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Title Inventory search for suitable species Book No. _____

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Need to finalize species selection. Criteria are:

- Octocoral species (better extraction success)
- Specimens available for range of collection dates (≥ 100 yo,
 ~ 50 yo, ≤ 25 yo)
- Well-annotated genome is available for species (or close relative)

Andrea suggested a few with high-quality genomes:

- *Eunicea knighti*
- *Leptogorgia sarmentosa*
- *Gorgonia ventralina*

Searched digital inventory for the above genera, with the stipulation of "dry" preservation and storage.

See digital lab notebook for detailed breakdown of specimen availability across species and collection dates.

Insufficient collections for *Leptogorgia* species

The following species could work: *E. flexuosa*, *E. tourneforti*, *G. ventralina*. Manually searched collections to get a sense of how much material is available for each.

E. flexuosa:

- All dried specimens retained rust brown color (not bleached during preservation)
- Abundant material from 1880s-1910s collections. 15-20 individuals, most quite large (~ 1 ft in diameter)
- Quite a bit of material from mid-1900s (1945-1959), w/ ~ 15 large individuals.
- A *ton* of material from collections between 1960-1972, ~ 30 large specimens, some very large. This could be an ideal "middle" timepoint.
- The only "modern" collections I see are 6 specimens in the last 25 years, and they're quite small (\sim a finger in size).

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E. tourneforti:

- May be better *Eunicea* species to work with, since its more closely related to the species for which we have an available high-quality genome, *E. knighti* (Sarmiento et al. 2025). (see digital notebook for phylogeny)
 - Branches are much thicker than *E. flexuosa*, which could be beneficial for subsampling
 - Several specimens from turn-of-the-century, all of good size (6-12 in., branching)
 - ~30 specimens from 1960s - 1970s, all with plenty of material. As in *E. flexuosa*, this could be an ideal "middle" time point.
 - 12 from last 25 years. All ~~are~~ small, but thicker than *E. flexuosa* moderns.
- ~~← little material from turn-of-century, and several are very small.~~

G. ventalina:

- Little material from turn-of-century, and some are very small. Growth form is also quite delicate: iacy, sp less material. I would be quite worried about destructively sampling these.
- As in other species plenty of specimens: material from 1960s-1970s.
- Very little modern material. I only saw 2-3 specimens from the early, early 2000s.

Conclusions

Insufficient range of collection dates for any *Leptogorgia* species, and both the very old and very recent collections of *G. ventalina* are potentially too small for subsampling. This leaves one of the *Eunicea* species, using the *E. knighti* genome as reference. Both *E. flexuosa* and *E. tourneforti* have sufficient material from a range of collection dates. However, *E. tourneforti* is more closely related to our reference, *E. knighti*; has more specimens and material available for the critical modern reference; and is thicker, potentially providing more material for tissue extraction. Because of these considerations, I think *E. tourneforti* is the best choice!

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Chose the "timepoints of specimen collection dates based on availability and collection location. Since both the old (>100yo) and modern (<25yo) specimens are limited, I will be subsampling all of these (~10 specimens each). There is a high number of specimens collected in the 1960s - 1970s and these specimens are quite large, providing abundant material, so this will be my "midpoint". Since both the oldest and recent collections were mostly performed in the Florida/ Bahamas region of the Caribbean (see digital notebook for mapping of geographic distribution), I will also endeavor to select 1960s-1970s specimens from the same region.

Selected ~10 specimens from each collection period.

<u>1880s-1910s</u>	<u>1960s-1970s</u>	<u>2000s-2010s</u>
USNM 50368	USNM 51728	USNM 100609
USNM 1207569 *	USNM 51730	USNM 100610
USNM 14366 *	USNM 51732	USNM 1007393
USNM 14366 *	USNM 51727	USNM 1018355
USNM 14399	USNM 51729	USNM 1606824
USNM 19054	USNM 51857	USNM 1606826
USNM 42137	USNM 51858	USNM 1740336
USNM 52026 *	USNM 51859	USNM 1740363
USNM 11801630	USNM 51860	USNM 1740390
USNM 50603	USNM 51861	USNM 1740407
USNM 52295	USNM 51862 51892	

* There are two entries in the inventory site for the catalog number USNM 14366, but only one bag in the collections with this number

* Couldn't find two of the old specimens - Emailed Andrea their numbers.

Current sample sizes: 1880s-1910s : n = 8

1960s-1970s : n = 11

2000s-2010s : n = 10

See details on specimen collection date/location in digital labnotebook. To Page No. _____

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Title Subsampling

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Pulled all specimens selected (see last entry, pg. 3).

Subsampling Protocol:

1. Clean benchtop and gloves with bleach wipe.
2. Clean tools with bleach wipe, then DI wipe, then 95% EtOH wipe.
Repeat between each sample, changing wipes every 3-5 specimens.
3. To subsample, cut a ~2mm thick round of tissue from stalk of specimen.
Since these specimens are quite thick, this yielded a good amount of tissue.

Subsampled all specimens, placing tissue in labelled 2mL tubes

Notes:

- USNM 143666 and USNM 14399 both have multiple individuals in a single specimen bag. In both cases, individuals were not separately labelled, so I just subsampled one of those at the top of the bag.

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Began tissue digestions. Digestions were unfortunately split over two days because I couldn't access LAB after I finished the last of the subsampling.

Digestions : extractions performed with Qiagen DNEasy Kit. Protocol modified slightly for use with dried coral specimens.

Digestion Protocol:

1. Begin with small cut/crushed quantity of dried tissue in a 1.5mL or 2mL snap-cap tube.
2. Add 180µl Buffer ATL and 40µl proteinase K. Mix by vortexing and incubate for ~48hr on a heated shaker, set to 50°C and 80 rpm.

Round 1 of digestions began incubating @ 07/09/25, 5:00pm:

Round 1 catalog (USNM) numbers:

14366	52295	100610	1740363
14399	51728	10073913	1740390
19054	51730	1018355	1740401
42137	51732	1606824	
52026	51892	1606826	
1180630	100609	1740336	

Round 2 digestions began incubating 07/10/25 @ 10:00am

Catalog numbers:

50603	51729	51859
50638	51857	51860
51727	51858	51861

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Completing E.tourneforti extractionsQiagen DNEasy Extraction Protocol (after digestion):

2. Add 200 μl Buffer ATL. Mix thoroughly by vortexing and spin down the sides.
3. Add 200 μl 95% ethanol. Mix thoroughly by vortexing and spin down.
4. Pipet mixture onto a DNEasy mini spin column placed in a 2ml collection tube. Centrifuge @ ≥ 8000 rpm for 1 min.
Discard the flow-through and collection tube.
5. Place column in new 2ml collection tube. Add 500 μl Buffer AW1.
Centrifuge @ ~~4,000~~ $\geq 8,000$ rpm for 1 min.
Discard the flow-through and collection tube.
6. Place column in a new collection tube. Add 500 μl Buffer AW2.
Centrifuge @ 14,000 rpm for 3 min.
Discard the flow-through and collection tube.
7. Transfer spin column to a new, labelled 1.5ml or 2ml tube.
8. Elute the DNA by adding 50 μl Buffer AFE to the center of the spin column membrane. Incubate @ room temp. for 1 min.
Centrifuge 1 min @ $\geq 8,000$ rpm.
9. Repeat Step 8, for a final elution volume of 100 μl.

Completed Round 1 extractions @ 3:00pm, July 11, 2025 (stored in fridge)
 Completed Round 2 extractions @ 10:00am, July 12, 2025 (stored in fridge)

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Title Zymo Cleanup.

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Completing Zymo DNA cleanup

Zymo Genomic DNA Clean + Concentrator -10 Protocol

1. Add 5 volumes of CHIP DNA Binding Buffer to each volume of DNA sample. Mix thoroughly and spin down.

e.g. to 100 μl DNA, add 500 μl DNA Binding Buffer.

2. Transfer mixture to a Zymo Spin Column (IC-XC) in a collection tube.

3. Centrifuge 30s @ 12,000 rpm. Discard flow through.

4. Add 200 μl DNA Wash Buffer to the column. Centrifuge 1min @ 12,000 rpm. Discard flow through.

5. Repeat Step 4.

6. Add ≥10 μl DNA Elution Buffer (or nuclease-free water) directly to column matrix and incubate @ room temp for 1 min. Transfer column to a 1.5 ml tube and centrifuge ≥ 30 sec to elute DNA.
labelled

NOTES: Heat DNA Elution Buffer to 60°C before use to improve yield (aliquoted to 2ml tube first)

Eluted each sample in 50 μl of buffer.

Performed Zymo cleanup on both Round 1 and Round 2 extractions @ 1:00pm, July 12, 2025. Stored cleaned DNA in fridge for weekend.

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Using Qubit Fluorometer to quantify dsDNA in *E.tourneforti* cleaned extractions

Qubit 1X dsDNA High Sensitivity (HS) Protocol

- 1.1 Set up required # of assay tubes for standards and samples (2 required standards)
- 1.2 Label tube lids - do NOT label sides of tubes
- 1.3 Add 10µl of each Qubit standard to the appropriate tube.
- 1.4. Add 1-20µl of each user sample to the appropriate tube. (I was recommended to use 2µl sample)
- 1.5 Add the Qubit 1X dsDNA working buffer to each tube such that the final volume is 200 µl.

Note: 190 µl solution to each standard

198 µl solution to each sample (if using 2µl sample)

1. Note recommended to aliquot out appropriate volume of working solution first, to avoid contaminating whole stock.
- 1.6 Mix each sample vigorously by vortexing 3-5 seconds
- 1.7 Allow tubes to incubate @ room temp for 2 minutes.

2.1 On the "Home" screen of the Qubit, select the "1X dsDNA High sensitivity (HS)" assay icon. The "Read Standards" screen is displayed. Press "Read Standards : run samples" to proceed.

2.2 Insert the tube containing Standard #1 into the sample chamber, close the lid, and select "Run Standards". Once complete, remove.

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2.3 Repeat 2.2 with Standard #2

2.4 Select "Next" from the "Standards Complete" screen. When prompted, load the tubes. Select the units for the output sample concentration (I will choose [ng]), then select "Next".

2.5 In the "Sample volume" screen, enter the volume of sample added to the assay tube (1-20 mL),

Note: The sample volume changes the assay accuracy range. A different sample volume or assay may be required if the sample concentration is outside what the assay can accurately quantify at the chosen volume.

2.6 Insert a sample tube into the chamber, close the lid, and press "Run samples." Once complete, remove.

2.7 Repeat Select "Add samples" to read more samples, and repeat step 2.6.

(Just realized the above instructions for machine operation (2.x) are for the Qubit Flex Fluorometer, and I think LAB has a Qubit 4 Fluorometer. Protocol is effectively the same though)

Nitrogen

Protocol copied from Thermo Fisher Qubit 1x dsDNA HS Assay Kit User Guide Manual, Catalog Number Q33230, Q33231, Pub. No. MAND0174SS.

Full LAB Protocol available on next page!

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**MATERIALS & REAGENTS:**

- DNA or RNA to be quantified
- DNA or RNA Qubit kit (HS or BR)
- 0.5 mL Agilent or GeneMate tubes (or comparable thin-walled tubes)

PROTOCOL:**Step 1 - Setting up your assay tubes**

- 1.1 Ensure your DNA/RNA samples are fully thawed and homogenous.
- 1.2 If using a kit that includes separate dye and buffer (not a 1X kit), allow the kit to come to room temperature before use.

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For running Qubit quantification on *E.tourneforti* extractions, will need to relabel samples (so labels will fit on Qubit lids)

Round 1 Extractions:Round 2 Extractions

<u>Catalog number</u>	<u>Qubit label</u>	<u>Catalog Number</u>	<u>Qubit label</u>
USNM 14366	A01	USNM 50368	B01
USNM 14399	A02	USNM 50603	B02
USNM 19054	A03	USNM 51727	B03
USNM 42137	A04	USNM 51729	B04
USNM 51730	A05	USNM 51857	B05
USNM 51732	A06	USNM 51858	B06
USNM 51782 51728	A07	USNM 51859	B07
USNM 51892	A08	USNM 51860	B08
USNM 52295	A09	USNM 51861	B09
USNM 100609	A10		
USNM 100610	A11		
USNM 1007393	A12		
USNM 1019355	A13		
*USNM 1180630	A14		
USNM 11606824	A15		
USNM 11606826	A16		
USNM 17403310	A17		
USNM 1740363	A18		
USNM 1740390	A19		
USNM 1740407	A20		

DNA Concentrations:

<u>Tube</u>	<u>ng/μL</u>	<u>Tube</u>	<u>ng/μL</u>	<u>Tube</u>	<u>ng/μL</u>	<u>Tube</u>	<u>ng/μL</u>
S1	37.57	*A04	15.0	A10	too high	A16	too high
S2	14064.10	A05	20.9	A11	too high	A17	too high
		A06	too high	A12	too high	A18	too high
*A01	0.173	A07	35.2	A13	44.3	A19	too high
*A02	33.9	A08	too high	*A14	1.41	A20	too high
*A03	6.03	*A09	7.08	A15	too high		

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DNA concentrations (cont.) :

<u>Tube</u>	<u>ng/μL</u>	<u>Tube</u>	<u>ng/μL</u>	<u>Tube</u>	<u>ng/μL</u>	$\bullet = \geq 100\text{ng spec.}$
B01	26.5	B04	48.9	B07	43.9	
B02	2.28	B05	43.7	B08	56.0	
B03	27.0	B06	28.5	B09	52.0	

Generally, almost all of my extractions have a good amount of DNA!

If I assume I'll have ~40 μ L DNA left after QCs for library prep, I'll need a concentration of 10 ng/ μ L to meet the recommended minimum of 400 ng DNA for the Native Barcoding Library Prep kit. Only 5 of my samples have below 10 ng/ μ L:

<u>Tube</u>	<u>Conc.(ng/μL)</u>	<u>Catalog #</u>	<u>Coll. Date</u>
A01	0.173	USNM 14306	1886
A03	6.03	USNM 19054	1898
A09	7.08	USNM 52295	1912
A14	1.41	USNM 1180630	1912
B02	2.28	USNM 50603	1912

Unfortunately though, all 5 of those low-quantity samples are from my "old" timepoint, specimens collected $\geq 100\text{yo}$. I only have 8 total specimens from that range of collection dates, and I want an n=5 for sequencing. The other 3 old samples are:

<u>Tube:</u>	<u>Conc.(ng/μL)</u>	<u>Catalog #</u>	<u>Coll. Date</u>
A02	33.9	USNM 14399	1886
A04	15.0	USNM 42137	1897
B01	26.5	USNM 50368	1880

I may also w

I'll need to do additional extractions on the 5 with low concentrations. I may also want to do an additional extraction for USNM 42137 (A04, 15.0 ng/ μ L) because it's only marginally higher in concentration than desired.

