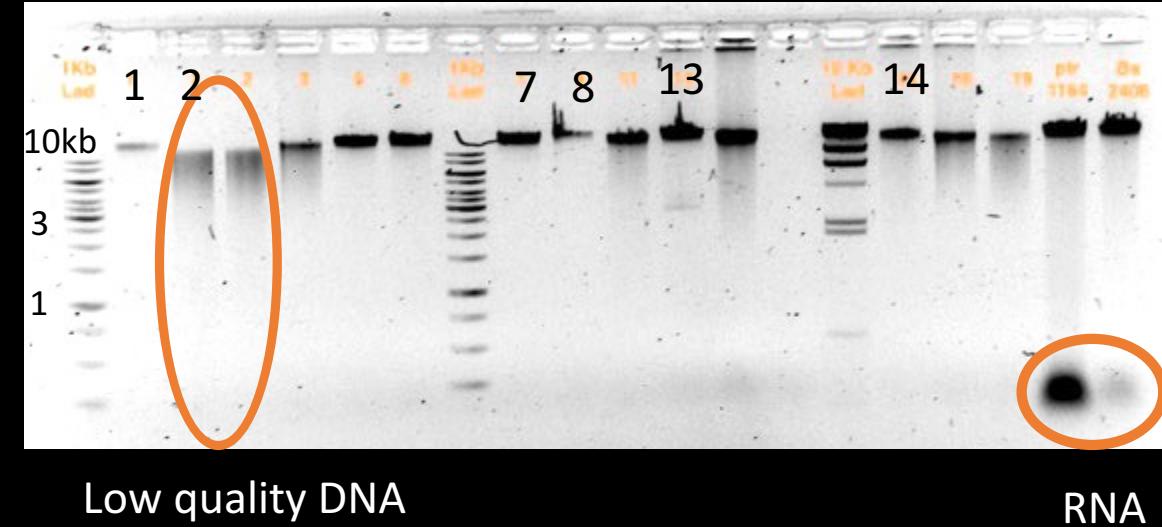
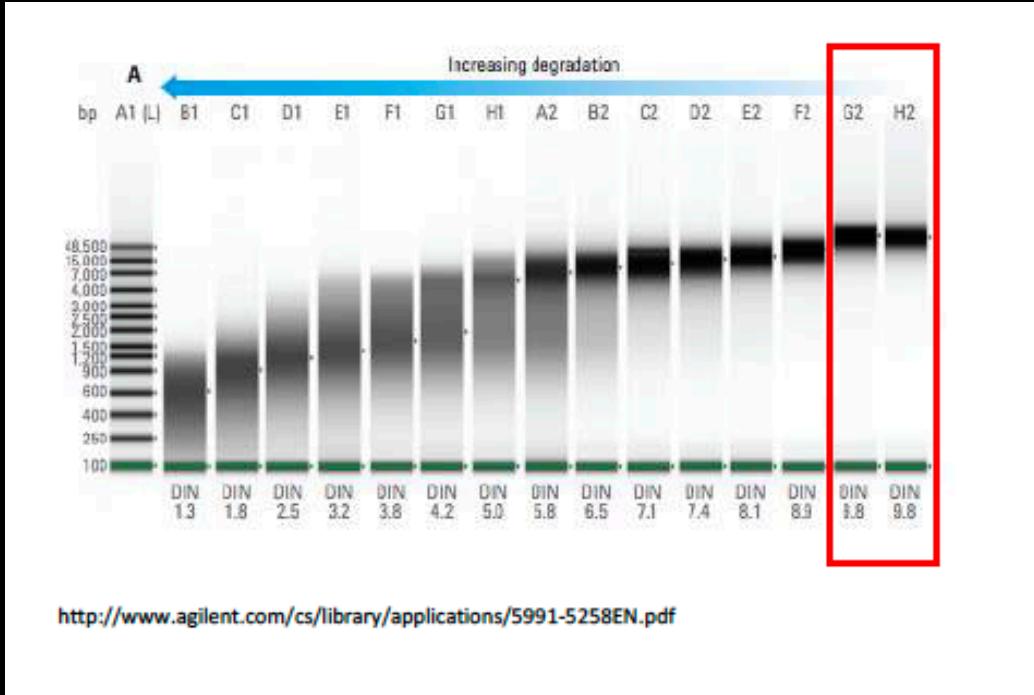
DNA quality measured by Nanodrop



