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Picogram input multimodal sequencing (PiMmS)

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ABSTRACT

This protocol describes a method for isolating and PCR amplifying low-bias long read (8-16 kb) genomic shotgun libraries from small-bodied invertebrates e.g. for de novo genome assembly. It can deliver diverse libraries and contiguous assemblies (100s of kb to several Mb contig N50s are typical) from specimens containing only a few hundred picograms of DNA, such as single meiofaunal specimens (e.g. tardigrades, nematodes, turbellarians). At the same time, it allows users to synthesize and PCR amplify full-length cDNA libraries from the same specimen used for genomic DNA sequencing, permitting evidence-driven genome annotation. The method is compatible with both Oxford Nanopore and PacBio Hifi sequencing, and experienced users will usually be able to complete a batch of specimens within a single week of full-time labwork.

MATERIALS

Bespoke buffers

TNES: 400 mM NaCl, 50 mM Tris-HCl pH 8.0, 100 mM EDTA pH 8.0, 0.5% SDS

To make 10ml:
0.8 mL 5M NaCl
500 µL 1M Tris-HCl pH 8.0
2 mL 0.5M EDTA pH 8.0
500 µL 10% SDS
MB-grade water to 10 mL

Note - the SDS tends to precipitate out of solution when stored at room temperature. A gentle warming in a hot water bath (or beaker of microwave-heated water) should quickly allow dissolution.

2 M DTT aqueous solution

Dissolve 2 g DTT in MB-grade water to a final volume of 6.48 mL. Dispense into 20 µL aliquots in 0.2 mL tubes and store at -80 C until use; avoid repeated freeze-thaw cycles.

RNA elution buffer: 10 mM Tris-HCl pH 7.5, 0.1 mM EDTA (optional: **1:50** RNAse inhibitor)
To make 1mL:
0.2 µL 0.5M EDTA pH 8.0
10 µL 1M Tris-HCl pH 7.5

DNA elution buffer: 10 mM Tris-HCl pH 8.5, 0.1 mM EDTA
To make 1mL:
0.2 µL 0.5M EDTA pH 8.0
10 µL 1M Tris-HCl pH 8.5

Wash Buffer I: 20 mM Tris-HCl, pH 7.5, 500 mM LiCl, 0.1% LiDS, 1 mM EDTA, 5 mM DTT

Wash Buffer II: 20 mM Tris-HCl, pH 7.5, 500 mM LiCl, 1 mM EDTA

Low-Salt Buffer: 20 mM Tris-HCl, pH 7.5, 200 mM LiCl, 1 mM EDTA

Recommended Plastic Consumables

- DNA LoBind Tube 1.5ml **Eppendorf Catalog #022431021** or
- Ultra High Recovery Microcentrifuge Tubes 1.5 mL **StarLab Catalog #E1415-2600**

- 0.2 ml 8-Strip Non-Flex PCR Tubes Natural Individually Attached Flast Caps (Xtra-Clear) **StarLab Catalog #I1402-3700**
- MicroAmp™ Optical 96-Well Reaction Plate **Thermo Fisher Catalog #N8010560**

- MicroAmp® Optical Adhesive Film **Applied Biosystems Catalog #4360954**

Required Molecular Biology Consumables

- Molecular Biology Grade Water **Fisher Scientific Catalog #10154604**
- RNAlater **Sigma-aldrich Catalog #R0901-100ML**
- Proteinase K, Molecular Biology Grade - 2 ml **New England Biolabs Catalog #P8107S**
- Ethanol Absolute (200 Proof) Molecular Biology Grade Fisher BioReagents™ **Fisher Scientific Catalog # 16606002**
- NaCl (5 M), RNase-free **Thermo Fisher Catalog #AM9759**
- Oligo dT25 Magnetic Beads - 5 ml **New England Biolabs Catalog #S1419S**