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I also have quite a few samples with concentrations too high to be accurately read by the Qubit Assay. While this is not a problem - quite the opposite! - I'll still need to obtain accurate concentration estimates for these. To do this, I need to make dilutions for each of these samples (probably 10X?) then quantify the dilutions.

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Yesterday, several samples had too high of a DNA concentration to be accurately quantified. I'm re-quantifying these today by making dilutions first.

Want a 10X dilution:

$$C_1 V_1 = C_2 V_2$$

$$x \cdot 1\text{ml} = \frac{1}{10} x \cdot y$$

$$10\text{ml} \cdot x = x \cdot y$$

$$10\text{ml} = y$$

So final volume should be

10 ml, with a DNA vol. of 1 ml.

Need to dilute with 9 ml

buffer

Will dilute in Zymo Elution Buffer, since this is what I used to elute the DNA.

For the following samples, diluted 10X in elution buffer (1 ml DNA + 9 ml buffer). Then re-ran Qubit Quantification, following protocol included on page 10 of this lab notebook.

<u>USNM Catalog #</u>	<u>Tube label</u>	<u>ng/ml (10X)</u>	<u>ng/ml (true)</u>
USNM 51732	A06	8.94	89.4
USNM 51892	A08	8.63	86.3
USNM 100609	A10	16.1	161
USNM 100610	A11	8.47	89.7
USNM 1007393	A12	11.6	116.0
USNM 1606824	A15	16.7	167.
USNM 1606826	A16	18.9	189.
USNM 1740336	A17	16.3	163.
USNM 1740363	A18	15.2	152.
USNM 1740390	A19	12.6	126.
USNM 1740407	A20	13.3	133.

Standard #1: 36.17

Standard #2: 14557.00

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Several of the older samples had low DNA concentrations ($<10\text{ng}/\mu\text{l}$).

To obtain enough DNA for library prep ($\geq 400\text{ng}$) I need to extract more DNA from these specimens.

To do this, I'll do several extractions for each of these specimens, then pool each specimen's extractions before Zymo Cleanup to concentrate the multiple extractions into one higher-concentration elution.

For each of the below specimens, subsampled 4 times as described on the subsampling protocol provided on page 4 of this lab notebook.

<u>Catalog Number</u>	<u>Subsample Labels</u>
USNM 14366	14366A, 14366B, 14366C, 14366D
USNM 19054	19054A, 19054B, 19054C, 19054D
USNM 52295	52295A, 52295B, 52295C, 52295D
USNM 1180630	1180630A, 1180630B, 1180630C, 1180630D
USNM 50603	50603A, 50603B, 50603C, 50603D

During subsampling I was able to see where on the specimen I had previously sampled tissue. I subsampled this time from the same stalk/finger, to avoid ~~per~~ preserve undamaged portions of the specimens.

Tissue Digestion

Digested the above specimens as detailed in the Digestion Protocol on page 5 of this lab notebook.

Digestions began incubating @ 3:00pm, 07/15/25.

* Realized while entering concentrations into spreadsheet that I may have mislabelled USNM 51728 as USNM 51782

Extractions completed @ 1:00pm, 07/17/25, as detailed in the Qiagen Extraction Protocol on page 6 of this lab notebook.

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Completed re-extractions of the following samples, with multiples of 4:

USNM 14366

USNM 19054

USNM 52295

USNM 118063D

USNM 56603

Used Zymo Cleanup Kit to clean and concentrate extractions, followed the Zymo Cleanup Protocol found on page 7 of this lab notebook, with the following modifications:

To concentrate 4 100 μ l extractions into a single cleaned extraction, during steps 2-3 of the protocol (adding sample to spin column and centrifuging), performed several iterations for each specimen.

The spin column is too small to hold the full volume of 4 \times 10 μ l DNA samples and the corresponding 2000 μ l of CHIP Binding Buffer. Instead, added 1000 μ l of sample DNA + CHIP Buffer and centrifuged; discarded flow through, and added ~~an addition~~ repeated until all extracted DNA of a given specimen had been sent through the specimen's assigned Zymo Spin column.

I then proceeded as normal, eluting in 50 μ l of heated Elution Buffer.

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Title Qubit of re-extractions

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Quantified DNA concentration of my 5 re-extractions using Qubit fluorometer. Followed Qubit Protocol found on page 10 of this lab notebook.

<u>Catalog Number</u>	<u>Qubit Label</u>	<u>Concentration (ng/μl)</u>
USNM 14306	C01	0.372
USNM 19054	C02	3.57
USNM 52295	C03	9.02
USNM 1180630	C04	1.78
USNM 50603	C05	14.9

Standard #1: 37.13

Standard #2: 14435.49

Ok, this isn't terrible! the 50603 concentrated re-extraction has ≥ 10 ng/μl, which is enough to meet the recommended 400ng minimum for minION. USNM 52295 is almost at this threshold, and if I can combine it with my first extraction of this specimen ($\Sigma = 7.08$ ng/μl) during e.g. bead cleanup, we'll have enough!

That would bring me to my desired minimum number of samples ($n=5$) for ≥ 100 μg specimens!

Now I need to run gels on everything to check fragment size.

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To check DNA integrity, ran gel electrophoresis.

Gel-making protocol

1. Tape sides of gel mold and insert combs. For ~~the~~ integrity check on genomic DNA, used 12-well molds that had an additional well for ladder.
2. Melt one 50mL Falcon tube of agarose gel (1.5% SB agarose) in beaker by microwaving in ~15s intervals, swirling intermittently to mix. Once fully melted, ~~check for any~~ allow to cool until the beaker is comfortable to handle.
10,000X
3. Add 2mL gel red to melted agarose, swirling to mix, then pour agarose into gel mold
4. Allow to cool until fully set. Will be slightly opaque, ~30min.

IF using immediately, remove combs and begin use (see next protocol)
 IF storing, leave combs in and refrigerate in a sealed container.

Electrophoresis Protocol

1. If necessary, make aliquot of "dosed" loading dye, by mixing 1000mL 2X Loading Dye with 1mL 10,000X gel red.
2. Place gel, with combs and tape barriers removed, in electrophoresis chamber. Ensure it is fully covered by buffer, and wells are by the neg. (black) node.
3. For each sample to be run, mix 2mL "dosed" Loading Buffer to 2mL DNA.
4. Load into wells, taking care not to puncture gel or eject sample from well.

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5. Load equal volume of Fast DNA Ladder in ladder well.

6. Run gel @ 100-120 volts for 60 min.

For 07/18/25 run, gels were set up as below:

Gel 1:	14366 14399 19054 42137 51730 51732 (10x) 51782 (10x) 51892 (10x) 52295 100609 (10x) 100610 (10x) 1007393 (10x) ladder	Gel 2:	+ 1018355 + 1180630 + 1606824 (10x) + 1606826 (10x) + 1740336 (10x) + 1740363 (10x) + 1940390 (10x) + 1740407 (10x) + 50368 + 50603 + 51727 + 51729 ladder
--------	--	--------	--

Gel 3:	51857 51858 51859 51860 51861 14366 (2nd) 19054 (2nd) 52295 (2nd) 1180630 (2nd) 50603 (2nd) ladder
--------	---

Gel 3 note:
in this sketch I mistakenly drew everything shifted by 2. The two empty wells are actually on the far side, away from the ladder! Order is correct though

10x = used 10x dilution to avoid high-concentration DNA "clogging". Same dilutions as used for Qubit.

2nd = 2nd extraction of this specimen

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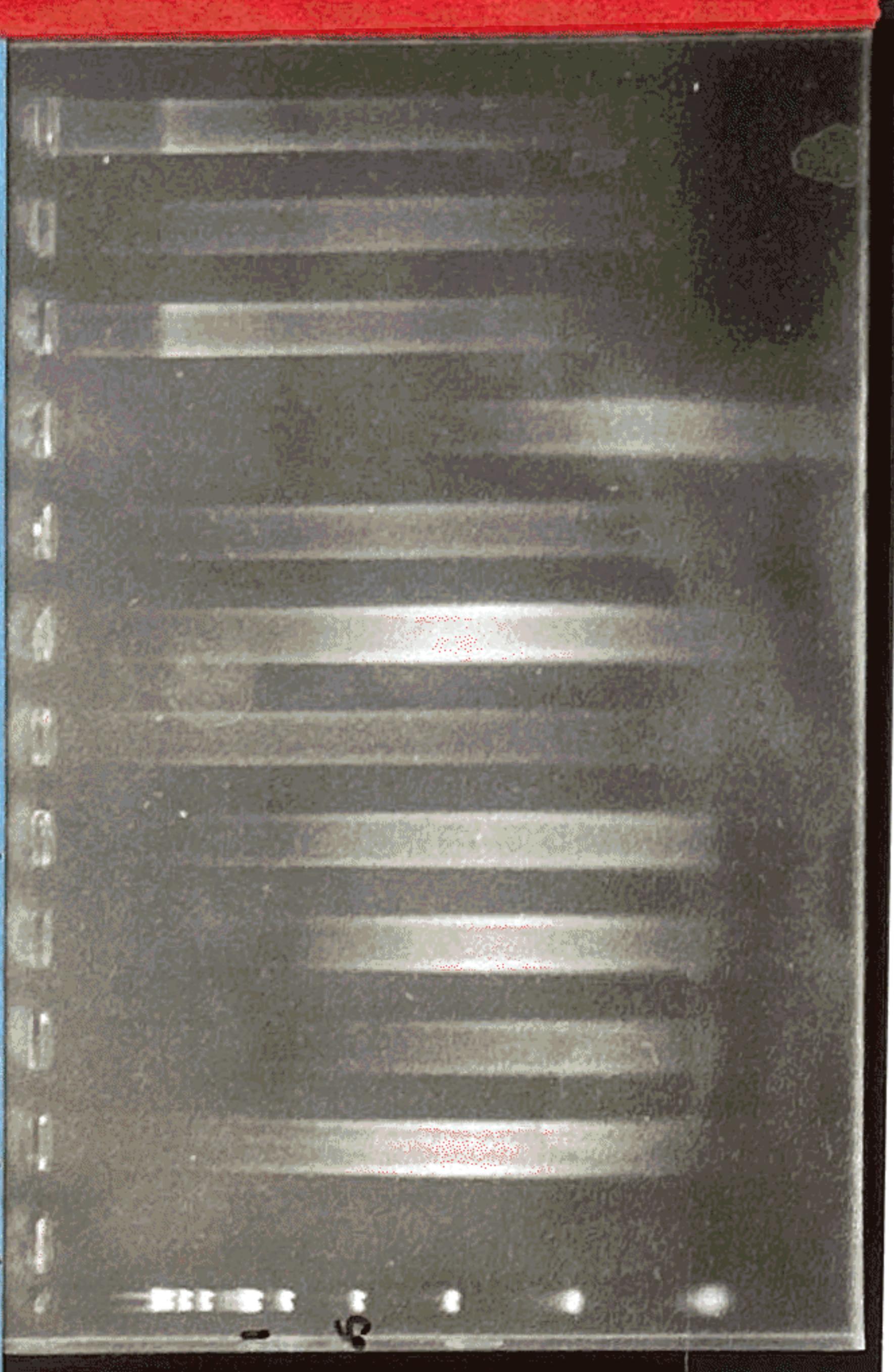
Recorded by

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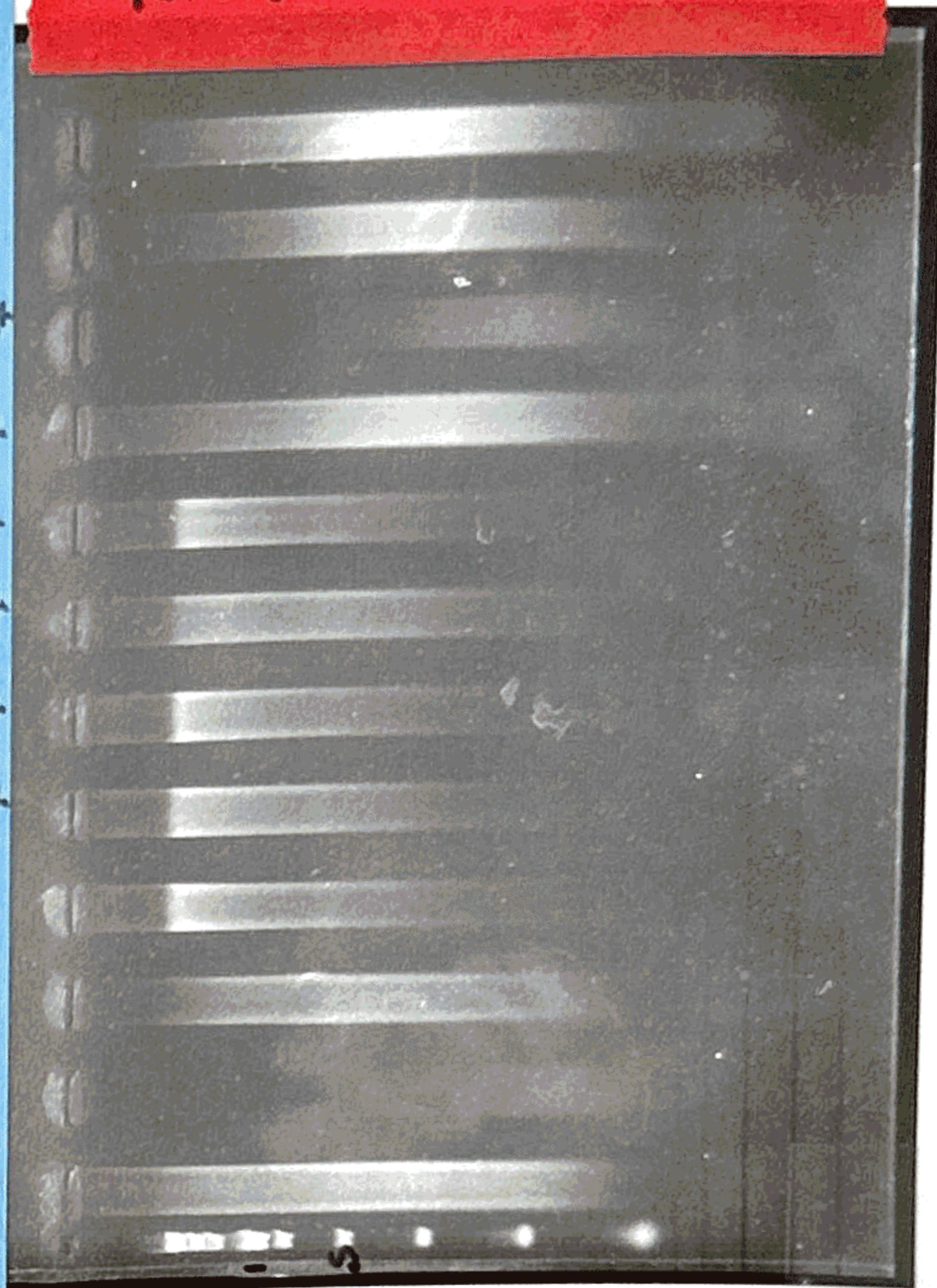
Round 1
14366 - 1007393

14366
14399
19054
42137
51730
51732 10x
51782
51892 10x
52295
100609 10x
100610 10x
• 1007393 10x
ladder



Round 2
1018355 - 51729

1018355
1180630
• 1606824 10x
1606826 10x
1740336 10x
1740363 10x
1740390 10x
1740407 10x
50368
50603
• 51727
51729
ladder



Round 3
51857 - 50603

51857
51858
51859
51860
• 51861
14366 (2nd)
19054 (2nd)
52295 (2nd)
1180630 (2nd)
50603 (2nd)
ladder



- A few lanes were too faint to really see (blue dots). Can re-run 1606824 undiluted, and 1007393 undiluted
- Most of the old stuff (14366, 19054, 52295, 1180630, 50603, 14399, 42137, ? 50368) seems ok. A few have HmW DNA, most range from 1kb - 0.15 kb. 42137 is the only one with only short fragments.
- Modern stuff also looks ok, especially the 1740#### samples, which I think correspond to the 2019 samples. These have a lot of HmW DNA!
- Surprisingly, the 00s stuff seems most degraded (51857 - 51861, 51727). May want to consider pulling different collections.