D is how it suppose to look like when $c[\text{nanodrop}] \sim c[\text{qubit}]$

Schalamun et al., biorxiv soon

DNA integrity measured by agarose gels or TapeStations

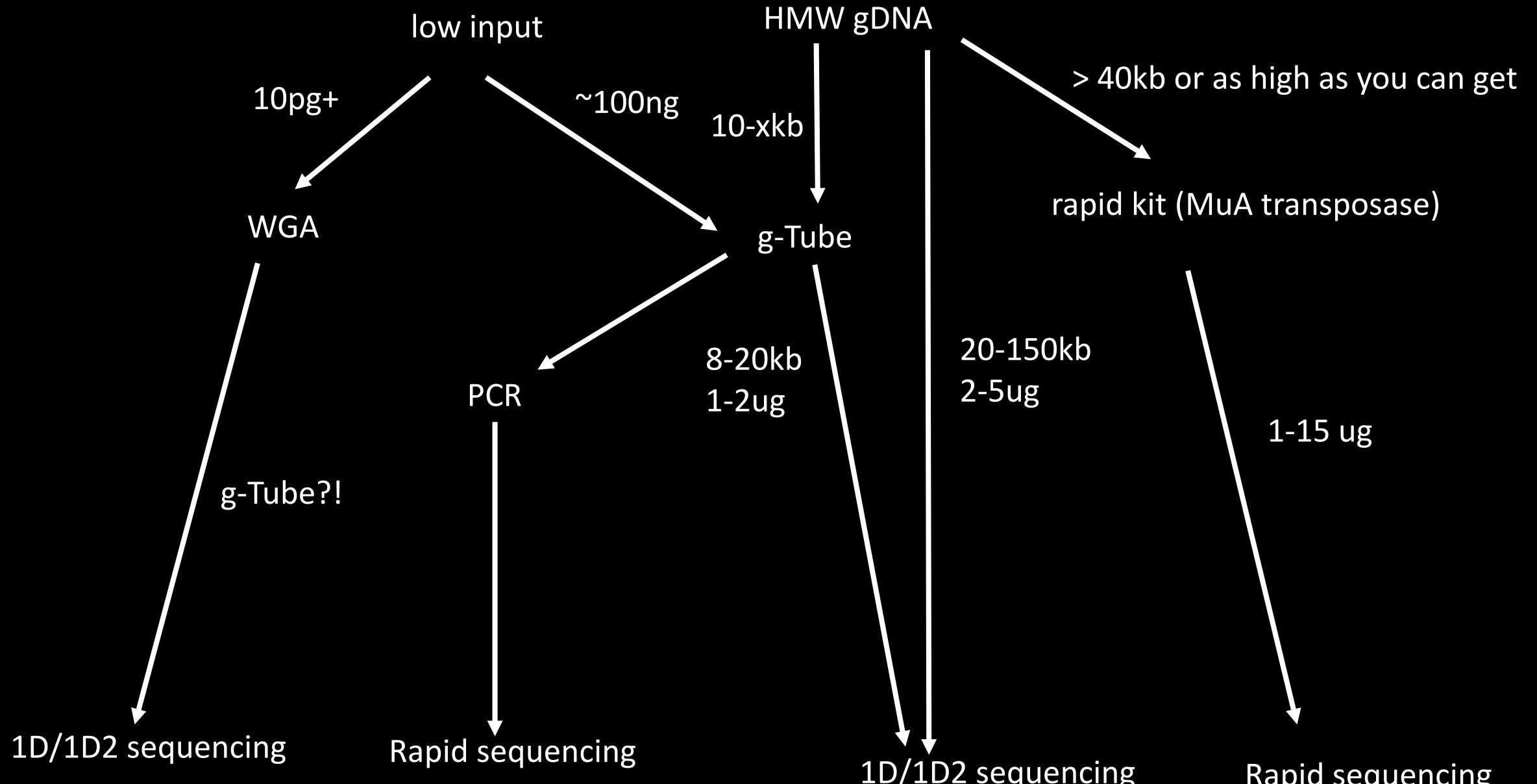


200kb
125
95
73
49
24

Only a PFGE will show you
the real size distribution of
your DNA

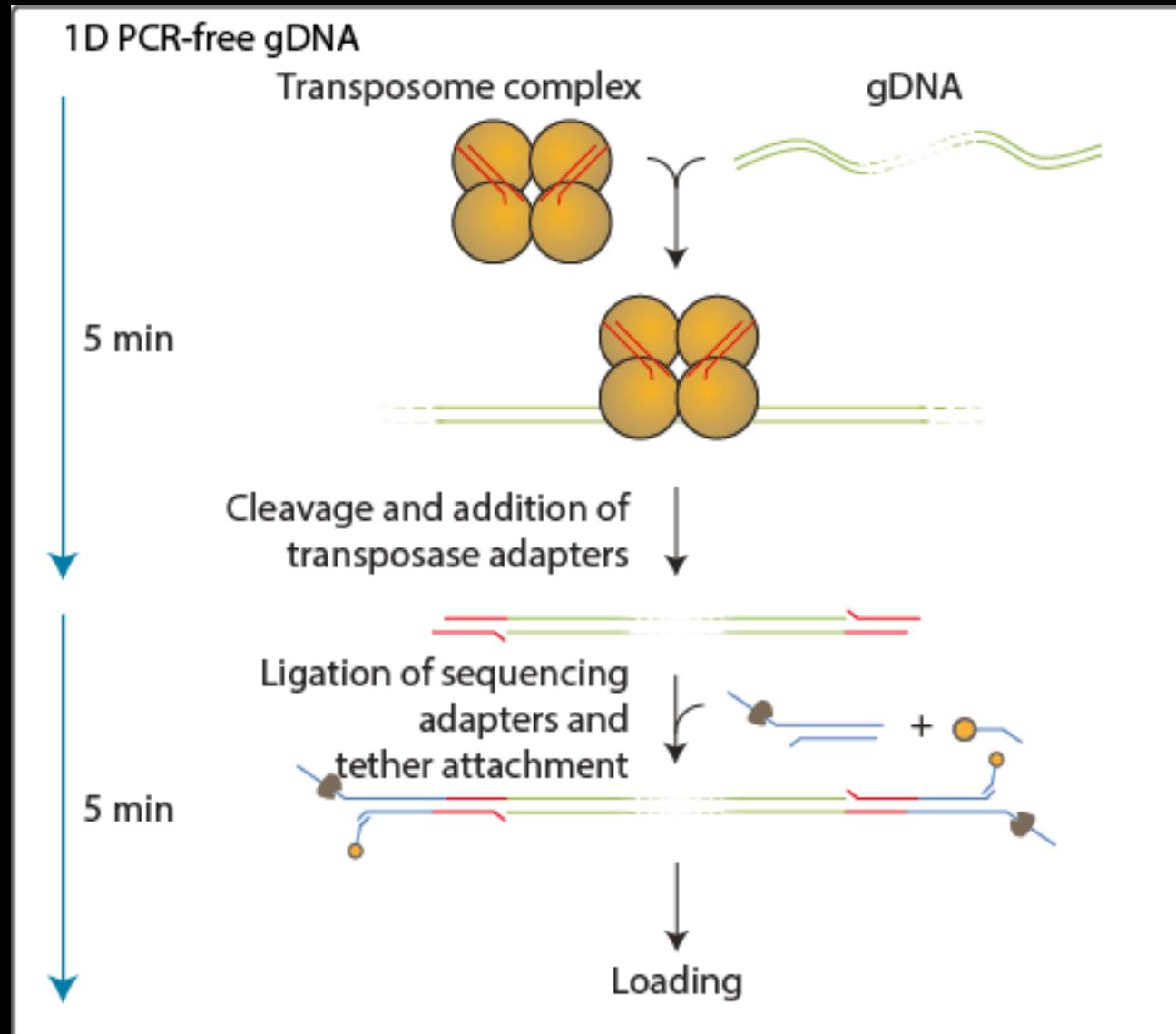
see also Josh Quick, F1000, 2017
ANU Nanopore workshop 2017