or

- Dynabeads™ Oligo(dT)25 **ThermoFisher Scientific Catalog #61002**
- Yeast tRNA (10 mg/mL) **Thermo Fisher Scientific Catalog #AM7119**
- Deoxynucleotide (dNTP) Solution Mix **New England Biolabs Catalog # N044**
- RNase Inhibitor, Murine - 15,000 units **New England Biolabs Catalog #M0314L**

or

- SUPERaseIN RNase Inhibitor **Thermo Fisher Scientific Catalog #AM2696**
- Superscript IV **Thermo Fisher Scientific Catalog #18090050**
- ET SSB - 50 ug **New England Biolabs Catalog #M2401S**
- Betaine 5M Solution Molecular Biology Grade Ultrapure **ThermoFisher Scientific Catalog #J77507.VCR**
- ProNex® Size-Selective Purification System **Promega Catalog # NG2002**
- NEB USER® Enzyme **New England Biolabs Catalog #M5505S/L**
- 20X EvaGreen **Biotium Catalog #31000**
- Terra™ PCR Direct Polymerase Mix **Takara Bio Inc. Catalog #639271**
- Megaruptor 3 Shearing Kit **Diagenode Catalog #E07010003**
- NEBNext Ultra II Ligation Module - 96 rxns **New England Biolabs Catalog #E7595L**
- NEBNext FFPE DNA Repair v2 Module **New England Biolabs Catalog #E7360L**
- RNase A 10mg/ml, DNase and Protease-free **Thermo Scientific Catalog #EN0531**
- Qubit dsDNA HS (High sensitivity) Assays **Thermo Fisher Scientific Catalog #Q32851**
- Genomic DNA ScreenTape **Agilent Technologies Catalog #5067-5365**
- Genomic DNA Reagents **Agilent Technologies Catalog # 5067-5366** or

- gDNA 165kb Analysis Kit 275 Samples **Agilent Technologies Catalog #FP-1002-0275**
- D5000 ScreenTape **Agilent Technologies Catalog #5067-5588**
- D5000 Reagents **Agilent Technologies Catalog #5067-5589** or
- High Sensitivity D5000 ScreenTape **Agilent Technologies Catalog #5067-5592**
- High Sensitivity D5000 Reagents **Agilent Technologies Catalog #5067-5593**

or

- Bioanalyzer High Sensitivity DNA Kit **Agilent Technologies Catalog #5067-4626**
- 0.75% Agarose 1-10 kb size selections or 4-20kb High Pass S1. 10/pkg. **sage science Catalog #BLF7510**
- SMRTbell prep kit 3.0 **PacBio Catalog #102-182-700** or
- Ligation Sequencing Kit V14 **Oxford Nanopore Technologies Catalog #SQK-LSK114**

Custom Oligonucleotides (formatted as comma-delimited IDT bulk inputs)

NxtR2_dT24VN,GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTTTTTTTTTTTTTTTTTTTTTTTTVN,100nm,STD
NxtR1_TSO_4dU,/5BiosG/TCG/ideoxyU/CGGCAGCG/ideoxyU/CAGA/ideoxyU/G/ideoxyU/GTA/ideoxyU/AAGAGACArGrGrG,100nmR,STD
TruSeqR1_i7_[idxnm],/5Phos/CAAGCAGAAGACGGCATACGAGAT[idx]GTGACTGGAGTTCAGACGTGT,100nm,STD
TruSeqR2_i5_[idxnm],/5Phos/AATGATACGGCGACCACCGAGATCTACAC[idx]ACACTCTTTCCCTACACGAC,100nm,STD
NxtR1_i7_[idxnm],/5Phos/CAAGCAGAAGACGGCATACGAGAT[idx]GTCTCGTGGGCTCGG,100nm,STD
NxtR2_i5_[idxnm],/5Phos/AATGATACGGCGACCACCGAGATCTACAC[idx]TCGTCGGCAGCGTC,100nm,STD