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Title Gel re-runs

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A couple samples showed no visible DNA in my first rounds of gel electrophoresis, so I'm going to rerun.

Two of these samples (1007393, 1606824) were first run using 10x diluted DNA, so I'll rerun with the undiluted extraction.

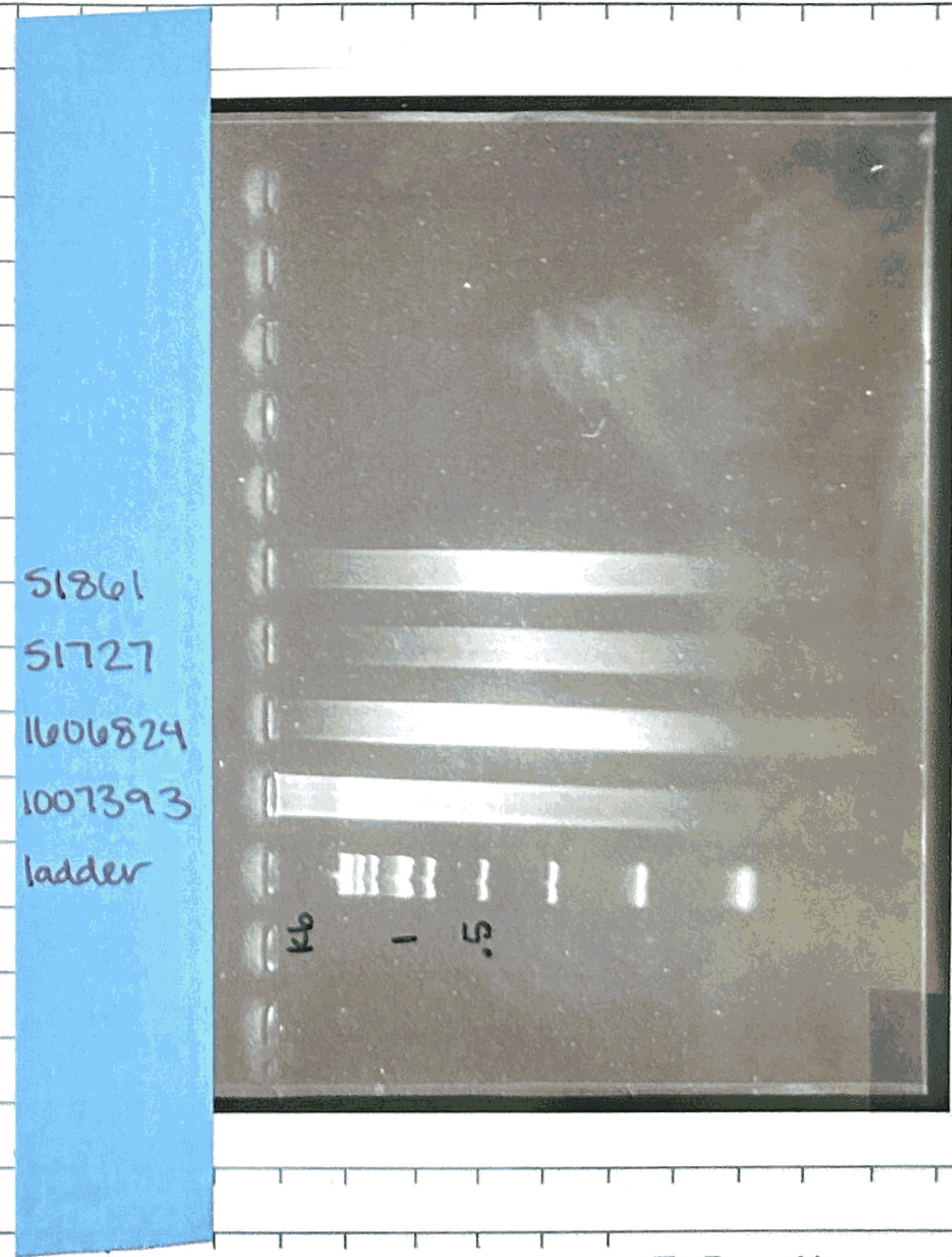
For the other two samples (51727, 51861), Qubit showed plenty of DNA (27 ng/ml, 52 ng/ml), so I'm not sure why nothing showed up on the first gels. Maybe user error (e.g. DNA floated out of well)? Either way, I want to try again to get a sense of size distribution.

Followed gel making and electrophoresis protocols found on pages 18-19 of this lab notebook. Used 2μl of *undiluted* DNA extraction for all samples.

Gel setup:

Gel:

51861	
51727	
1606824	
1007393	
ladder	



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Title Kapa Bead Cleanup - Titration Trial

From Page No. _____

I'll be using KAPA Pure Beads to size-select and clean (and, potentially, concentrate) my DNA extractions before library prep.

I'm not sure exactly what bead ratio will work best to remove ≤ 350 bp fragments from my samples, so I'll be trying a "titration" trial of several ratios.

For this trial I'll be using two mid-age samples with high DNA concentrations but a wide range of fragment sizes ("smear"):

USNM 51732, 89.4 ng/ml, smeared around 0.3 kb

USNM 51892, 86.3 ng/ml, smeared around 0.15 kb

The KAPA Pure Beads recommends a bead-to-sample ratio of 0.9X - 0.7X to retain fragments ≥ 250 bp - ≤ 350 bp. I'll test three ratios across this range.

	0.7X	0.8X	0.9X	} label table
USNM 51732	A.7	A.8	A.9	
USNM 51892	B.7	B.8	B.9	

Followed the KAPA Pure Beads Protocol on the next page (#2, Cleanup of Fragmented DNA in NGS Workflow), with the following notes / modifications:

- Mixed fresh aliquot of 80% EtOH using nuclease-free water
- For each rxn, used 5ml DNA and diluted to 20ml using Zymo Elution Buffer (5ml DNA + 15ml Buffer).
- With 20ml DNA, bead volumes are as follows:

0.7X: 20ml DNA + 14ml beads

0.8X: 20ml DNA + 16ml beads

0.9X: 20ml DNA + 18ml beads

- First incubation (2.4) for 10 minutes

- Dried beads (2.13) for 4 min

- Re-eluted DNA (2.15) in 20ml Zymo Elution Buffer

- Incubated eluting DNA (2.16) for 8 minutes

* Note that eluting in 20ml means my DNA samples (e.g. 5ml) are essentially 25% dilute. *

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KAPA Pure Beads

Technical Data Sheet

Protocols

1. Genomic DNA Purification (Cleanup)

Prior to library construction in NGS workflows, it may be beneficial to perform an upfront genomic DNA cleanup. For cleanup, buffer exchange, and/or concentration of high-quality genomic DNA prior to library construction, a KAPA Pure Beads-to-sample volumetric ratio of 3X is recommended.

The detailed protocol below is an example of a 3X cleanup of genomic DNA in 100 µL. Please pay special attention to steps 1.15 and 1.16 (elution of DNA off beads). To ensure optimal recovery, *these steps may be performed at an elevated temperature: 37°C for 10 min.* Elution buffer may be pre-heated for this step and/or the elution performed in a thermocycler or heating block. The heated elution is not required for the

- 1.13 Dry the beads at room temperature for 3 – 5 min, or until all of the ethanol has evaporated. *Caution: over-drying the beads may result in reduced yield.*
- 1.14 Remove the plate/tube(s) from the magnet.
- 1.15 Resuspend the beads in an appropriate volume of pre-heated elution buffer at 37°C and/or perform the elution incubation (step 1.16) in a thermocycler or heating block set to 37°C. The appropriate elution buffer may be either 10 mM Tris-HCl, (pH 8.0 – 8.5) or PCR-grade water, depending on the downstream application.
- 1.16 Incubate the plate/tube(s) for 10 min to elute the DNA off the beads.
- 1.17 Place the plate/tube(s) on a magnet to capture the beads. Incubate until the liquid is clear.
- 1.18 Transfer the clear supernatant to a new plate/

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Yesterday I trialed several ratios of KAPA Pure Beads to determine which works best for size-selecting my samples.

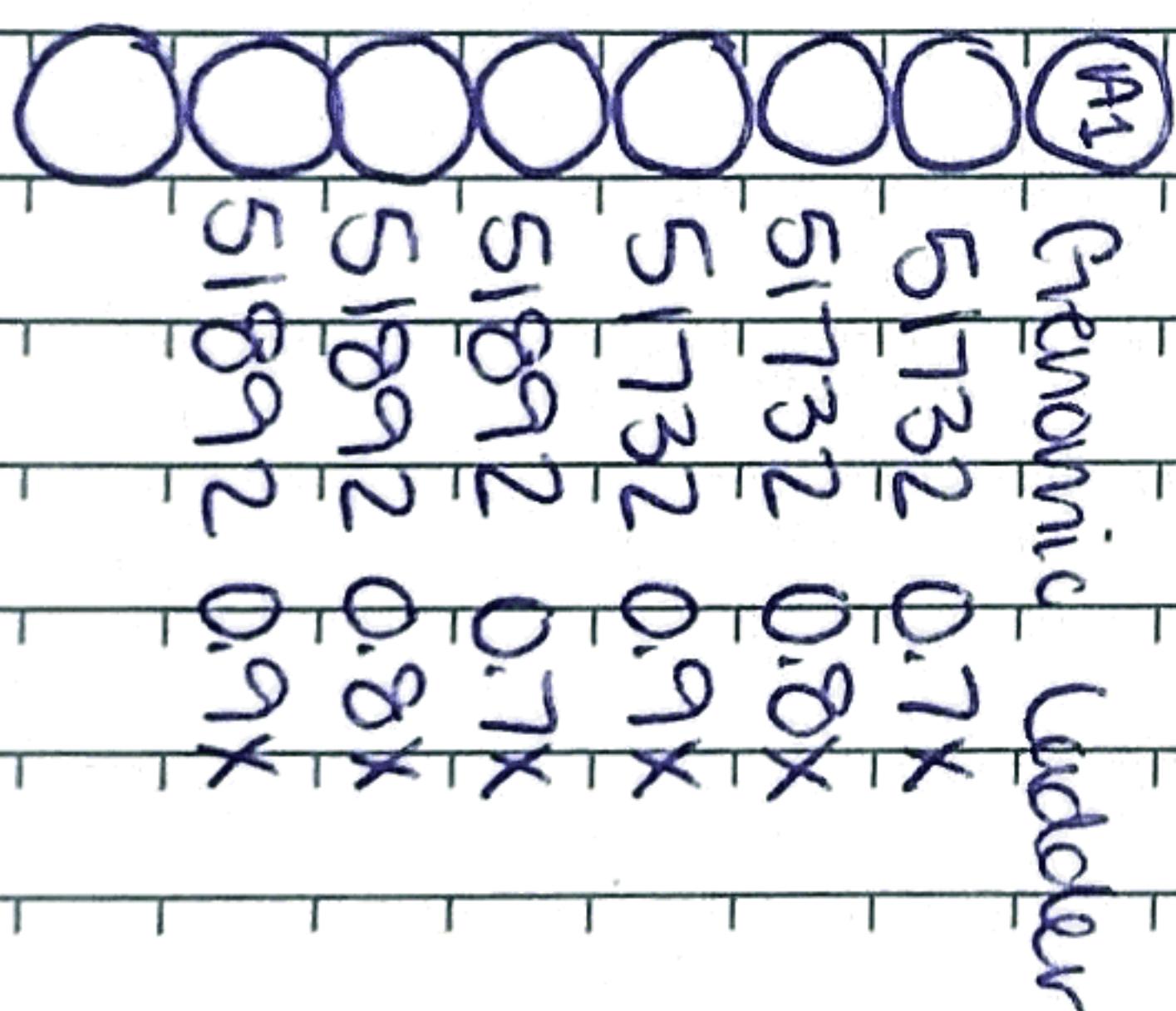
Now I'm running TapeStation on these titrated bead cleanups to see what range of fragment sizes was retained through cleanup.

Used Genomic Tape, and followed Genomic DNA Protocol (on next page)

Machine is an Agilent 4200, serial # DEDD DEDIA03520

Note: Tape I'm using expired April 2023, but should still be usable.