Nanopore DNA sequencing decision tree for beginners



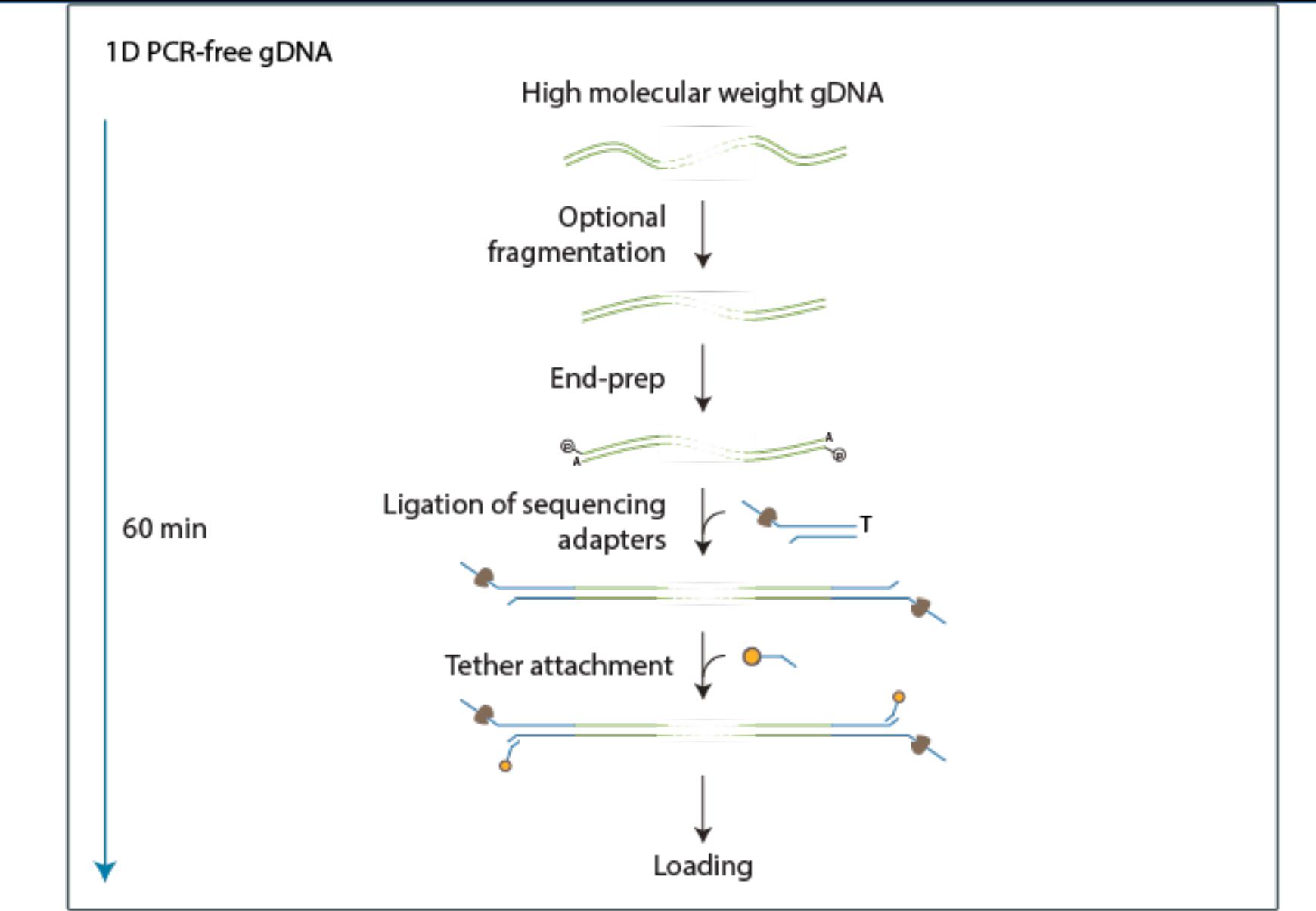
inspired by Josh Quick, F1000, 2017

Intro Rapid library prep!!!

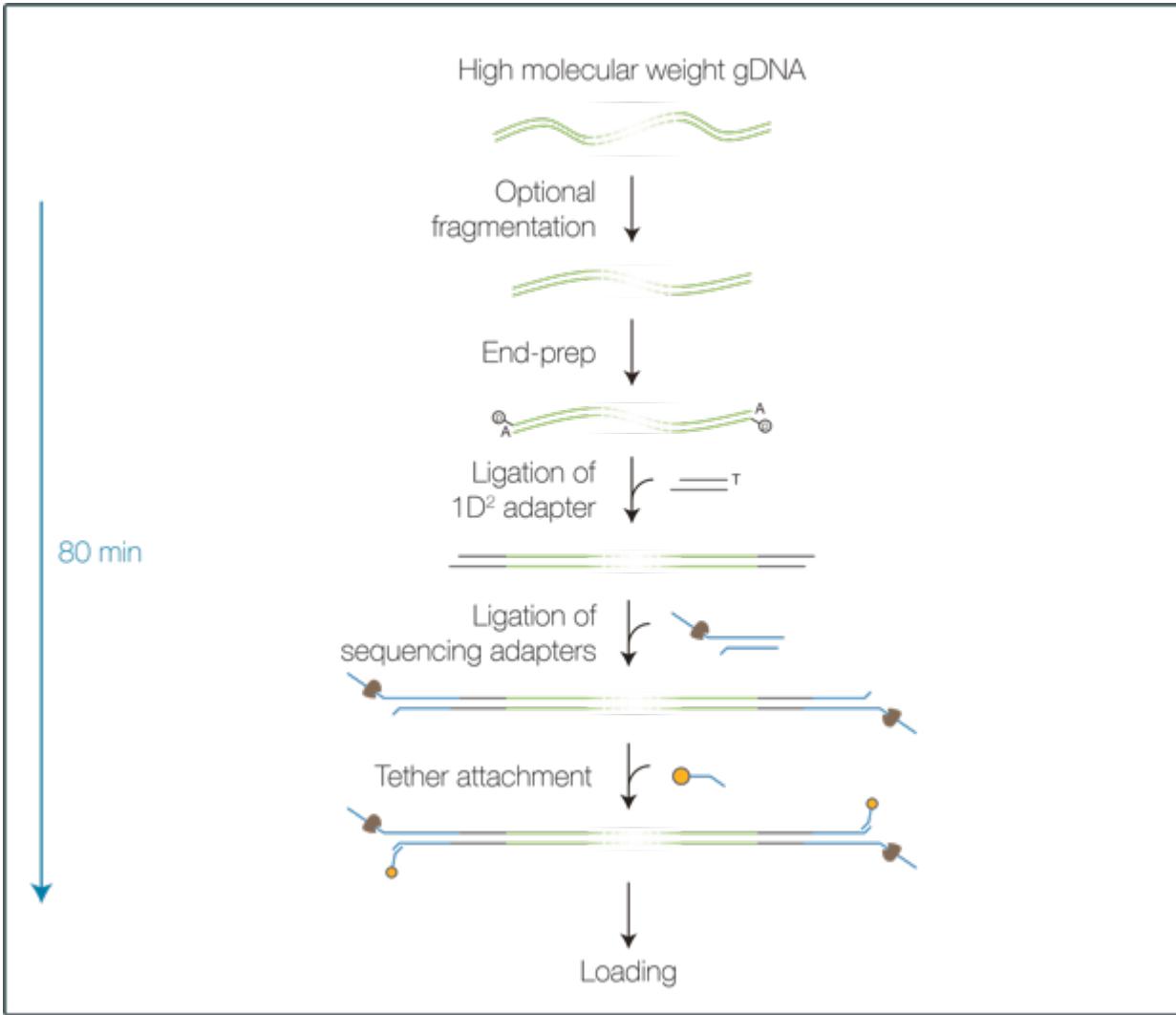


https://community.nanoporetech.com/protocols/rapid-lambda-control-sqk-rad002/v/rse_9024_v2_revo_21nov2016/overview-of-the-rapid-sequ

Intro 1D library prep!!!