Where [idxnm] and [idx] are the particular index names and index sequences to be used. Custom index designs are compatible with these indexing oligos, but the IDT for Illumina unique dual indexes (UDI) used in the TruSeq DNA and Nextera DNA indexing primers available here are also suitable choices:

https://support-docs.illumina.com/SHARE/AdapterSeq/Content/SHARE/AdapterSeq/AdapterSequencesIntro.htm

It is recommended to resuspend all oligos at 200 uM stock concentration in DNA EB. A convenient calculator for this can be found at:

https://www.thermofisher.com/uk/en/home/life-science/oligonucleotides-primers-

probes-genes/custom-dna-oligos/oligo-technical-resources/oligo-calculators.html

Make a 100 uM working solution for NxtR1_TSO_4dU, and a 10 uM working solution for all other oligos. Store aliquots at -20 C when not in use.

Recommended equipment

Equipment	
Concentrator Plus	NAME
Centrifugal vacuum concentrator	TYPE
Eppendorf	BRAND
5305000568	SKU
https://www.eppendorf.com/gb-en/eShop-Products/Centrifugation/Concentrator/Concentrator-plus-p-5305000568	LINK

or similar

Equipment	
Centrifuge 5425 R	NAME
Refrigerated Centrifuge	TYPE
Eppendorf	BRAND
5406000364	SKU
https://www.eppendorf.com/gb-en/eShop-Products/Centrifugation/Microcentrifuges/Centrifuge-5425-5425R-p-5406000364	LINK

or similar

Equipment

12-Tube Magnetic Separation Rack	NAME
Magnetic Separator	TYPE
New England Biolabs	BRAND
S1509	SKU
https://www.neb.uk.com/products/neb-catalogue/protein-analysis,-exp-purification/12-tube-magnetic-separation-rack	LINK

or similar

Equipment

DynaMag™-96 Side Magnet	NAME
Magnetic Separator	TYPE
Invitrogen	BRAND
12331D	SKU
https://www.thermofisher.com/order/catalog/product/12331D	LINK

or similar

Equipment

C1000 Touch™ Thermal Cycler with 96–Deep Well Reaction Module	NAME
Thermal Cycler	TYPE
BioRad	BRAND
1851197	SKU
https://www.bio-rad.com/en-us/sku/1851197-c1000-touch-thermal-cycler-with-96-ndash-deep-well-reaction-module?ID=1851197	LINK

or similar modern heated-lid thermal cycler (deep wells preferable)

Equipment

Megaruptor® 3

NAME

Diagenode

BRAND

B06010003

SKU

https://www.diagenode.com/en/p/megaruptor-3

LINK

Equipment

4200 TapeStation System

NAME

Electrophoresis tool for DNA and RNA sample quality control.

TYPE

TapeStation Instruments

BRAND

G2991AA

SKU

https://www.agilent.com/en/product/automated-electrophoresis/tapestation-systems/tapestation-instruments/4200-tapestation-system-228263

LINK

Alternatively, 4150 or 2200 TapeStation models can also be used.

Alternatively for cDNA but not gDNA sizing, one may use:

Equipment

2100 Bioanalyzer

NAME

Fragment Analysis system

TYPE

Agilent

BRAND

G2939BA

SKU

https://www.agilent.com/en/product/automated-electrophoresis/bioanalyzer-systems/bioanalyzer-instrument/2100-bioanalyzer-instrument-228250

LINK

Optionally, for the most accurate sizing of sequencing libraries:

Equipment	
FEMTO Pulse	NAME
DNA fragment size analyzer	TYPE
Agilent	BRAND
M5330AA	SKU
https://www.agilent.com/en/product/automated-electrophoresis/femto-pulse-systems/femto-pulse-system/femto-pulse-system-365750	LINK