Samples loaded in Optical Tube 8x strip, in the following orientation:



Some TapeStation Results (see all details and full report in digital lab notebook):

Catalog #	Ratio	Conc. (ng/μL)	% reads 300-60,000 bp	Conc. reads 300-60,000 bp (ng/μL)
USNM 51732	0.7X	24.5	91.88	22.51
USNM 51732	0.8X	23.3	90.21	21.02
USNM 51732	0.9X	15.8	87.65	13.85
USNM 51892	0.7X	27.7	90.52	23.26
USNM 51892	0.8X	23.1	89.59	20.70
USNM 51892	0.9X	20.6	87.97	18.12

Reminder: expect ~27ng/μL in each sample

All ratios show similar % longer reads retained, but 0.7X retains most total DNA quantity.

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Genomic DNA ScreenTape Assay for TapeStation Systems

Genomic DNA ScreenTape Assay Operating Procedure

- 1 Allow Genomic DNA Reagents to equilibrate at room temperature for 30 minutes.
 - 2 Launch the Agilent TapeStation Controller software.
 - 3 Flick the Genomic DNA ScreenTape device and insert it into the ScreenTape nest of the TapeStation instrument.
 - 4 Select required sample positions in the TapeStation Controller software.
 - 5 The required consumables (tips, further ScreenTape devices) are displayed in the TapeStation Controller software.
 - 6 Vortex reagents and samples. Spin down before use.
 - 7 Prepare ladder.
 - For 1 or 2 ScreenTape devices: pipette 10 µL Genomic DNA Sample Buffer (●) and 1 µL Genomic DNA Ladder (○) at position A1 in a tube strip.
 - For more than 2 ScreenTape devices¹: pipette 20 µL Genomic DNA Sample Buffer (●) and 2 µL Genomic DNA Ladder (○) at position A1 in a tube strip.
 - 8 For each sample, pipette 10 µL Genomic DNA Sample Buffer (●) and 1 µL DNA sample in a tube strip or 96-well sample plate¹.
- ¹ An error can occur if tube strips and/or fail-safe to 96-well consumables are used.

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After confirming which bead-to-sample ratio I want to use (0.7x), performed bead cleanup and concentration of all samples.

Using KAPPA Pure Beads, and following protocol provided on pages 22-23 of this lab notebook.

This will be slightly more complicated, though, because many ~~trace~~ of my DNA extractions have slightly different remaining volumes due to differences in pipet loss, # of gels run, dilutions performed, # of extractions, and whether samples were included in the titration bead trial.

I'm also combining and concentrating the following:

*Extractions 1 : 2 for: 14366, 19054, 52295, 1180630, 50603

• Extraction and titrated bead cleanups: 51732, 51892

~~To accurately determine~~

I want to use all extracted DNA I have, so I can't just take an equal volume from each sample. Instead, I'll measure the total extraction volume of each sample as I transfer it to 8x strip tubes. I'll then calculate, for each sample, what bead volume to use to maintain a bead-to-sample ratio of 0.7x.

Catalog #	DNA (μL)	Beads (μL)	Catalog #	DNA (μL)	Beads (μL)	Catalog #	DNA (μL)	Beads (μL)
14366*	90	63	14399	55	38.5	1606824	50	35
19054*	100	70	42137	50	35	1606826	55	38.5
52295*	100	70	51730	70	49	1740336	55	38.5
1180630*	110	77	51782	45	31.5	1740363	50	35
50603*	110	77	100609	45	31.5	1740390	45	31.5
51732•	100	70	100610	45	31.5	1740407	65	45.5
51892•	90	63	1007393	45	31.5	50368	50	35
			1018355	55	38.5	51727	50	35
Strip-tube A			Strip-tube B			Strip C		

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Catalog#	DNA(μL)	Beads(μL)
51729	45	31.5
51857	45	31.5
51858	45	31.5
51859	45	31.5
51860	40	28
51861	30	21

After pipetting all samples into 8X tube strips (in the same order as shown in volume tables on this and the previous pages), filled 4 8X tube strips with the corresponding volumes of ICAPTA Pure Beads (temp-acclimated and vortexed). This allowed me to quickly add the appropriate volume of beads to all samples using a multi-channel pipette.

Strip D

Eluted all samples in 30μL of Zymo Elution Buffer.

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Following KAPA Bead cleanup of all DNA extractions (pg. 26, 27 of this lab notebook), used Tape Station to evaluate DNA integrity and quantity, quantity.

Used genomic DNA tapes, and followed genomic DNA protocol (found on page 25 of this lab notebook)

Machine is Agilent 4200, serial # DEDIAH03520

Samples loaded in following order:

Tape #1

○ ○ ○ ○ ○ ○ ○	(A2)	○ ○ ○ ○ ○ ○ ○	(A1)	Ladder
100609	51728	51730	42137	14399
100610	51892	51891	51732	50603
1007393	1008355			1180630
				52295
				19051
				14366

Tape #2

○ ○ ○ ○ ○ ○ ○	(A2)	○ ○ ○ ○ ○ ○ ○	(A1)	Ladder
51861	51860	51859	51857	51727
				1740363
				1740407
				1606826
				1606824

See all Tape Station report details in digital lab notebook

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Title Transfer DNA extractions for storage

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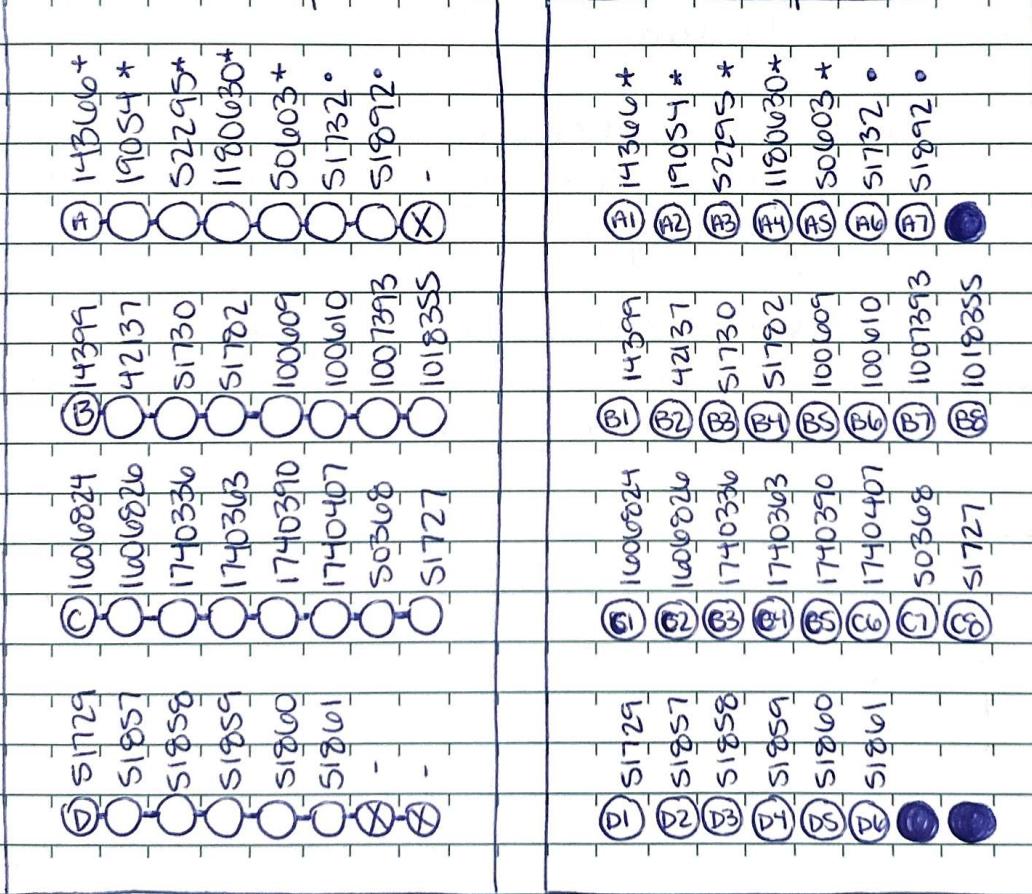
Transferred all DNA extractions from their 8x strip tubes (0.2ml) to individual 0.2ml tubes

As I begin library prep, I'll need to pull and thaw small sets of specific samples. To avoid constant freeze-thaw of all samples, they need to be in individual, separate tubes, not the strip tubes they're currently in.

Pulled all DNA extractions from freezer, thawed in fridge for ~20 min. then spun down and placed on ice.

Current setup:

New setup:



These are all "C" prefixes

Labelled new 0.2ml tubes on cap and sides. Didn't change the "location" of any samples. Transferred full volume of each extraction from old strip-tube spot to new, labelled 0.2ml tube. Stored again in -20°C freezer.

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08/11/25

Performing library prep on extracted, cleaned, and QC'd DNA for Nanopore sequencing

As a reminder, will be multiplexing 4 samples per MinION flow cell:

$$\begin{array}{l} \text{MinION output (max 48Gb)} : 30 \text{ Gb} \\ \text{E. tourneforti genome} : 600 \text{ Mb} \\ \text{E. tourneforti genome (10X)} : 6 \text{ Gb} \end{array} \quad \begin{array}{l} \xrightarrow{\quad} \frac{30 \text{ Gb}}{6 \text{ Gb/sample}} = 5 \text{ samples} \\ \xrightarrow{\quad} \text{(max)} \end{array}$$

To achieve desired coverage of full genome, conservatively, I can run 5 combine 5 samples max in a single run. I chose 4 samples per cell, to allow room for error.

I also want to group samples for multiplexing by DNA integrity and quantity, since library prep includes selecting a pooled library quantity based on average fragment length.

Using the TapeStation peak fragment size as a proxy for average fragment size of each sample (reasonable, due to normal distributions of fragment lengths), I selected 4 samples with highest fragment size for Group 1:

Catalog #	peak bp	LP tube #
16068210	1906	1
1740336	1698	2
1740363	1696	3
1740407	1798	4

Following the Oxford Nanopore Technologies Ligation Sequencing gDNA - Native Barcoding kit 96 V14 (SOK-NBD114.96) protocol
(V NBE_9171_v114_revS_02Jul2025 document)

Print-out of protocol found in back of this lab notebook, with notes.

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Using Short Fragment Buffer (SFB). No LoBind PCR plates, so using 0.2ml 8x strip-tubes.

Following the final step of adapter ligation, will need to prep two versions of Final Library - one at approx appropriate volume \rightarrow DNA content for Flongle flow cell, and one for MinION.

Notes:

Native Barcoding 96 Kit

REF SQK-NBDI14.96

LOT NBDI1496.40.0007

Flongle Expansion

REF EXP-FSE002

LOT FSE002.30.0005

B

Catalog #	Ext. Conc.(ng/ml)	µl for 400ng	Barcode #
1606826	408	1 µl	01
1740336	368	1.1 µl	02
1740363	343	1.2 µl	03
1740407	260	1.5 µl	04

Before beginning library prep..

- Ensure there is an available thermal cycler with the following program: 20°C for 5min, 65°C for 5min.
- Pre-heat an incubator plate to 37°C

Qubit after Barcode-Ligation

Standard 1: 39.06

Standard 2: 16073.91

Barcode-ligated DNA: 1.62 ng/µl

Qubit after Adapter-Ligation

Standard 1: 30.11

Standard 2: 14133.11

Adapter-ligated DNA: 2.56 ng/µl

Library has very low final concentration. This is likely because, while I started with 400ng DNA from each sample, only a fraction of this is

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carried over after End-Prep. At Step 7 of Barcode Ligation, for each sample 0.75 μl of end-prepped DNA is used, along with 3μl nuclease-free H₂O. Protocol is likely optimized for multiplexing many samples, but I'm using just 4.

I think I should be able to, instead, carry over a full 3.75μl end-prepped DNA at this step, and exclude additional nuclease-free water. I'm going to try another round of library prep using the same samples, and make this change to retain more DNA.

I'll call the first round of library prep with these samples "Library 1", and the second round, with the change to retained DNA, "Library 2".

Library 2 Notes:

Catalog #	Ext. Conc. ng/μl	μl for 400ng	Barcode #
1606826	408	1 μl	05
1740336	368	1.1 μl	06
1740363	343	1.2 μl	07
1740407	260	1.5 μl	08

During Barcode-ligation, Step 7, excluded nuclease-free H₂O, and used 3.75μl end-prepped DNA (instead of the recommended 0.75μl)

Qubit after Barcode-ligation

Standard 1: 33.38

Standard 2: 13411.37

Barcode-ligated DNA: 13.7 ng/μl

Qubit after Adapter-ligation

Standard 1: 30.11

Standard 2: 14133.11

Adapter-ligated DNA: 16.5 ng/μl

Took both Library 1 and Library 2 through step 18 of Adapter Ligation; clean up. Did not dilute to Final Library - will need different dilutions for Flongle? minION.

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Title Flongle Run, Group 1, Library 1

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I want to confirm successful library prep and compare the two "versions" of library prep FFO for my Group 1 samples. To do this, will first run a small portion of ^{each} library on Flongles.

Flongle runs for ~24 hrs, so doing just library 1 today.

Library 1 has a concentration of 2.56 ng/ml, and an average fragment size of ~1700bp.

For Flongle, final library should be 5-10 fmol DNA in 5ul EB.
5-10 fmol is equivalent to 5.2ng - 10.5ng of 1700bp DNA.

For Library 1, prepped Flongle Final Library as follows:

- 3ul Library 1
- 2ul EB

Final volume. 5ul, final DNA quantity of 7.68ng, or 7.33 fmol

Followed Oxford Nanopore Technologies Native Barcoding 96 kit protocol for Flongle Flow Cell Loading. Print-out of this protocol can be found at back of this lab notebook.

Flongle Sequencing Expansion: REF EXP-FSE002
 LOT FSE002.30.0005

Flongle Adapter: SN A100007434

Flongle Flow Cell: RELOT 33001524
 SN B028104085
 # AYW935

Performed Flongle Flow Cell check: 59 pores
 After loading library: 44 pores

Run for 24hr.

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After pulling the completed Flongle run of Group 1, Library 1, performed pore check on all remaining flow cells.

As a reminder, the pore check of the used Flongle flow cell showed:

<u>SN</u>	<u>Flow cell ID</u>	<u># pores</u>
B028104085	A4W935	59

Checked all 11 remaining Flongle flow cells. As a reminder, <50 pores would fall below warranty. Maximum # of pores is 126.

<u>SN</u>	<u>Flow Cell ID</u>	<u># pores</u>
B028104112	AXS 867	37 *
B028103996	AXS 408	40 *
B028103978	AYL 256	35 *
B028103949	AYL 126	80
B028104129	A4W 871	61
B028104068	AYK 267	59
B028103992	AYK 963	89
B028104122	A4W 685	59
B028104029	A4X 067	79
B028104142	AYL 029	44 *
B028104167	AYL 248	60

* = below warranty

Wow, so 4/12, or 1/3 of the Flongle flow cells we received are below warranty. Will need to contact Nanopore to request replacements.