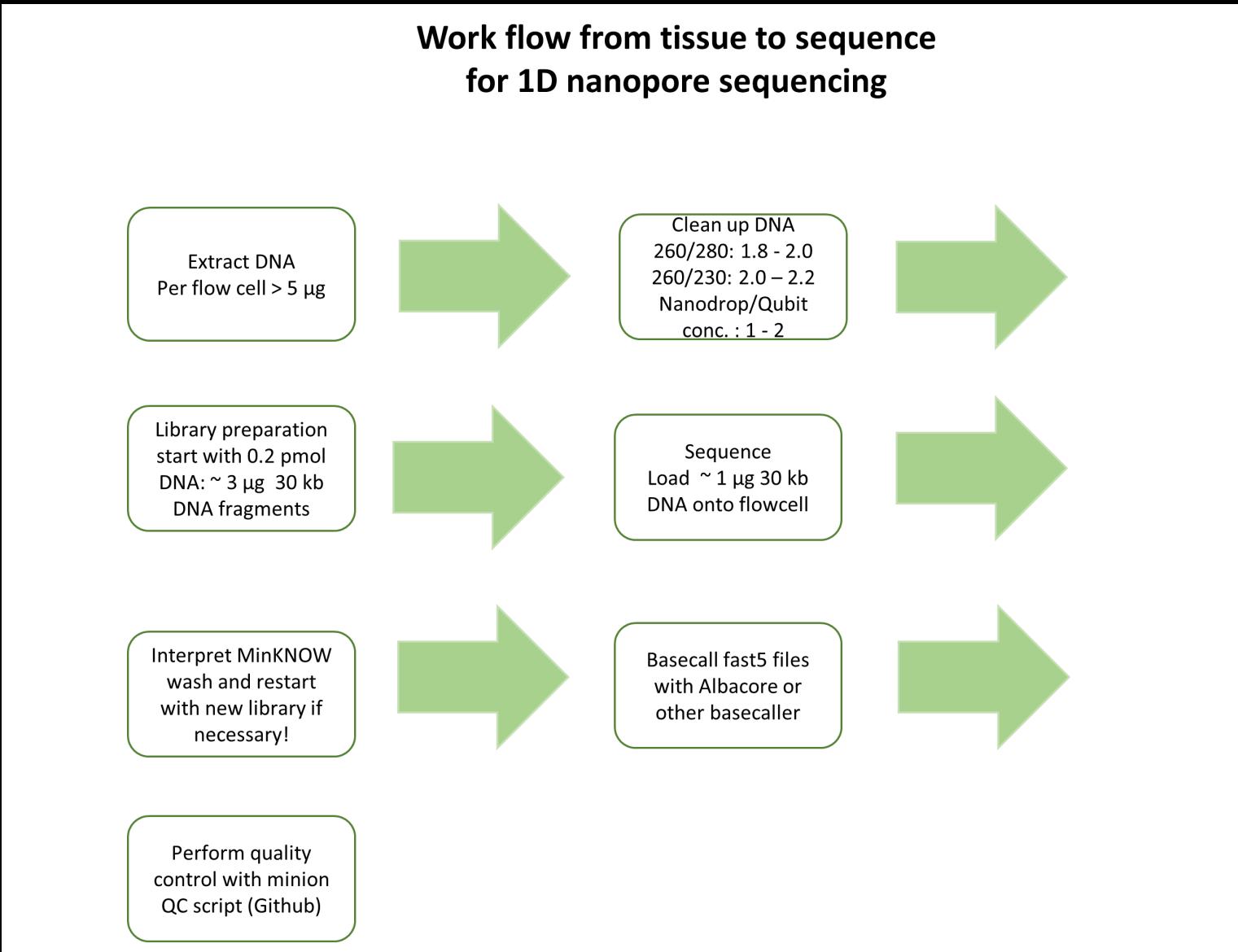
Intro 1D² library prep!!!



https://community.nanoporetech.com/protocols/1d%5E2-genomic-sequencing/v/lsd_9032_v11_revi_23mar2017/introduction-to-the-1d-2-s

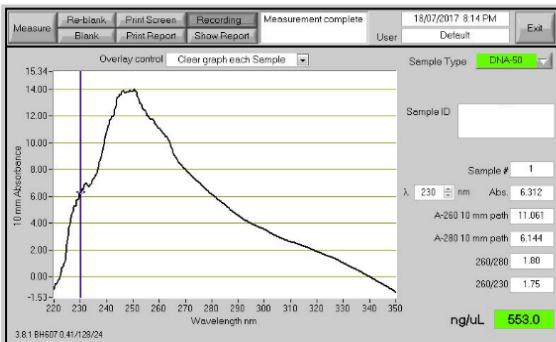
A case study of setting up MinION sequencing for a difficult species