Equipment	
Qubit 4	NAME
Fluorometer	TYPE
Invitrogen	BRAND
Q33238	SKU

or older models (Qubit 3 or 2), as appropriate to your lab

Equipment	
BluePippin Size Selection System	NAME
Electrophoretic size selection system	TYPE
Sage Science	BRAND
BLU0001	SKU
https://store.sagescience.com/	LINK

or else

Equipment	
PippinHT DNA Size Selection System	NAME
Electrophoretic size selection system	TYPE
Sage Science	BRAND
HTP0001	SKU
https://store.sagescience.com/	LINK

1 Isolate specimen for extraction

Animals or tissue samples should be placed individually into the bottom of a 1.5 mL high recovery microcentrifuge tube. Samples may be:

-Live
-Flash-frozen at -80 C (appropriate for small specimens), on LN2/dry-ice chilled absolute ethanol.
-Fixed (I have had success with RNALater following manufacturer's storage recommendations, or with 100% ethanol with immediate storage at -20 C or below)

Minimize the volume of culture/environmental medium or fixative transferred with the specimen to <5 µL (the less the better). For small aquatic animals a P2 micropipette is appropriate; for larger, a fresh glass pasteur pipette; for terrestrial samples, sterilized fine forceps. With <1 mm sized animals it is advisable to verify that the specimen has indeed been transferred under a stereoscope before proceeding. If flash freezing, isolate into labelled tubes as described before freezing, and store on dry ice until ready to use.

2 Specimen lysis

Make a master mix of TNES lysis buffer (leaving an appropriate excess of dead volume when batching multiple specimens). For each specimen, this will consist of:

195 µL TNES (pre-warmed if needed to resuspend)
5 µL Proteinase K
2 µL DTT, 2M aqueous solution

Pre-warm capped microcentrifuge tubes of <1.4 mL of this lysis buffer in a heat block set to to 55 C.

When ready, add 200 uL lysis buffer to the specimen-containing tube, vortex briefly, and incubate at 55 C to lyse. Normally specimens are fully lysed within 30-60 minutes. A periodic capped inversion to mix may speed lysis.

Note

To preserve RNA and HMW DNA integrity, it is essential that the lysis buffer penetrate tissues & inactivate endogenous nucleases quickly. For small specimens and soft tissues this is rarely a challenge. However, for larger (2 mm+) specimens, arthropods, or other cuticle-bound fauna, it is advisable, immediately after adding lysis buffer, to manually smash the specimen using a pellet pestle. For minute cuticle-bound fauna (such as tardigrades), piercing the cuticle of a fixed specimen e.g. with a fine-tip hypodermic needle may be helpful prior to adding the lysis buffer. For especially tough or larger specimens, it is advisable to cryo-disrupt the sample chilled in LN2 before adding the lysis buffer, e.g. using an appropriate dewar https://3dprint.nih.gov/discover/3dpx-010819. For samples >50 mg, proteinase K or overall volumes may require upscaling.

3 RNA-dT bead hybridization

While the specimens are lysing, allow a stock vial of oligo-dT magnetic beads to come to room temperature. Vortex to fully resuspend the settled beads, then for each specimen, take 10 µL bead suspension to a fresh eppendorf tube (allowing excess volume when batching). Pellet the beads on a magnetic separation rack (example given below). Discard the supernatant, and resuspend the pellet two times in 500 µL Wash Buffer II. After the second wash, resuspend the pellet in TNES, to the same volume taken from the stock container.

Verify that specimens are fully lysed before proceeding. Often, the lysate will be slightly viscous (reflecting the presence of HMW DNA). It is advisable to vortex the lysate for 15-30 seconds prior to proceeding for most applications, as the oligo-dT beads can become difficult to pellet and resuspend in lysates containing very HMW DNA. Vortexing will gently shear the DNA, but typically not below 20 kb.

For larger specimens, after vortexing, if you see refractory insoluable material, centrifuge the lysate to pellet this fraction (1 minute, up to 5,000 g speeds). After the insoluable fraction has been pelleted, transfer most of the lysate supernatant to an identical labelled 1.5 mL tube, using a wide-bore pipette tip, and taking care not to transfer any of the pelleted material.