Another 5 (including the cell used for Group 1, Library 1), have pore counts "just" above warranty, ~60 pores.

Requested replacements for the out-of-warranty cells 08/13/25 from Nanopore support, case # 01333308

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Title Flongle run, Group 1, Library 2

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Group 1 Library 1 was composed of the below 4 extractions, prepared using the Native Barcoding 96 protocol as written. This prep yielded a low final DNA concentration/quantity, so I did another round of library prep using the same extractions but a slightly modified library prep protocol (see pg. 31-32 of this lab notebook). This second library prep will be referred to as Group 1, Library 2.

Group 1 extractions: 16006826, 1740336, 1740363, 1740407

To compare the two library prep protocols, and confirm the modified protocol doesn't affect sequencing success, running Group 1, Library 2 on a Flongle flow cell.

Library 2 has avg. fragment length ~1700bp and a concentration of 16.5ng/ml.

Flongle requires 5-10fmol DNA in 5ul DNA EB. For my fragment size, this corresponds to 10.47ng DNA for 10fmol. Will max out DNA. Library concentration is too high to accurately pipette equivalent of 10ng. Instead, will make final library of 20fmol (21ng) DNA in 10ul EB, and only use 5ul in Flongle loading.

21ng of DNA from 16.5ng/ml $\rightarrow \frac{21}{16.5} \rightarrow 1.27 \text{ ml library 2}$

8.73 ml EB

10 ml total

Followed Flongle flow cell loading protocol found in back of this lab notebook.

[LOT] 33001524

Flongle flow cell : [SN] B028104122

[ID] A4W68S

pores on check: 59 pores

pores after run begins: 43 pores

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Title Pore check of MinION flow cells

From Page No. _____

Ran pore check on all MinION flow cells.

As a reminder, MinION flow cells have a maximum of 2048 pores, and warranty guarantees 800 pores.

<u>SN</u>	<u>Flow cell ID</u>	<u># active pores</u>
B024226778	FBD08455	1652
B024226909	FBD36390	1528
B024226802	FBD09922	1428
B024226790	FBD39370	1482
B024226535	FBD42232	1519

Nice! All 5 MinION flow cells are well above warranty.

Note from 08/25/25: All MinION flow cells from same LOT: 11004308

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Title Native Barcoding Prep, Group 1, Library 3 Book No. _____

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After my preliminary, Pangle runs (Group 1 Library 1, and Group 1 Library 2), I'm a little worried about how much MinION output I'll get, so I'm trying going to switch to 3 samples per flow cell.

I've also looked at some options for library prep adjustments:

- Exclude FFPE Repair. FFPE repair, in part, will repair cytosine deamination ($C \rightarrow U$) ~~transfor~~ damage. Since I'm interested in potentially examining patterns of deamination, I'd like to exclude this step. It may cause problems, though, since the DNA damage (e.g., nicks) may affect sequencing.
- Begin with 1000ng gDNA for each sample, instead of 400ng, when multiplexing ≤ 4 samples.

Catalog #	[] ng/ml	µl for 1000ng	Label/barcode
1606826	408	2.5	09
1740336	368	2.7	10
1740363	343	2.9	11

End-prep Master Mix (no FFPE)

<u>1X</u>	<u>4X</u>	
Ultra II Buffer	1.75µl	7µl
Ultra II Mix	0.75µl	3µl

add 2.5µl MM to each tube

Qubit 1 (Barcodes ligated)

S1: 37.40
S2: 9926.48
L3: 22.8 ng/ml

Qubit 2 (adapters ligated)

S1: 31.61
S2: 11333.17
L3: 26.2 ng/ml

Assume length of 1000 bp, since last Pangle runs have showed shorter fragment lengths than TapeStation. $5\text{-}10\text{ fmol} = 3\text{-}6.2\text{ ng DNA}$.

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Need 6.2ng DNA in 5uL EB. Too low to pipette my high-concentration DNA. Instead, mixed:

$$\frac{6.2 \text{ng DNA}}{5 \mu\text{L EB}} = \frac{31 \text{ng DNA}}{25 \mu\text{L EB}}$$

31ng of 26.2ng/μL DNA is
 $\frac{31}{26.2} = 1.2 \mu\text{L DNA.}$

So mixed 1.2uL library 3 in $(25 - 1.2) = 23.8 \mu\text{L EB.}$

Then used 5uL of diluted library 3 to load Flongle

Flongle Flow Cell:
 [SN] B028104068
 [LOT] 33001S24
 [ID] A4K267

59 pores on flow cell check

Ran for full 24 hours.

Notes after run completed:

Excluding, FFPE during end-repair resulted in very low yield (~70mb compared to ~120mb), likely because unrepaired "nicked" DNA clogged the pores quickly.

Supported by low pore occupancy throughout run, and quicker pore loss.

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Summary of Group 1 Libraries 1-3
Title Native Barcoding Prep, Group 1, Library 4

Project No. _____

Book No. _____

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Summary from last 3 library preps : tweaks :

- Beginning with and retaining more DNA through prep doesn't have a noticeable negative effect on sequencing, but DOES result in more a library with much higher DNA content. This is very important for being able to load the MINION flow cell multiple times (with washes), so for all future library preps I will :
 - begin with 1000ng DNA input from each sample (or as much as I have, if <1000ng available)
 - following end-prep, retain 3.75μl end-prepped DNA during Native Barcode ligation Step 7, instead of the recommended 0.75μl.
 - Note that, to get 1000ng DNA in 12μl input will need a concn. of $>83.3\text{ng}/\mu\text{l}$. If input DNA is too dilute, will need to concentrate (e.g. using Zymo).
- I realized I don't need to use the DNA Control Sample (DCS), since its purpose is to inform, during, in the event of failed sequencing, whether the cause was failed library prep. I'm running libraries on Flongles to confirm successful library prep (without risking a MINION flow cell), so I don't need the DCS. Instead, this volume can be used for input DNA. For all future library preps I will :
 - Exclude the 1μl DCS from each sample prep during DNA Repair and End-Prep Step 4. Instead, make up 100ng of each input DNA sample in 12μl.
- Based on Flongles' po rate of pore degradation and sequencing, I think MINION cells may yield less output than I originally estimated. As such, I will only multiplex 3 samples per flow cell.
- Excluding FFPE during end repair affects sequencing. This reagent will be used in all future library preps.

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Now that I'm satisfied with the tweaks to the Native Barcoding gDNA protocol I've made, I will incorporate them all for a library prep of Group 1 (Library 4) that will hopefully be good to sequence on MinION.

As a reminder, I'm using Native Barcoding 96 . gDNA protocol (Version NBF-9171-v114-revS02Jul2025), with the following modifications:

DNA Repair ? End-Prep:

[Step 4] aliquot 1000ng DNA per sample (not 400ng)

[Step 6] exclude DCS, making up remaining 1ml with sample DNA or nuclease-free water.

Native Barcode ligation:

[Step 7] use 3.75μl End-prepped DNA for each sample, and exclude the nuclease-free H₂O

Catalog #	ng/ml	μl for loading	μl for 12ml	Label/Barcode
1606826	408	2.5	9.5	12
1740336	360	2.7	9.3	13
1740363	343	2.9	9.1	14

End-Prep Master Mix	1x	4x	
NEBNext FFPE DNA Repair Buffer	0.875μl	3.5μl	- Add 3μl /sample
Ultra II End-prep Reaction Buffer	0.875μl	3.5μl	
Ultra II End-prep Enzyme mix	0.75 μl	3 μl	
NEBNext FFPE DNA repair mix	0.5 μl	2 μl	

Qubit 1 (Barcodes ligated)

Standard 1: 38.64

Standard 2: 16901.25

Library 4: 14.9 ng/ml

Qubit 2 (Adapters ligated)

Standard 1: 39.46

Standard 2: 10449.45

Library 4: 340.6 ng/ml

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Title Flongle run, Group 1, Library 4. Book No. _____

From Page No. _____

Running Group 1, Library 4 on a Flongle to confirm successful library prep.

Need 10fmol DNA in 5ul EB.

Assume 1700bp avg. length, $10\text{ fmol} = 10.5\text{ ng DNA}$

Library Library 4 is 36.6ng/ml.

$$\frac{10.5 \text{ ng DNA}}{5\text{ul EB}} = \frac{42 \text{ ng DNA}}{20\text{ul EB}} = \frac{42 \text{ ng DNA}}{\frac{36.6 \text{ ng/ml}}{\text{in}}} = \frac{1.15 \text{ ul Library 4}}{(20 - 1.15) - 18.85\text{ul EB}}$$

Then use 5ul of the [1.15ul Library 4 + 18.85ul EB] mix to load Flongle

Flongle Flow Cell

[SN] B028104167

[LOT] 33001524

[ID] A4L248

60 pores on check

33 pores after loading

Ran for ~24hrs.

This Flongle run had very poor results - low output and quickly degrading pores.

After discussion with Dan, though, he thinks it was a problem with the flow cell itself, not the library. I'll go ahead with the Library 4 prep and try it on a MinION.

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Ok, today's the day! I have a library I'm happy with, that also has a lot of DNA for multiple cycles of wash/reload.

Will be running Group 1 Library 4 on a MinION flow cell.

Following MinION loading protocol found in the Native Barcoding 96 gDNA protocol (version NBE_9171_v114_rcv5_02jul2025).

Make a diluted Final Library 4:

Though TapeStation told me these DNA extractions were ~1700bp on average, the Flongle run showed a read distribution centered around ~700bp. This is the length estimate I'll use for mol → mass conversions.

Protocol recommends 100fmol in 12μl EB for ≤ 1kb DNA.

Using NEB calculator, 100fmol of 700bp DNA is 43.1ng DNA

Library 4 is 36.6 ng/ml, so $\frac{43.1 \text{ng DNA}}{36.6 \text{ng/ml}} = 1.17 \mu\text{l Library 4}$

Actually, I'll need more diluted Library 4 later for wash/reload, so I'll just mix several loads - worth now.

$$\frac{43.1 \text{ng}}{12 \mu\text{l}} \rightarrow \frac{129.3 \text{ng DNA}}{36 \mu\text{l EB}} \quad \frac{129.3 \text{ng DNA}}{36.6 \text{ng/ml}} = \boxed{\begin{array}{l} 3.5 \mu\text{l Library 4} \\ + \\ 32.5 \mu\text{l EB} \end{array}}$$

Loaded MinION flow cell according to protocol, and began run.

MinION Flow Cell

SN

B024226802

Lot

11004308

ID

FBD 09922

1428 pores on check

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* We noticed after the first 10 minutes of the run that the pore occupancy was low (roughly 50% of available pores were sequencing).

Dan suggested this may be due to loading too little DNA, so we decided to try spiking in more library at a higher concentration, without washing the flow cell.

I made a dilution of Library 4 at double the recommended concentration:

Recommended: 100 fmol = 43.1 ng

[Doubled]: 200 fmol = 86.2 ng

so need 86.2 ng in 12 μl EB.

<u>86.2 ng DNA</u>	=	<u>2.4 μl Library 4</u>
<u>36.6 ng/ml</u>		
+ add (12 - 2.4) =		<u>9.6 μl EB</u>

Prepared the second load as instructed in protocol (SB, LIB, and higher-concentration Library 4 dilution).

Paused MiSeq run @ ~17 minutes and added full 75 μl of second library to the SpotON port, dropwise (opened priming port first, to create required negative pressure). Library was "sucked" into cell as desired.

Un-paused run, and it resumed as normal. Pore occupancy steadily increased.

By minute 30, ~75% of available pores were sequencing - the spike of second library was successful!

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At 22.5 hrs into the Group 1 Library 4 MinION run, there was noticeable plateau of cumulative output, and pore loss (from 1383 active pores, dropped to 474 active pores)

At 22.5 hrs, paused the run, and washed the flow cell using Flow Cell Wash kit, according to the Oxford Nanopore Technologies Flow Cell Wash Kit protocol (document version OFC-9120-v1-revS-25jul2025)

After wash, reloaded the MinION flow cell (FBD09922) with prepared library. I again made the library with 2x the recommended concentration (200 fmol DNA, instead of 100 fmol). After reloading, resumed the run.

After resuming the run, the flow cell was back to 845 pores (though, on pore scan, 293 of those were "Reserved", so I'm not sure how many of the recovered pores will actually be sequencing).

Before wash, pore activity showed:

15.6% of pores sequencing, ~~+/-~~ 15.7% available

so 30% of pores still live, and ~50% pore occupancy of those available

After wash/reload, pore activity showed:

30.8% sequencing, 20.2% available

so 51% of pores still live, and ~40% pore occupancy of those available

Notes:

- Barcodes are unbalanced (#12: 1.3M, #13: 1.5M, #14: 2.5M), so I messed up barcode ligation ?? Maybe not fully mixed? Maybe sample concentrations are inaccurate?

- $\frac{1}{2}$ life of pores is ~11 hr. @ 0 hr, 1383 pores. @ 11 hr, 704 pores

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At 1 day, 10 hours in to run (~12hr after first wash), performed a second wash on the minION flow cell.

First, needed to get a library of 200fmol. I previously diluted a portion of Library 4 to 100fmol / 12μL, in a total of 36μL. I used 12μL of this for the first load, so I now have 24μL of diluted library. I also still have some undiluted Library 4. I need to add some undiluted Library 4 to my dilution to raise the concentration to 200fmol (since I observed higher pore occupancy with more input DNA).

Calculate how many μL of undiluted Library 4 to add:

$$A = \text{conc. of Library 4} = 36.6 \frac{\text{ng}}{\mu\text{L}}$$

$$B = \text{conc. of dilution} = \frac{100 \text{ fmol}}{12} = \frac{43.1 \text{ ng}}{12 \mu\text{L}} = 3.58 \frac{\text{ng}}{\mu\text{L}}$$

X = volume of Library 4

V = volume of dilution = 24 μL

$$\frac{\text{cum. DNA ng}}{(\text{BV} + A \cdot X)} = 2B \Rightarrow X(A - 2B) = BV \Rightarrow X = \frac{BV}{A - 2B}$$

$$X = \frac{BV}{A - 2B} = \frac{(3.58 \frac{\text{ng}}{\mu\text{L}})(24 \mu\text{L})}{(36.6 \frac{\text{ng}}{\mu\text{L}}) - 2 * (3.58 \frac{\text{ng}}{\mu\text{L}})} = \frac{85.92 \text{ ng}}{29.44 \frac{\text{ng}}{\mu\text{L}}} = 2.92 \mu\text{L}$$

Added 2.92 μL of undiluted Library 4 to the minION dilution to bring concentration up to $\frac{85.92 \text{ ng}}{12 \mu\text{L}} = 200 \text{ fmol} / 12 \mu\text{L}$. This is twice the concentration recommended for minION loading.