Eucalyptus

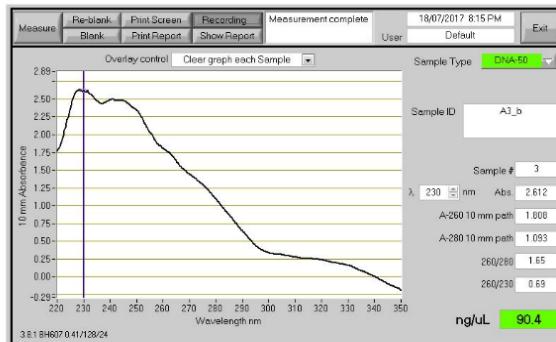


Getting clean and long DNA

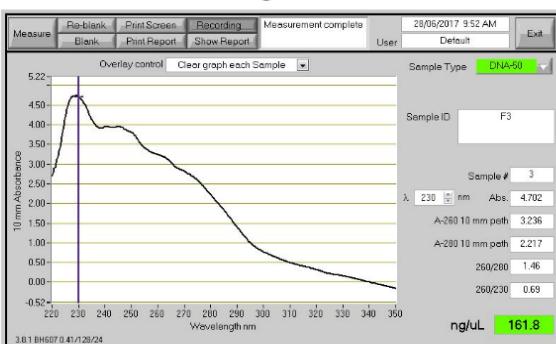
A



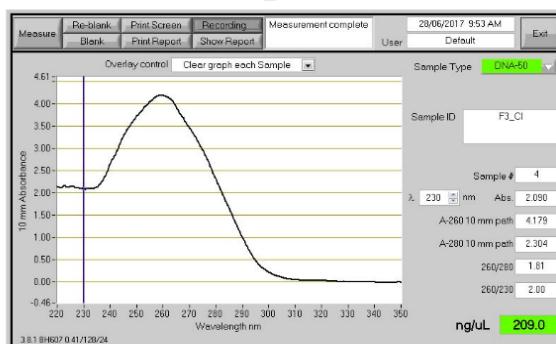
B



C

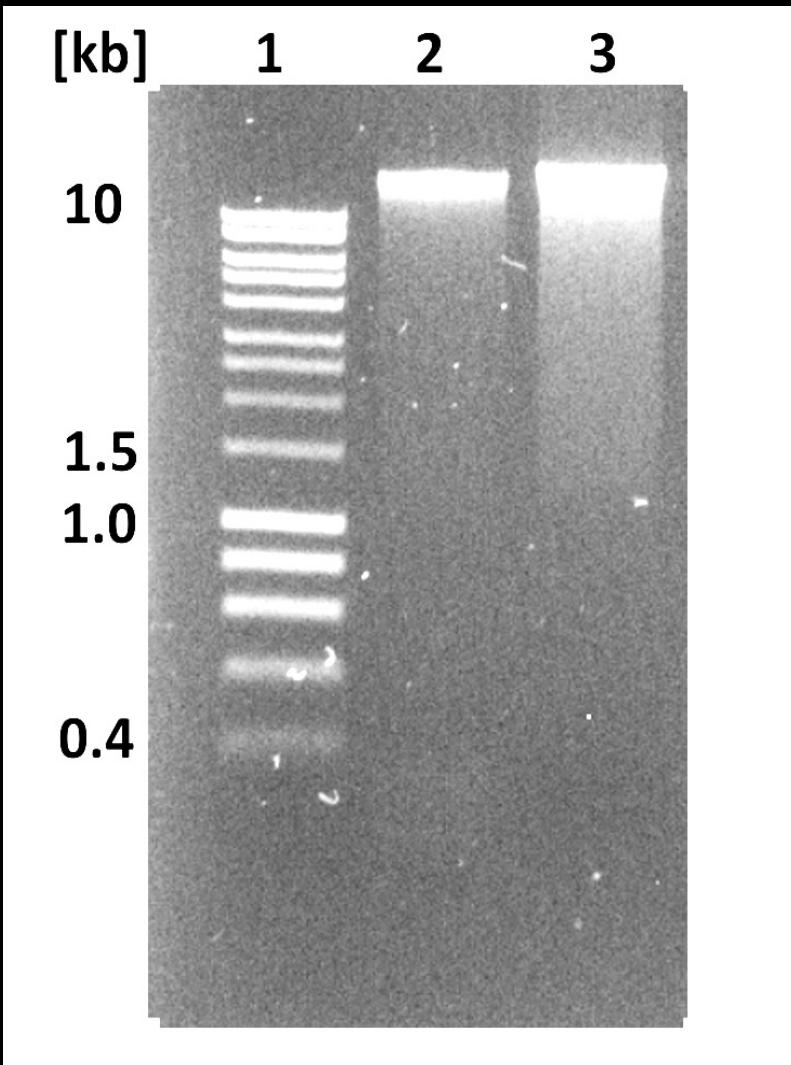


D



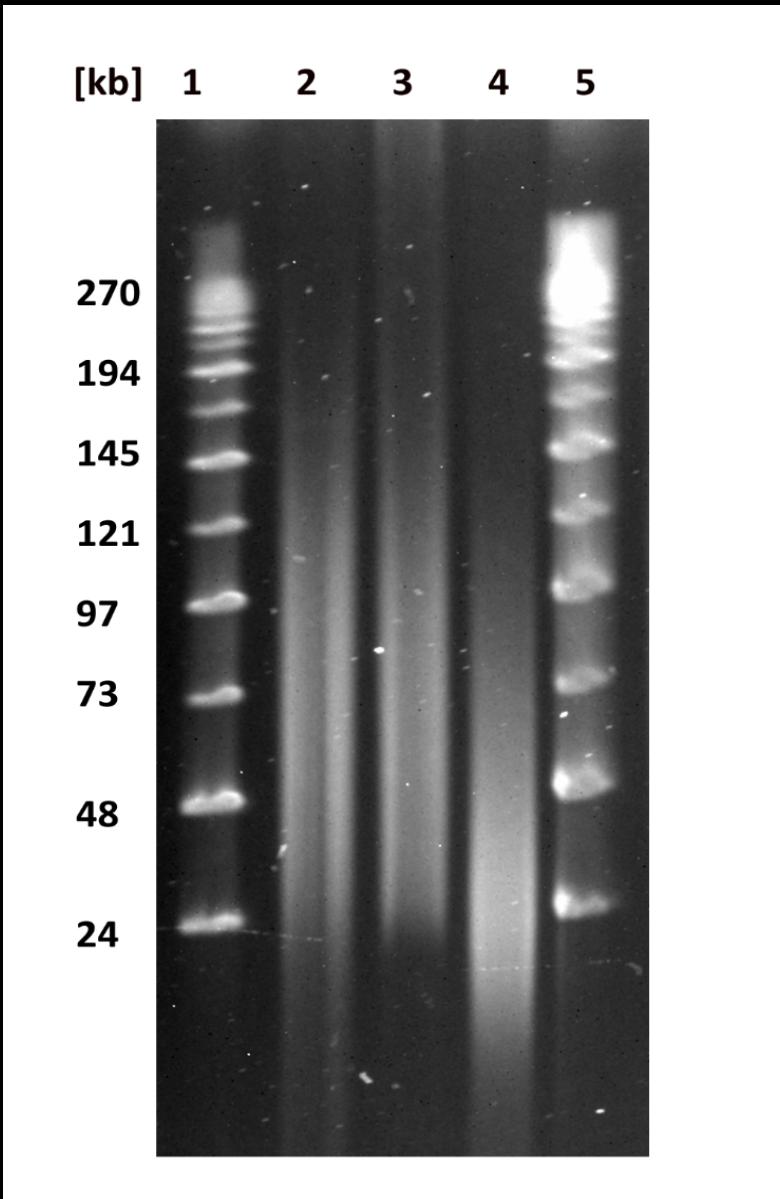
Sample	Qubit [ng/ μ L]	Nanodrop [ng/ μ L]	260/280	260/230	Yield [Gb]	Yield _{Q7} [Gb]
10	178	203	1.8	2.1	6.0	5.9
27	142	188	1.8	2.3	7.8	7.4
5	57	80	1.7	1.0	0.7	0.7