To each tube of clarified lysate, add 10 uL of the washed oligo-dT beads in TNES suspension. Cap and invert to mix, and let rest at room temperature for 10 minutes to allow the poly-A mRNAs to hybridize.

4 RNA and HMW DNA separation

After hybridization, briefly spin down the lysate & homogenized beads in a mini-centrifuge, and set on a magnetic separator rack. Wait until the lysate fully clarifies and a bead pellet with well-defined edges forms.

With a wide-bore tip, transfer the lysate supernatant to a doubly (side and lid) labelled tube to which 2.5 µL yeast tRNA (10 mg/mL) have been previously added. (This is a carrier molecule for

efficient DNA extraction from small specimens; with large animals that you know will form a visible DNA pellet, it can be omitted.)

Resuspend the bead pellet in 200 µL fresh TNES .You may need to pipette-mix or even vortex thoroughly to resuspend. Set the tube on the magnetic separator again and allow the beads to pellet. Take this supernatant and add it to the previous tube to make a total ~400 µL lysate containing the genomic DNA. Cap and set aside at 4 C.

Genomic DNA extraction continues at step 14.

5 RNA purification and elution

> Note
>
> It is important in this section not to let beads dry - work in serial rather than parallel when batching specimens. Lysate trapped on tube lids during handling could be a source of cross-sample contamination. When in doubt, spin down tubes before opening between washes to control this.

Set a microcentrifuge tube heat block to 80 C before beginning.

1. Using a P1000 micropipette, resuspend the pelleted beads in 500 µL Wash Buffer I. Reset on separator to form a pellet.
2. Repeat the above step. For small (<2 mm) specimens, this second wash can be omitted.
3. Repeat again but using Wash Buffer II. The beads will collect as a dissolute smear rather than a tightly defined pellet owing to the lack of detergent; this is normal.
4. Perform a second Wash Buffer II wash, as above. This second wash can again be skipped with small specimens.
5. Wash one last time in 500 µL Low Salt Buffer (LSB) and pellet again.
6. With your desired elution buffer ready (see below), remove all traces of LSB. Take the tube from the rack and fully resuspend the beads in elution buffer, taking care to catch all beads stuck to the tube wall.
7. Take the capped tube to the 80 C heat block, and set the capped tube there to heat and break the oligo-dT-RNA hybridization. If batching specimens, work in no more than one pair at a time. Once on the block, immediately set a timer for 2 minutes.
8. After 90 seconds have passed, remove a tube to a magnetic separator rack. Let the beads pellet fully, then take all the supernatant to a fresh labelled tube kept on ice or a cold block. Work quickly to avoid re-hybridization. Tubes should not be allowed on the heat block longer than 2 minutes.

With small specimens, RNA concentrations are often below the detectable QC threshold (and there is not much to waste). Therefore, I prefer to elute the entire RNA extract directly in 15.4 µL Reverse Transcription Master Mix I (see step 6), allowing me to proceed immediately to cDNA synthesis.

However, if you want a pure poly-A RNA sample, elute in up to 20 µL of RNA elution buffer, and QC

or split as appropriate to your application.

6 PolyA-transcriptome cDNA synthesis

Two master mixes are required, with as usual an excess volume when batching specimens. Per reaction, construct on ice:

Reverse Transcription Master Mix I (RT1, 15.4 µL per reaction)
9.2 µL RNA elution buffer or previously eluted RNA template, as appropriate (max. 100 ng polyA RNA input)
3 µL dNTP mix, 10 mM each
3 µL NxtR2_dT24VN Oligo-dT primer, 10 µM
0.2 µL RNAse inhibitor

Reverse Transcription Master Mix II (RT2, 14.6 uL per reaction)