Then used this new dilution to wash and reload the Group 1 Library 4 flow cell.

541 pores (129 reserved, 412 available) before wash, 693 pores (255 reserved, 438 available).

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Not sure I'm going to get much more out of this flow cell, but I want to try one more wash and reload. Paused run @ 2 days in.

On this last wash/reload I want to try getting pore occupancy higher, so I'll try 3x the recommended concentration - 300 fmol in 12ul EB. That means I again need to increase the concentration of my minION dilution of library 4.

I want 300fmol = 129.3ng DNA in 12ul of EB

I currently have the following DNA:

MinION dilution: 7ul of $7.18 \frac{\text{ng}}{\mu\text{l}}$, so 50.26ng DNA

undiluted library 4: 3ul of 36.6ng/ μl , so 109.8ng DNA

To get 129.3ng DNA in 12ul EB: $(y + 36.6 \frac{\text{ng}}{\mu\text{l}}) + (7\mu\text{l} + 7.18 \frac{\text{ng}}{\mu\text{l}}) = 129.3 \frac{\text{ng}}{\mu\text{l}}$

7ul of minION dilution = 50.26ng DNA

2.16ul of Library 4 = 79.05ng DNA

2.84ul EB

12ul

129.31ng DNA

Combined the above quantities to obtain 300fmol in 12ul EB. Then washed and reloaded the flow cell with this, and resumed run.

Before wash, 420 pores (128 reserved, 412 & 292 available), after wash there were 475 pores (208 reserved, 267 available). So no appreciable difference.

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Project No. _____

Title Native Barcoding, Group 2 Library 1 Book No. _____

From Page No. _____

Library prep for Group 2 using Native Barcoding 96 v14 kit. This is first attempt at library prep for this set of individuals (Library 1).

Again will be including 3 specimens in a single sequencing round. This group will be 3 individuals from the selected subset of "mid-age" specimens:

Catalog #	Year	Conc. (ng/ml)	µl for 1000ng	Label / Barcode
51861	1960	94.1	10.63	#37 15
51892	1960	131	7.30	#47 16
51732	1960	157	6.37	#563 17

Performed prep as outlined in ONT Native Barcoding 96 V14 protocol for gDNA. (document version NBE-9171-v14-revS-02jule2025), with the following modifications:

- Excluded DCS
- Began with 1000ng input DNA from each sample
- Retained 3.75µl end-prepped DNA through to barcode ligation for each sample.

Qubit 1 (Barcodes ligated)

standard 1: 37.15

Standard 2: 16818.29

G2L1: 14.1ng/mlQubit 2 (Adapters ligated)

standard 1: 39.17

Standard 2: 17680.37

G2L1: 20.0ng/ml

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Ran Group 2 Library 1 on a Flongle flow cell to confirm successful library prep.

Performed hardware check on Flongle adapter. Then inserted Flongle flow cell and performed flow cell check.

Flongle flow cell:

ID: AYW871

pores on first pore check: 61 pores

pores on this pore check: 57 pores

According to TapeStation peaks, these samples are ~700bp in avg. fragment length. Thus, the recommended Flongle loading amount, 5-10 fmol is roughly equivalent to 2.1 ng - 4.3 ng.

I loaded the Flongle with 10 fmol (4.3 ng) of Group 2 Library 1 in 5uL EB.

G2LI concentration too high to accurately pipette 4.3 ng, so (conc. = 20ng/ml), so instead mixed:

$$\frac{4.3 \text{ ng DNA}}{5 \mu\text{L EB}} = \frac{20 \text{ ng DNA}}{23.3 \mu\text{L EB}}$$

Added 1 uL G2LI (20ng) to
 $(23.3 - 1) = 22.3 \mu\text{L EB}$

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Title Native Barcoding, Group 2 Library 2

Book No. _____

From Page No. _____

Dunney Group 1 library 4 MINION run. I had some issues with pore occupancy. I was originally concerned that, by increasing the quantity of DNA retained through library prep, I'd "overloaded" the adapter ligation step, resulting in a high portion of un-adapted DNA. I thought this unadapted DNA might then be lowering the effective library concentration, resulting in low occupancy.

After some discussion in a Nanopore community forum thread, with David Eccles, I don't think adapters are the issue. Instead, David suggested the problem may lay with the short reads "leaving the pores hanging," and taking too long for a given pore to "find" a new read to sequence. His recommendation was to spike in some longer control DNA. My timeline is too short to order any additional reagent, but I can use the DCS provided in the Native Barcoding Kit (~3.4kb).

As such, with this re-introduction of DCS to the library prep protocol, I need to re-do the Group 2 library.

Catalog #	Conc. (ng/ml)	µl for 1000ng	µl for 11ml	Barcode
51861	94.1	10.63	0.37	18
51892	137	7.30	3.7	19
51732	157	6.37	4.63	20

Followed Native Barcoding gDNA protocol, using DCS, with the following modifications:

- 1000ng input DNA from each sample
- retain 3.75µl DNA through end-prep

Qubit 1

Standard 1: 31.32

Standard 2: 11873.41

G2L2: 17.7 ng/mlQubit 2:

Standard 1: 32.99

Standard 2: 14756.63

G2L2: 24.2 ng/ml

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Checked Group 2 Library 2 on a Flonge Flow cell

Flonge flow cell

[ID] AYX067

pores on pore check: 79 pores

Based on TapeStation peaks, samples are ~700bp, so $10\text{fmol} = 4.31\text{ng}$

$$\frac{4.31\text{ng}}{5\text{ml}} \times 5 \Rightarrow \frac{21.55\text{ng}}{25\text{ml}} = \frac{21.55\text{ng}}{24.2\text{ml}} = 0.89\text{ml}$$

so mixed 1ml G2L2 with 24ml EB (total 25ml)

Loaded Flonge with 5ml of this

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Title MinION run, Group 2 library 2 Book No. _____

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Run Group 2, Library 2 on a MinION flow cell.

Following MinION loading protocol found in the Native Barcoding 96, v14 gDNA protocol.

Load flow cell with 200 fmol DNA, after seeing higher pore occupancy with this amount (in comparison to 100 fmol).

Need 200 fmol DNA in 12 μl EB; G2L2 is 24.2 ng/μl, 200 fmol of 700 bp is 86.24 ng.

$$\frac{86.24 \text{ ng}}{24.2 \text{ ng/μl}} = 3.5 \text{ μl G2L2} \Rightarrow \text{use } 3.5 \text{ μl G2L2}$$

So mix 3.5 μl G2L2 with 8.5 μl EB.

Flow cell:

ID FBD39370

1482 pores on pore check

1400 pores after priming and loading

Started run @ 10:26pm 8/24/25. Based on MinION G1L4, expect half life of pores to be ~12 hrs, so I'll be back in office to minnow at around that time. If at $\frac{1}{2}$ pore loss by then, will likely wash.

@ 20 min into run, looking good! Bars are all stable, with 49.7% sequencing; 13.4% available (so ~80% of available pores are sequencing!).

Notes on run progress: washes will be added on next page =>

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Title MinION Run Group 2 Library 2 - NOTES

From Page No. _____

Washes

Wash #1 @ 13hr into run. Re-loaded with 200fmol G2L2. 224 pores → 867 pores

Wash #2 @ 23hr in. Re-loaded with 200fmol G2L2. 238 pores → 563 pores

Wash #3 @ 1 day 12hr in. Actually had to fully stop run to allow for a mandatory computer update (took ~5min), then re-started "new run", confirmed flow cell was still sequencing, washed, reloaded, and let run for remaining 3hr. 212 pores → 410 pores. Out of G2L2, so could only load ~100fmol (2μl G2L2 in 10μl EB).

Notes

Very poor run, lost pores *very* quickly. Weird pattern with the "channel disabled" pore state will need to look into what this state actually means.

After some research, seems I may have a defective "wildflower" flow cell will contact Nanopore to see about a replacement.

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Title Sequencing Reagents

Book No. _____

From Page No. _____

I realized I'm already half-through the priming/loading reagents included in the Native Barcoding v16 V14 kit (SB, LIB, FCF, FCT). This is because I'm using multiple rxns of priming/loading reagents for each MINION flow cell run, due to wash/reloads and Flongle runs.

MinIO Nanopore offers "sequencing expansions" with these reagents, so I'll need to purchase one (12rxns would allow for additional 3 MINION runs, assuming 4 total loads for each cell).

I'll also adjust the total volume of priming mix I make during each loading, since the provided protocol makes an excess.

As a reminder, I only need 1000 μl for priming a MINION flow cell.

Priming Mix	1X	0.85 X
Flow Cell Flush (FCF)	170 μl at 1162.5 μl	988.125 μl
BSA 20mg/ml	12.5 μl	10.625 μl
Flow Cell Tether (FCT)	30 μl	25.5 μl
	1205 μl	1024.25 μl

Will begin making an 0.85X mix of priming mix every time I load.

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Nanopore requests absorbance profiles from Group 2 extractions (though yes, I realized I should have already performed this QC step).

Loaded 3 blanks of 3ml Zymo Elution Buffer. CV % < 10%, so accepted.

Loaded HUB 51861, 51732, and 51892 (for each, 1μl extraction + 2ml Zymo Elution Buffer)

Full results saved in OneDrive, folders titled "epoch-group2*.xlsx" ^{files}

	260/280	260/230	ng/μl
51861	1.866	2.256	38.433
51732	1.845	2.169	62.533
51892	1.808	2.013	70.33

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Witnessed & Understood by me,

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08/27/25

Zymo
Title Concentrate 50368, 42137

Project No. _____

Book No. _____

From Page No. _____

Two of my 1800s 5ml extractions that I want to include in Group 3 are below the concentration needed to get 100ng in 1ml. I'm going to concentrate them to a lower volume / higher concentration, using the Zymo Genomic DNA Clean Concentrator Kit (see pg. 7 of this lab notebook for full protocol).

Catalog #	Tube	conc(ng/ml)	vol.(ml)	des.conc.	des.vol(ml)
50368	C7	68.3	25	91	18.76 ≈ 19
42137	B2	46.3	26	91	13.23 ≈ 14

$$\text{desired concentration (des.conc.)} = 100\text{ng} / 1\text{ml} = 91\text{ng/ml}$$

$$\text{desired volume (des.vol)} = (\text{conc.} + \text{vol}) / \text{des.conc.}$$

Eluted each sample in the "des.vol" of Zymo EB (heated to 60°C)

Made a 5ml 5x dilution of each (1ml DNA + 4ml Zymo EB)

Then quantified with Qubit (1x dsDNA HS protocol 1kit)

S1: 34.04

S2: 13568.74

50368: 7.40 ng/ml $\xrightarrow{5\times}$ 37 ng/ml

42137: 5.39 ng/ml $\xrightarrow{5\times}$ 26.95 ng/ml

and QC'd with Epoch spectrophotometer

260/280 260/230

50368: 1.917 23609

42137: 1.9 3.018

So.... Zymo did not concentrate my samples (must have lost too much during the clean). I actually cut concentration by quite a bit. I'll just have to work with what I got. :-)

I also might choose to fully exclude 42137 due to the high 260/230.

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First whack at library prep for a set of 3 samples from my oldest time point (Group 3). My attempt to concentrate 2 of these extractions failed pretty spectacularly, but I'll just have to work with what I've got. Selected samples with highest extraction concentrations (determined [dsDNA]) using either TapeStation or Qubit).

Determined maximum fmol DNA contained in 11 μl for each selected sample, to use equi-molar input from all during library prep. I hope this will help with the slightly "unbalanced" levels of sequenced barcodes in past preps.

<u>Catalog #</u>	<u>Tube #</u>	<u>[dsDNA] (ng/μl)</u>	<u>length (bp)</u>	<u>max.ng. 11μl</u>	<u>max.fmol. 11μl</u>
14399	B1	100	444	1100	4022
50368	C7	37	529	407	1248
50603	A5	42.4	357	466.4	2121

So 50368 is the limiting extraction (unsurprisingly) to maintaining equimolar inputs. I want to use 1250 fmol DNA from each sample for library prep, in 11 μl.

<u>Catalog #</u>	<u>Barcode</u>	<u>ng. for. 1250fmol</u>	<u>μl. for. 1250fmol</u>	<u>μl. to make. 11μl</u>
14399	21	341.9 ng	3.5 μl	7.5 μl
50368	22	407.3 ng	11 μl	0 μl
50603	23	274.9 ng	6.5 μl	4.5 μl

Prepared library according to Native Barcoding v6 V14 gDNA protocol, using above equimolar inputs for each sample, and using DCS, and retaining 3.75 μl end-prepped DNA through to barcode-ligation. I also cleaned used a 1X bead-to-sample ratio + also during both Ampure bead cleans, to avoid significant loss of my heavily-fragmented DNA.

I also bead cleaned the leftover end-prepped DNA, using 1X Ampure beads and eluting in 5 μl nuclease-free H₂O, for potential future use. Stored @ 4°C.

Standard 1: 34.61

G3L1 Barcoded: 3.24 ng/μl

Qubited both rounds together: Standard 2: 15943.60

G3L1: 9.74 ng/μl

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Witnessed & Understood by me,

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Invented by

Date

Recorded by

08/30/25

Title Group 3. Library 1 Flongle Run

Book No. _____

From Page No. _____

Ran ~~Group 1~~ Group 3 library, 1 on a Flongle to confirm successful library prep.

To get 10 fmol DNA in 5 μL EB:

for avg. length, $9.74 \text{ ng}/\mu\text{L} = 35.69 \text{ fmol}/\mu\text{L}$.

$\frac{10 \text{ fmol}}{5 \mu\text{L EB}} \xrightarrow{1 \mu\text{L DNA}} \frac{35.69 \text{ fmol}}{17.85 \mu\text{L EB}}$ 86 mix 1 μL G3LI with $(17.85 - 1) = 16.85 \mu\text{L EB}$. Then use 5 μL

Flongle Flow cell

ID AYL126

80 pores on original flow cell check (08/13/25)

74 pores on this flow cell check

↓

45 pores after loading and beginning run :

Looks like the library (or at least the white UB bars) are sort of "stuck" on the first half of the pores, closest to the loading port.