Shearing leads to short reads



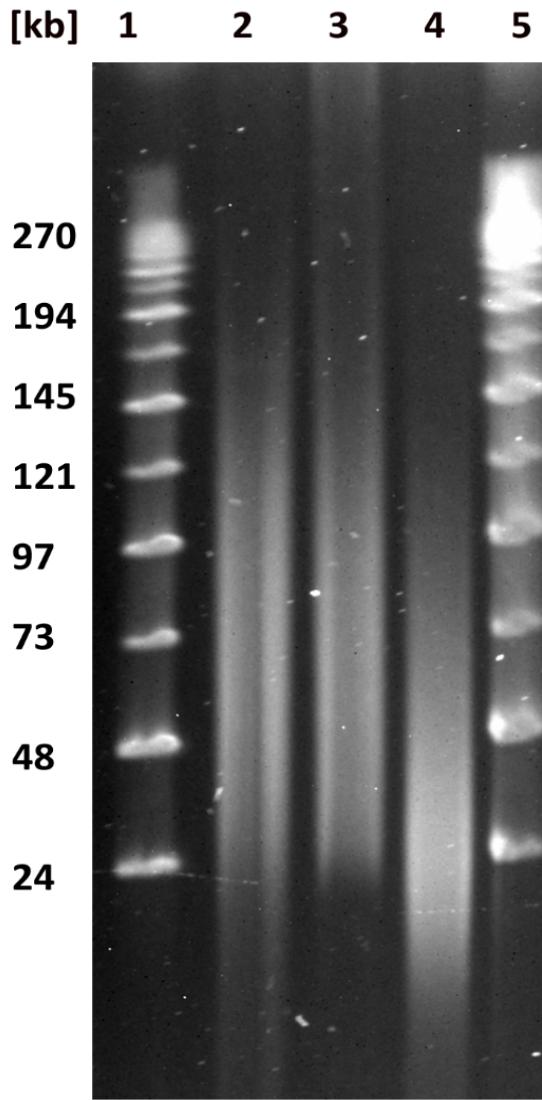
Sample	Size selection	$N50_{Q7}$ [kb]	$Mean_{Q7}$ [kb]	$Median_{Q7}$ [kb]	Yield [Gb]	$Yield_{Q7}$ [Gb]
10	NO	25.8	12.4	6.2	6.0	5.9
27	NO	26	13.2	7.5	7.8	7.4
9	sheared during extraction	9.2	4.9	2.5	3.5	3.5

Why would you use a g-Tube shearing? Not sure I would if I am after long reads.



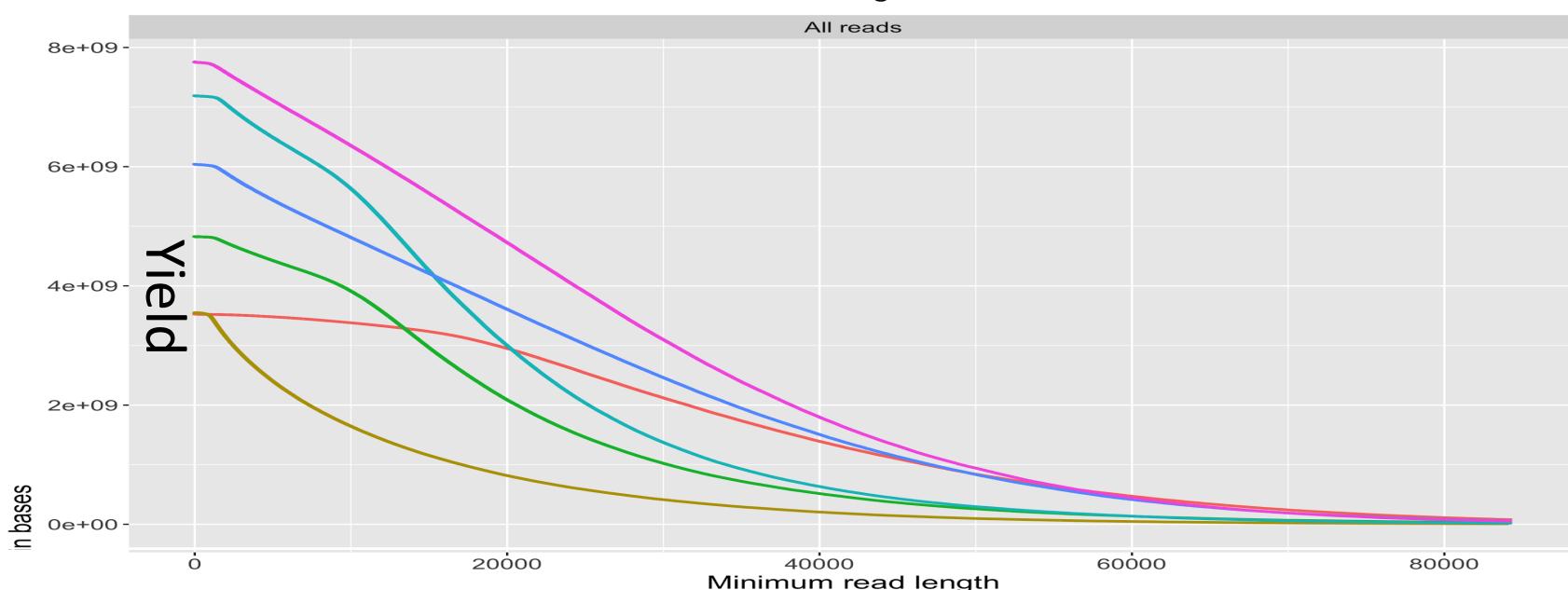
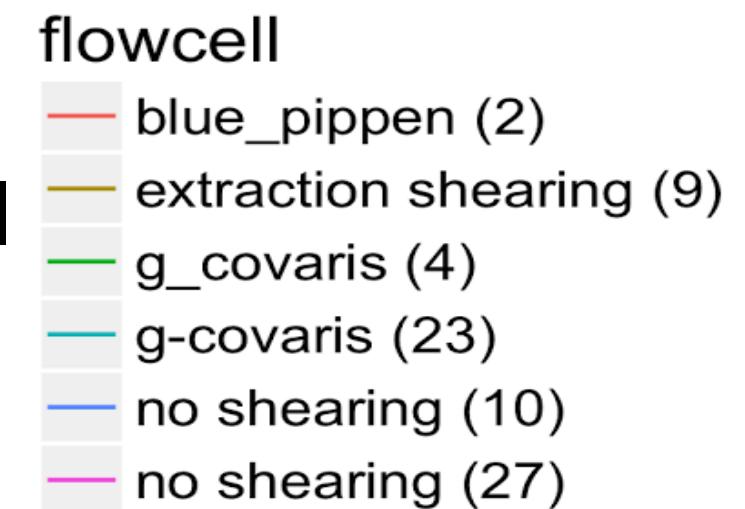
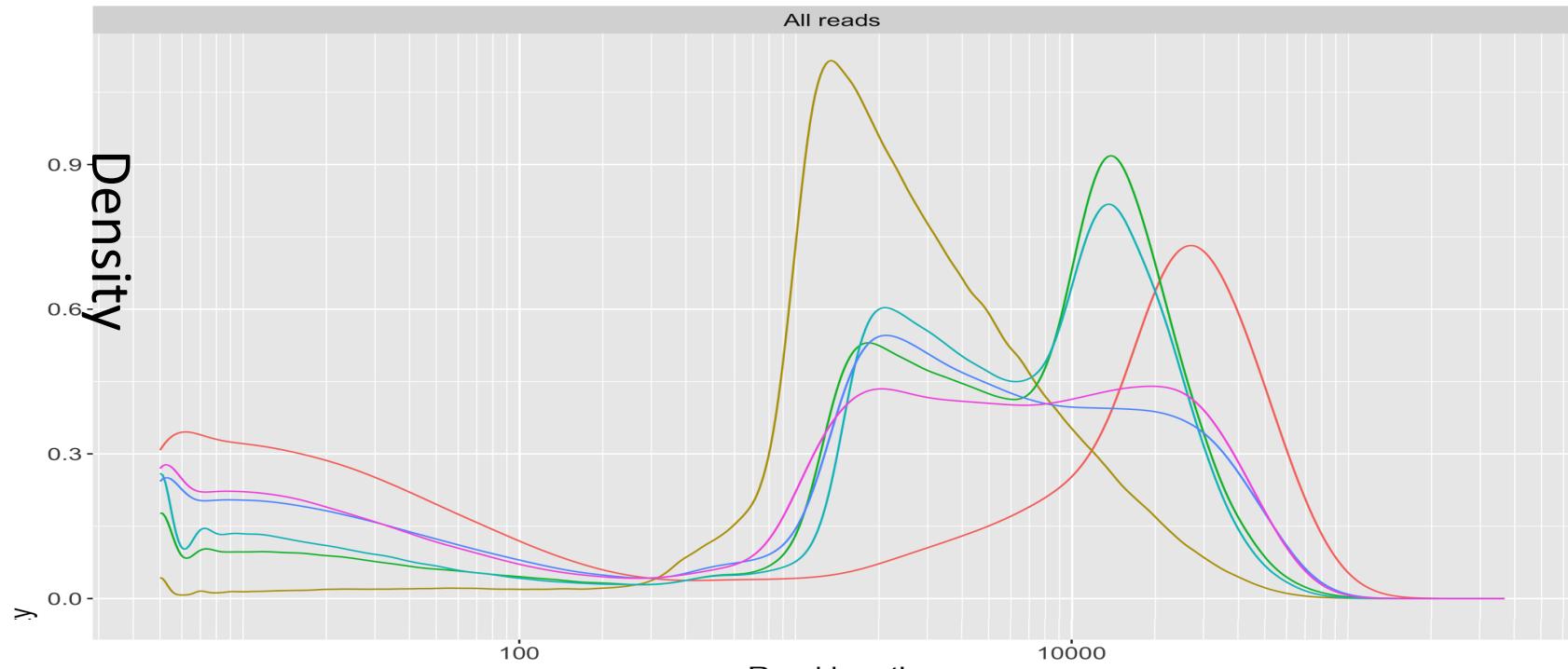
Sample	Size selection	$N50_{Q7}$ [kb]	Mean $_{Q7}$ [kb]	Median $_{Q7}$ [kb]	Yield [Gb]	Yield $_{Q7}$ [Gb]
10	NO	25.8	12.4	6.2	6.0	5.9
27	NO	26	13.2	7.5	7.8	7.4
4	g-covaris	18.4	11.8	9.5	4.8	4.7
23	g-covaris	17.9	11.2	8.5	7.2	7.0