After ~1 hr of running, I notice that the barcodes are very uneven - very few reads from Barcode 22, compared to 21 & 23. Could mean there was a problem during barcode ligation, or that extraction quantification/length estimate was inaccurate, leading to unequal inputs.

Barcode 22 is low enough that I'm not happy with running this library on a MinION (would effectively be 2 samples, not 3).

Unfortunately, I'm out of DNA for one of these extractions so I'll need to reextract and preprocess.

In the meantime, since re-extraction/processing will take a few days and I only have 2 weeks left, I'll prep and run another iteration of the low-yield Group 2.

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08/31/25

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My first MinION run of Group 2 (Group 2 Library 3) had very low output, so I'm going to do another prep and run of the same samples, to try to get more data.

Catalog #	tube	[dsDNA] (ng/ml)	length	Vol. remaining. ul	fmol. remaining
S1861	P6	94.1	647	6 ul	1417
S1892	A7	137	711	9.5 ul	2972
S1732	A8	157	724	13 ul	4576

During G2L2 run, the barcodes were not equally present. This could be due to slightly different lengths of each extraction, meaning wrong DNA wouldn't be completely equimolar.

S1861 is the limiting extraction, with the lowest remaining fmol of DNA, so will equalize to that. Want 1400 fmol DNA from each extraction to use in library prep. Should be in 11ul total volume, with made up with nuclease-free water.

Catalog #	Vol. 1400 fmol	ml. for. 11ml	Barcode
S1861	6 ml	5ml	24
S1892	4.5 ml	6.5ml	25
S1732	4.0 ml	7ml	26

Haven't been seeing expect DCS peak @ ~3.6 kb, so mixed and used new DCS dilution. Used 1X Ampure bead-to-sample ratio in both bead cleans.

Qubits:

S1: 36.01

S2: 15671.74

G2L3+barcodes: 7.50 ng/ml

G2L3+adapter: 15.3 ng/ml

With 15.3 ng/ml in 14ml, and an avg. length of ~700bp, I have ~500 fmol total library.

I can roughly do a 200 fmol load + 3 100 fmol wash/reloads, or a 200 fmol load + 200 fmol wash/reload + 100 fmol wash/reload.

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Title Pore Check for Replacement Flongle Flow Cells Book No. _____

From Page No. _____

Received 4 replacements for below-warranty Flongle flow cells (see page 34 of this lab notebook) sometime last week (8/25/25 - 8/29/25). Will run flow cell health checks on all replacement cells to confirm they're above warranty (>50 pores).

<u>Lot</u>	<u>SN</u>	<u>ID</u>	<u># pores</u>
33001548	B028107472	AZG145	62
33001548	B028107456	AXT258	61
33001548	B028107437	AXT655	82
33001548	B028107440	AXT657	87

All 4 replacement cells passed pore check!

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Title Re-extraction of 1800s samples

From Page No. _____

After using much of my 1800s DNA in a partially unsuccessful library prep (see page 57 of this lab notebook), I want extra for additional preps. I need to extract more DNA for 5 ~~sa~~ specimens:

<u>Catalog #</u>	<u>Collection Date</u>
50603	1912
19054	1898
42137	1897
14399	1886
50348	1880

Subsampled, as detailed on page 4 of this lab notebook, then began tissue digestion, as detailed on page 5.

Began digestion @ 7:00pm 09/01/25, will run for ~48 hr.

Notes:

Power outage interrupted digestion incubation. Incubator stopped @ ~9pm, 09/02/25, and I restarted it ~7am, 09/03/25, so digestions were sitting at room temp for ~10hr.

Spiked in 20µl proteK to each tube, will let digestions sit on shaking incubator for another 12-24 hr.

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Title Flongle Run, Group 2 Library 3

Book No. _____

From Page No. _____

Running Group 2 Library 3 on a Flongle to confirm successful library prep.

Need 10 fmol DNA in 5 μl EB. Avg. length is ~700 bp, and G2L3 library is 15.3 ng/ml

$$15.3 \text{ ng/ml} = 35.48 \text{ fmol/ml}$$

$$\begin{array}{cccc} \underline{10 \text{ fmol}} & \xrightarrow{1 \mu\text{l DNA}} & \underline{35.48 \text{ fmol}} & \text{so mix } \underline{1 \mu\text{l G2L3}} \text{ with} \\ \underline{5 \mu\text{l EB}} & & \underline{17.74 \mu\text{l EB}} & (17.74 - 1) = \underline{16.74 \mu\text{l EB}}. \end{array}$$

Flongle flow cell

ID AYK9163

89 pores on 8/13/25 check

86 pores on today's pore check

66 pores after loading

We noticed the Flongle flow cells lose a lot of pores during loading, and community posts suggest this is a) common, and b) related to the ONT loading protocol being too harsh. This time I tried using the community-developed gentle, negative-pressure loading protocol for Flongles. Still lost almost 25% of pores, but better than last time (G3L1 Flongle), when I lost almost 50% of pores (74 → 45).

I was also extra careful to seal the cover tape around all ports after loading.

Notes:

Barcode 24 is low, but that sample had a high proportion of reads in the G2L2 MinION run, so maybe this will help offset. Think it's ok to run on a MinION.

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Ran Group 2 Library 3 on a MinION flow cell. This is the second attempt to sequence these samples on a MinION flow cell.

Group 2 Library 3 has ~700 bp avg length (based on TapeStation), and a dsDNA concentration of 15.3 ng/ml = 35.5 fmol/ml.

Want to load 200 fmol in 12 μl of EB : $200 \text{ fmol} / 35.5 \text{ fmol/ml} = 5.6 \mu\text{l}$.

I want to conserve library for the additional wash/reloads so will use 5 μl.

So mixed 5 μl GLL3 with 7 μl EB

~~Flow Cell~~

ID FBD3b639b

1528 pores on 8/14/25 pore check

1532 pores on today's pore check

After removing Flongle adapter, ran hardware check before inserting flow cell and running new pore health check.

Will start sequencing run *before priming or loading*, to check for flow cell issues not captured by the # of pores.

OK, lots of issues:

- 1st attempt to run unloaded FBD3b639b errored out w/in 1 min ("run error")
- 2nd attempt also errored out immediately.
- Unplugged MinION, restarted computer, plugged back in and ran hardware check, then tried again to start a sequencing run w/ unloaded FBD3b639b. Again errored out immediately.
- Thought might be flow cell issue, so got new MinION flow cell: FBD42232
- Attempted run of unloaded FBD42232, errored out.
- Thought might be related to trying to run an unloaded cell, so primed and loaded FBD42232 as normal. Sequencing run again errored out.
- Contacted Nanopore Tech Support. In the meantime, transferred the loaded FBD42232 cell to fridge (4°C)

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Title _____ Book No. _____

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- ONT support could only recommend updating the MinION Software, but that requires admin access. Matt is out today, so can't update.
- On Katie's suggestion, tried using the older MinION sequencer, MK1B.
- Plugged MK1B in, ran successful hardware check.
- ~~Star~~ Inserted loaded FBD42232 (was sitting upright in packaging @ 4°C for 30min - 1hr)
- Sequencing run didn't error out! Performed first pore scan and began sequencing as normal

Note, did NOT end up using FBD36396, stored it in fridge as normal.

Flow cell actually used

ID FBD42232

1519 pores on 8/14/25 pore check

1372 pores on sequencing start

{RUN ON MK1B SEQUENCER}

Washes

[Wash #1] Planned to do this @ ~7hr into run, but museum power outage on the night of 09/02/25 prevented that. Instead, washed the next morning, ~16.5 hr. Loaded 4 μl G2L3 (~150 fmol), went from 246 pores to 754 pores.

Power outage didn't impact sequencing run, sequencer + PC is connected to back-up power.

[Wash #2] Paused run @ ~~21.5 hr~~ today, 21.5 hr for 1 day, 1 hr for wash #2. After wash 2, primed ; loaded with 4 μl G2L3 (~150 fmol). After wash #2, all pores saturated and unable to sequence ;. Not sure what could have caused this, since I made no changes to wash protocol. Detailed channel states panel showed field of uniform light blue, pore scan was uniform gray ("saturated"). Tried stopping run, removing cell,

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[Wash #2 (cont)]

reinserting, and starting new run. After removing + reinserting, showed 4 active pores, 84 zero, 1763 saturated,? 190 unavailable.

Theory: Before wash #2 (but after pausing run), I removed the flow cell from the MinION to take a picture of the bottom of the flow cell (requested by ONT support). I didn't think this would have any effect on the cell, since I kept all ports sealed, and cells are regularly inverted during shipping/handling. However, during sequencing, the heating of the flow cell + electrical current causes small bubbles to form under the pore cover. These don't normally touch the pores or affect sequencing, but maybe tilting the cell to remove it/reinsert it made the bubbles touch the pores, killing them? Not sure if bubble damage would cause a "saturated" signal though...

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Title Qiagen Extraction : Zymo Clean - 1800s re-extraction Book No. _____

From Page No. _____

re-

Finished extraction of the 5 1800s specimens using Qiagen DNEasy kit.
 Digestions began 09/01/25 7pm, pulled digestions 09/04/25 ~7am.
 See digestion details page 60 of this lab notebook.

Followed Qiagen DNEasy extraction protocol found on page 60 of this lab notebook.

Samples: 50368, 50603, 19054, 14399, 42137.

Notes: eluted in 100µl Qiagen AE. 50368 has lots of big sclerites.

After Qiagen extraction, immediately cleaned extractions using Zymo Genomic DNA Clean & Concentrate Kit. Followed protocol included on page 7 of this lab notebook.

Eluted in 50µl of 60°C Zymo EB.

Notes: 42137 had an almost black Qiagen extraction, but almost clear after Zymo!

Qubit of Zymo-cleaned extractions!

Used dsDNA 1X HS kit, followed protocol found on page 8 of this lab notebook, using 1µl of sample input.

Standard #1: 35.68

50368: 84.0 ng/µl

Standard #2: 16957.03

50603: 9.72 ng/µl

19054: 1.58 ng/µl

14399: 30.8 ng/µl

42137: 54.0 ng/µl

Next step would normally be a gel, but I'm short on time and want to conserve DNA.

Since I already know the KAPA bead-to-sample ratio I want to use, I'll go straight to bead clean / size-selection.

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Will clean ; size-select my 1800s re-extractions to remove the shortest fragments.
 Following protocol found on page 22-23 of this lab notebook.

- Notes:
- Mixed fresh 80% EtOH. Need $400\text{mL} \times 5\text{ samples} = 2000\text{mL}$, so mix 2200mL 80% EtOH $\Rightarrow 1760\text{mL}$ EtOH + 440mL nuc-free H₂O
 - Currently have 49mL of each sample (eluted Zymo in 50mL, used 1mL for Qubit)
 - Want 0.7x bead-to-sample ratio (should remove ~350 bp).
 Bead volume is $0.7 \times 49\text{mL} = 34.3\text{mL}$ KAPA Pure beads to each sample (50368, 50403, 19051, 14399, 42137)
 - Incubated on beads (step 2.4) for 15min to maximize DNA retention).
 - Eluted in 20mL to help concentrate (eluted in Zymo EB).

Qubit of cleaved DNA

Based on post-Zymo Qubits, expect 50368 : 42137 may have 20mL concentrations above the qubit threshold of 100 ng/mL, so will dilute to $\frac{1}{2}$ concentration (2mL sample + 2mL Zymo EB)

Performed Qubit quantification as normal (protocol on page 8 of this notebook)

Standard #1: 37.43

Standard #2: 16705.55

50368 (dil.): 67.6 ng/mL $\xrightarrow{\times 2}$ 135.2 ng/mL

50403 : 10.3 ng/mL

19051 : 0.520 ng/mL

14399 : 42.8 ng/mL

42137 (dil.): 27.2 ng/mL $\xrightarrow{\times 2}$ 54.4 ng/mL

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Title TapeStation of 1800s re-extractions

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Using TapeStation to evaluate size distribution + integrity of 1800s re-extractions. will also run some old extractions to check whether they've degraded since first being extracted.

This time since I expect my 1800s samples to have essentially no Hmw DNA, will use an HS DS5000 tape instead of a gDNA tape.

HS DS5000 tape requires 2μl of DNA sample. I want to conserve my extractions, and the HS DS5000 tape can handle very low concentrations, so I'll be using a 2x dilution for all samples. (1μl extraction + 1μl Zymo EB)

Followed HS DS5000 protocol found on the next page (pg. 68).

TapeStation layout

	1	2
A	Ladder	1740343
B	50368 (new)	51892
C	50603 (new)	51732
D	19054 (new)	14399
E	14399 (new)	50368
F	42137 (new)	50603
G	1606826	
H	1740336	

Will include full Tapestation report in digital lab notebook entry.

Notes: Looks like 50368 (new), 42137 (new), and 14399 (new) are by far the best 1800s re-extractions to use for sequencing.

Don't see an immediately obvious degradation issue comparing new to old extractions of 50368, 50603; 14399, will need to compare to OG tapestation.

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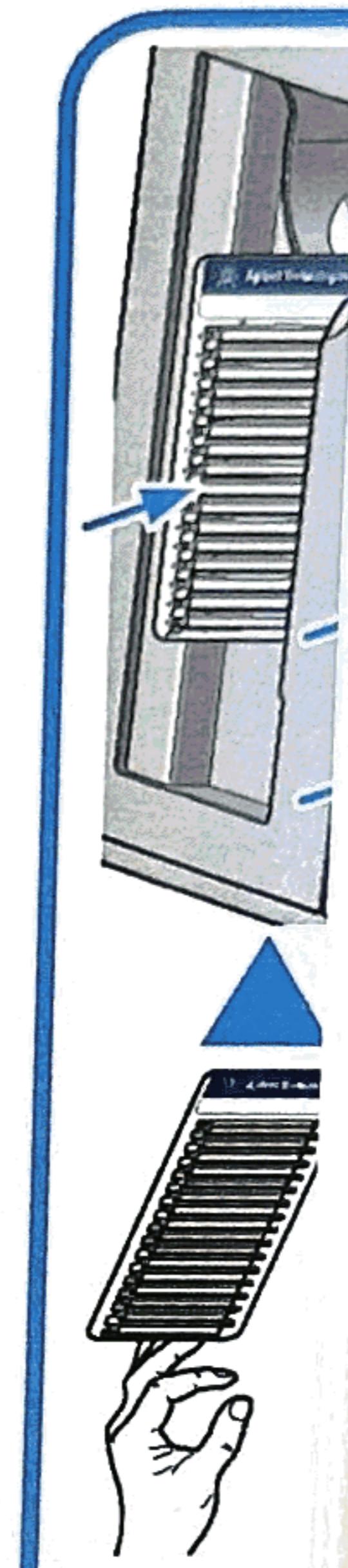
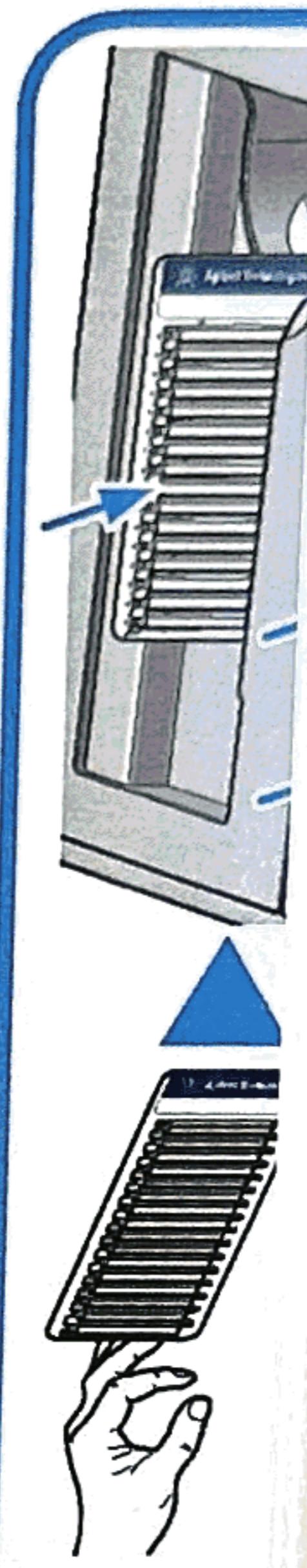
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High Sensitivity D5000 ScreenTape Assay for TapeStation Systems**High Sensitivity D5000 ScreenTape Assay Operating Procedure**

- 1 Allow High Sensitivity D5000 Reagents to equilibrate at room temperature for 30 minutes.
- 2 Launch the Agilent TapeStation Controller software.
- 3 Flick the High Sensitivity D5000 ScreenTape device and insert it into the ScreenTape nest of the TapeStation instrument.
- 4 Select required sample positions in the TapeStation Controller software.
- 5 The required consumables (tips, further ScreenTape devices) are displayed in the TapeStation Controller software.
- 6 Vortex reagents and samples. Spin down before use.
- 7 Prepare ladder.
 - For 1 ScreenTape device: pipette 2 μ L High Sensitivity D5000 Sample Buffer (●) and 2 μ L High Sensitivity D5000 Ladder (●) at position A1 in a tube strip.
 - For 2 ScreenTape devices: pipette 4 μ L High Sensitivity D5000 Sample Buffer (●) and 4 μ L High Sensitivity D5000 Ladder (●) at position A1 in a tube strip.
 - For more than 2 ScreenTape devices¹: pipette 15 μ L High Sensitivity D5000 Sample Buffer (●) and 15 μ L High Sensitivity D5000 Ladder (●) at position A1 in a tube strip.
- 8 For each sample, pipette 2 μ L High Sensitivity D5000 Sample Buffer (●) and 2 μ L DNA sample in a tube strip or 96-well sample plate¹.
- 9 Apply caps to tube strips and/or foil seals to 96-well sample plates.
- 10 Mix liquids using the IKA MS3 vortexer at 2000 rpm for 1 min.
- 11 Spin down samples and ladder for 1 min.

**High Sensitivity
D5000**



Many want to use sample dilution (in DNA + in H_2O)
to conserve sample. D5000 can handle low concentrations.

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Title Native Barcoding Prep, Group 4, Library 1 Book No. _____

From Page No. _____

library prep for Group 4, which is a slightly updated selection of 1800s samples.

From Qubit + TapeStation output for 1800s re-extractions, 50368, 42137, ?
14399 have highest concentrations and decent length distributions.

Catalog #	Qubit conc. ng/ml	Tapestation avg. length	conc. fmol/ml
50368	135.2 ng/ml	681 bp	322.3 fmol/ml
42137	54.4 ng/ml	555 bp	159.1 fmol/ml
14399	42.8 ng/ml	591 bp	117.6 fmol/ml

14399 has lowest concentration, so its the limiter in taking equimolar input from each extraction.

$$1 \mu\text{l of 14399} = 1 \mu\text{l} \times 117.6 \text{ fmol/ml} = 1293.6 \text{ fmol} \approx 1300 \text{ fmol}$$

So I want to use 1300 fmol of input from all 3 samples:

$$50368 : 1300 \text{ fmol} / 322.3 \text{ fmol/ml} = 4.031 \approx 4 \mu\text{l}$$

$$42137 : 1300 \text{ fmol} / 159.1 \text{ fmol/ml} = 8.171 \approx 8.2 \mu\text{l}$$

So for library prep:

Catalog #	μl for 1300 fmol	rem. volumetric	Barcode
50368	4	7	27
42137	8.2	2.8	28
14399	11	0	29

Followed ONT Native Barcoding kit v1.4 gDNA protocol, with the following modifications:

- changed input quantity for each sample (see above)
- Did 3 full barcode ligation runs, each with 375 μl of end-prepped DNA, then pooled.
- Low on SFB, so washed beads during barcode clean with fresh 80% ethanol

Qubit: Standard 1: 38.87

G4L1 barcodes: 34.0 ng/ml

Standard 2: 16411.03

G4L1 : 59.2 ng/ml

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Ran G4LI on Flongle flow cell to confirm successful library prep. This Following gentle, negative-pressure loading protocol created by Nanopore community members (print-out in back of notebooks).

I also want to try loading more DNA on the Flongle, as I've seen recommended in community forum posts (and in the gentle Flongle loading protocol), so instead of the recommended 10fmol DNA, I'll prep 30fmol in 5ml EB.

G4LI is 59.2ng/ml, with an average length of 609bp, so concentration is 157.8 fmol/ml

30 fmol = 157.8 fmol So mix 1ml G4LI with (26.3-1) - 25.3ml
5ml EB 26.3ml EB EB, then use 5ml for loading.

Flongle flow cell

ID AXT657

87 pores on 09/01/25 check

62 pores after loading

Still running on the MK1B sequencer, after error issues with the MKID (see pages 42-43)

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Title MinION run, Group 4 library 1 Book No. _____

From Page No. _____

Run Group 4 Library 1 on a MinION flow cell for sequencing.

I have a lot of DNA in this library, so I want to try loading 300 fmol DNA to try to increase pore occupancy more (it's been chronically low-ish, ~75%).

So I want 300 fmol DNA in 12 μl EB:

G4L1 is 59.2 ng/ml = 157.8 fmol/ml, so let's do 2 μl G4L1 + 10 μl EB.

Flow cell

ID FBD08455

1652 pores on 08/14/25 check

1645 pores on 09/05/25 check

Trying the MK1D again after the minknow software update.

- Hardware check passed
- MinION flow cell health check completed
- When setting up sequencing run, turned off pore reserve function, to front-load data acquisition.
- After update, there are more base modified basecalling options. Selected all options (6mC, 5mC, 3hmC in all contexts)
- Run sequencing on *un-primed, un-loaded* flow cell for Lemin to evaluate pore health.
- really weird run stats... only 316 pores available on pore scan, almost everything shows "no pore" ..
- Paused to prime + load.
- Okay, after priming + loading, resumed run + initiated pore scan - 1430 pores.

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G4U minION notes, continued

- Seeing some "wild flower"ing in the channel states panel. Barely on
- Occupancy is looking good in first ~15min! e.g. 47% sequencing, 4.8% available, so $(47 / 47 + 4.8) = 90.1\%$! That's my highest proportion yet of available pores that are actively sequencing.
- There's a notably higher level of adapter than in previous runs - ~7%, compared to previous 1% - 2% in minION runs. This might be expected though, since library is shorter than previous libraries, which means the proportion of read:adapter:adapter:read will be higher.

Wash #1 @ 4.5 hr in, after ~ $\frac{2}{3}$ pore loss (510 pores at the 3.5hr scan). Very fast pore loss, mostly to the "unavailable" category. Re-loaded w/ 300fmol again. 783 pores after wash #1

Wash #2 @ 19.5 hr in, 84 pores remaining. Re-loaded with 300fmol, and there are 381 pores after wash/reload.

Wash #3 @ 2 days 1hr in, 40 pores remaining. Re-loaded with 300fmol.

Run errored out when I tried to resume, so I had to restart the Minknow software and start a new run. 185 pores after run started.

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Title Native Barcoding, Group 4 Library 2 ? DCS Book No. _____

From Page No. _____

Library prep for Group 4 Library 2, since G4L1 had low MinION output. ONT support has also requested 1 try prepping and running a DNA control sample library for 1-2hr to check flow cell function, so I'll be doing 2 preps.

Following Native Barcoding v14 gdNA protocol with the following modifications:

- Use below input quantities
- For all samples, did 3 full barcode ligation rxns before pooling
- Washed beads during barcode clean with 80% ethanol. (fresh)

DCS Library

Use full 12μl of diluted DCS as input. DCS is 10ng/μl so diluted DCS is 3.3ng/μl. Will use Barcode 33.

G4L2

Catalog #	[dsDNA]	volume left	length	ng left	fmoles left
50368	135.2ng/μl	15μl	681	2028ng	4834 fmol
42137	54.4 ng/μl	9.5 μl	555	516.8ng	1512 fmol
14399	42.8 ng/μl	6.5 μl	591	278.2ng	764.2 fmol

SD limiting extraction is 14399. Will use ~800fmol from all extractions.

Catalog #	ng for 800fmol	μl for 800fmol	μl for 11ml	Barcode
50368	335.6 ng	~2.5 μl	8.5	30
42137	273.5 ng	~5 μl	6	31
14399	291.2 ng	~6.8 μl or 5 μl	4.5 μl	32

End-repair Master Mix (5X)

Qubits

FFPE Buffer	4.375 μl	Standard 1: 48.12
Ultra II Buffer	4.375 μl	Standard 2: 15478.64
Ultra II Mix	3.75 μl	DCS barcode: 0.528 ng/μl
FFPE Mix	2.5 μl	DCS library: 0.776 ng/μl
		G4L2 barcode: 11.1 ng/μl
		G4L2 library: 22.0 ng/μl

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Run Flongle flow cell with G4L2 to confirm library prep.

Again, will use 30 fmol input DNA and gentle, negative pressure loading protocol
(print-out in back of lab notebook)

G4L2 has avg sample length of 609 bp, concentration of 22 ng/ml \Rightarrow
58.64 fmol/ml

30 fmol DNA	\Rightarrow	58.64 fmol	so mix 1ml G4L2 with (9.77 ml ⁻¹) =
5 ml EB		9.77 ml	8.77 ml EB, then use 5ml.

Flowcell

1D AXTGSS

82 pores on 09/01/25 check

84 pores on 09/08/25 check

50 pores after loading

Again, back to running on MK1D.

Notes:

- Good occupancy on start though, ~80% after 45min.
- Already seeing accumulation of "unavailable" pores

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Title MinION Run G4L2 t DCS and G4L2

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Book No. _____

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Sequencing G4L2 on a minION flow cell. Also running DCS library first to check flow cell function.

DCS Library

DCS library is 3.6 kb long and $0.776 \text{ ng}/\mu\text{l} = 0.35 \text{ fmol}/\mu\text{l}$. Will load full 12 μl of DCS library, though it will be way below the recommended loading quantity of 100 fmol.

Flow cell

ID FBD363916

1528 pores on 08/14/25 check

Note that this is the flow cell I originally tried during the "run error" problems (see pgs 62-63)

MK1D passed hardware check after removing Flongle adapter.

* Another "run error" on flow cell check!

- Removed? replaced flow cell? reran check - again, "run error"
- Tried switching back to MK1B sequencer
- MK1B hardware check passed
- Flow cell check passed! 1512 pores found

Started sequencing DCS with following settings:

- All modified basecalling
- No reserved pores

* Pore scans every 30 min

1423 pores after run start

Stopped DCS run after 2 hrs, after 4 pore scans. 1309 pores available on last pore scan.

WOW, this run had really strong output, almost no pore loss in first 2 hours, etc.

Clearly other runs are having sample issues

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G4L2 Library

Now using the same flow cell to sequence G4L2. (flow cell FBD 36396)

First washed flow cell using normal Flow Cell Wash protocol. Then loaded with 200 fmol in 12 μl EB:

G4L2 = 22 ng/μl, @ 600 bp avg. length, so 58.64 fmol/μl.

$$\frac{200 \text{ fmol}}{58.64 \text{ fmol/μl}} = 3.4 \text{ μl}$$

so mixed 3.4 μl G4L2 with
(12 - 3.4) = 8.6 μl EB.

After priming + loading, started run with the following settings:

- all modified basecalling
- pore check every 1.5 hrs
- no reserved pores

+ wash, prime, load

1124 pores available after run start, so the DCS run cost me ~300 pores ≈.

Notes

- Again, relatively high adapter content (5%, compared to 1-2% from earlier runs), though I again think this is at least partially due to the shorter reads.
- Keep in mind that this is the same flow cell used to run the DCS library, so any reads with the 33 barcode are library that wasn't removed during the flow cell wash.

Wash #1 @ 8hr 40min into the run, (though this is technically the 2nd wash of this flow cell). 180 pores left. Reloaded with 200 fmol of G4L2. 6009 pores after wash.

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Wash #2 @ 22 hr 15min. 130 pores left, Reloaded with 200 fmol.
 Had a channel blockage when trying to load the 200 μl priming mix,
 just before loading. Unable to add the 200 μl w/o overflowing the SPOTON
 port. Ended up fixing it by drawing up a bunch of library beads that
 were clogged up by the waste port. I was then able to load as normal.
 This means the second load of priming mix was actually only ~150 fmol
 ~150 μl, and the first load of 200 μl priming, was sitting on the pores for
 10 - 15 min, not the instructed 5 min.
463 pores available after the wash.

Wash #3 @ 1 day 8 hr in. 132 pores left. Reloaded with 1.5 μL G4L2 +
 5 μL Flonde G4L2 + 5 μL FEB (should be $1.5 + 58.64 \text{ fmol}/\mu\text{L} + 30 \text{ fmol} = 118 \text{ fmol}$)
332 pores after wash + reload.

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