Blue Pippen is great for long reads! But....

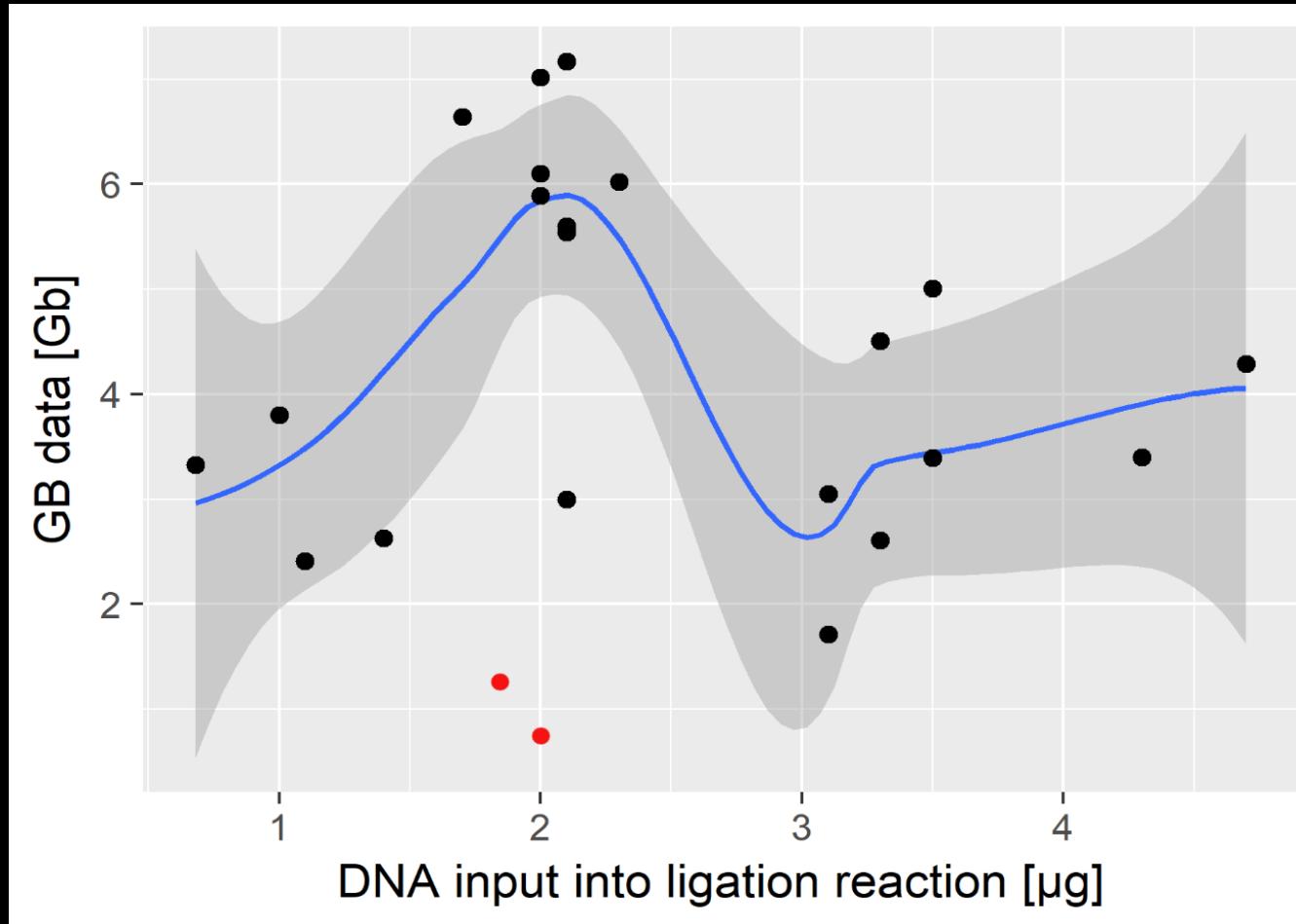


Sample	Size selection	$N50_{Q7}$ [kb]	$Mean_{Q7}$ [kb]	$Median_{Q7}$ [kb]	Yield [Gb]	$Yield_{Q7}$ [Gb]
10	NO	25.8	12.4	6.2	6.0	5.9
27	NO	26	13.2	7.5	7.8	7.4
2	Blue Pippin	35.1	26.5	23.9	3.5	3.5

Add another step to the procedure
Not always reliable
Much DNA required

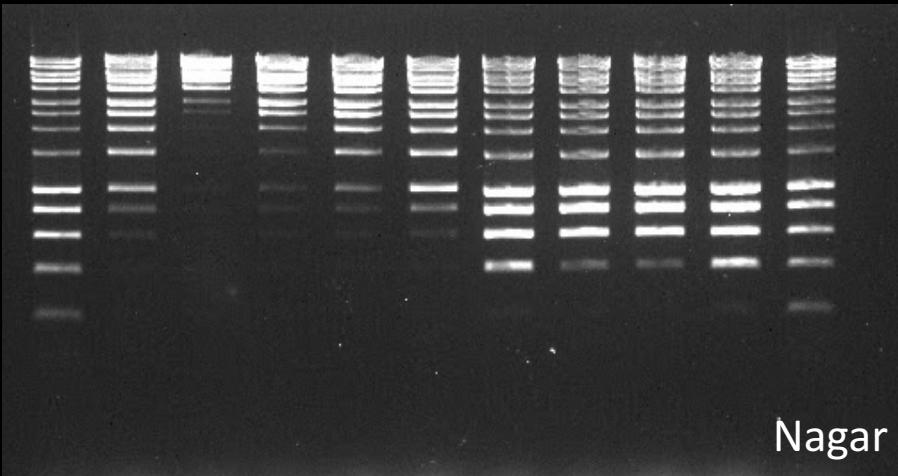


DNA input into the adapter ligation step is must crucial for output!
at least it was for us.



Other tricks

Use home made SPRI beads for size selection



Heat library @ ~37oC for 10 mins at the final elution step

Experience with careful run to run evaluation is most important

Check out protocols.io @

<https://www.protocols.io/groups/minion-user-group-with-fungi-and-plants-on-their-mind>

135 Members, 10k+ views, active discussions of protocols.

Please contribute!!!!

<https://tinyurl.com/nanopore-tools>

for a every other months updated list of nanopore tools

Schalamun et al., biorxiv soon
Nagar et al. biorxiv sometime
+ others

IN's and OUT's of DNA extraction

IN's	OUT's
Use double strand specific DNA dyes to measure concentration e.g. Qubit	Ban the vortexer!
If $c[\text{qubit}] \sim c[\text{nanodrop}]$ the absorbance curve and the absorbance ratios at 260/280 (~1.8) and 260/230 (2.0-2.2) are good indications of DNA purity	Avoid excessive pipetting or use wide mouth pipettes
Use SPRI, e.g. AMPure, at 0.45vol for removal of smaller DNA fragments (< 1kb)	If the goal is 'whale length' reads avoid column purifications
Use 'old skool' phenol cholorform based extraction methods for high molecular weighth DNA purification a.k.a. Sambrook and Russell are your friends	You gonna hate small DNA fragments (< 1kb). Get ride of them!
Clean DNA is your friend!	Never! Never freeze DNA!
Use nuclease free water for short term storage at 4oC (couple of weeks)	Do not use EDTA in your final resuspension buffer e.g. TE buffer.
Use 10mM Tris pH 8.5 for long term storage at 4oC (up to years) and for re-suspension of high molecular DNA	

IN's and OUT's of library prep

IN's	OUT's
Use SPRI, e.g. AMPure, at 0.45vol for removal of smaller DNA fragments (< 1kb)	Avoid excessive pipetting or use wide pore pipettes.
Use RAPID kit with high molecular weight DNA (>200kb) for 'whale length' sequencing reads.	Ban the vortexer!
Shear high molecular weight DNA into a specific size range for input into the 1D ligation kit. This can be done with covaris g-tubes, silica DNA clean up columns, megasdisrubter, syringe needles.	You gonna hate small DNA fragments (< 1kb). Get ride of them!
Use 1D ligation with sheared DNA (5-50kb) for highest throughput and basecalling quality.	Nanopores hate air and so will you. Avoid introducing them at all cost!
When priming the flow cell open all ports, set your pipette to a small volume when removing air initially, add appropriate priming solution and close all ports once done.	Do not leave ports and SpotON open for prolonged times (>1 mins) to avoid evaporation and introduction of air.
Have your library completely ready when performing the second priming step with the SpotON open. Load your library immediately.	Do not always believe the official protocol. Think! E.g. Reduce the amount of 'mixing by pipetting' during library preparation. Mix by flicking and inversion instead.
Close all ports properly during the sequencing run.	Do not over dry SPRI beads when handling longer DNA fragments (> 10kb). About one minute airdry will be enough once all ethanol has been removed by pipetting.
Stop your sequencing run when less than 30% of your pores are in strand within one hour of starting your run. This will save our flow cell as it indicates a bad library preparation.	
You can heat samples to 40-50°C for 5-10 minutes to aid elution of the SPRI beads.	
One library preparation is designed for 0.2 pmoles of DNA molecules. The amount of input in ug will vary according to average fragment size e.g. 0.2 pmoles for 8kb is about 1ug.	