- 6 µL 5X SuperScript IV buffer
  6 µL Betaine, 5 M
  0.3 µL ET SSB (NEB)
  0.3 µL NxtR1_TSO_4dU template-switching oligo 100 µM
- 1.5 µL DTT, 0.1 M
- 0.5 µL SuperScript IV reverse transcriptase

> **Note**
>
> If using polyA mRNA that has been separately eluted in RNA EB, mix 9.2 uL RNA template to the 6.2 uL remaining RT1 reagents by pipette individually, then cap and heat at 72 C for 2 minutes, and shock-cool on ice. This step is important to break any RNA secondary structures and anneal the dT primer. If eluting the RNA from the beads directly into premade RT1, this step can be omitted.

Mix the RT1 containing the template RNAs on ice by pipette to RT2; the latter is viscous so 10+ pipette cycles can be required.

Then take the cDNA synthesis master mix to a thermal cycler and incubate on the following programme:

1. 53 C / 12 minutes
2. 42 C / 120 minutes
3. 80 C / 10 minutes

7 Primary cDNA cleanup

Collect a 1 mL aliquot of ProNex size selection beads from 4 C storage and let it come to room temperature before use.

After the reverse transcription programme has finished, to each tube add 1 uL NEB USER enzyme.

Mix (cap, vortex, then spin down) and incubate a minimum of 15 minutes at 37 C. This step digests the template switching oligo (TSO), including any TSO-primed cDNAs (an undesirable side-reaction).

Add 1 uL Proteinase K, mix as before, and incubate for at least 15 minutes at 55 C. This step digests a protein (putatively BSA) in the RT buffer that can interfere with ProNex bead cleanup - if it is omitted, beads are strongly adherent and difficult to resuspend.

Finally, to the 32 uL master mix, add 42 uL ProNex beads (~1.3X), vortexed to fully resuspend before use. Pipette thoroughly (>10x) to mix. Let sit 10 minutes to precipitate the cDNA, and then pellet the beads/cDNA against a 0.2 mL tube magnetic separator (I prefer side-oriented magnets).

Once the tubes have clarified and the beads have formed a pellet with tight edges, draw off and discard the supernatant, and replace with 180 uL ProNex wash buffer using a P200 micropipette. Wait 30 seconds, then draw off and discard the wash buffer with a P200 set to 200 uL. Repeat this wash one more time, taking care to fully pipette all wash buffer away. Wait up to 5 minutes for any residual wash buffer to evaporate, if necessary drawing away any residuum with a P10. The formation of cracks in the pellet is not particularly detrimental.

Finally, fully resuspend the bead pellet in 12.75 uL DNA elution buffer. After two minutes you may optionally re-pellet the beads and transfer the cDNA-containing supernatant to a fresh tube/strip. I prefer to retain the cDNA in the same tube, pelleting immediately before drawing any supernatant off for downstream amplification - the beads are inert and it saves on plastic. Store at 4 C for up to one week, or -20 C for longer.

8 Proxy PCR for cDNA library amplification cycle number estimation

Because different tissues/organisms vary so widely in size & RNA content, the number of PCR cycles required to amplify enough cDNA to sequence can be unpredictable. Furthermore, the input amounts required to meet the lower detection threshold of fluorescence quantification techniques can represent a substantial proportion of the available cDNA. For this reason, I avoid quantifying cDNA directly, instead using real-time PCR to empirically estimate for each sample the optimum cycle number for amplification.

Make a master mix as follows, vortexing and spinning down before use:

Proxy PCR Master Mix (PPCR, final volume 25 uL per reaction)

- 12.5 uL 2X Terra Direct PCR buffer
- 0.5 uL Terra Direct PCR enzyme
- 1.25 uL EvaGreen (20X in water)
- 1.25 uL NxtR1_i7_[idxnm] indexing primer 10 uM
- 1.25 uL NxtR2_i5_[idxnm] indexing primer, 10 uM
- 7.5 uL MB-grade water

The indices you choose for the master mix are arbitrary, and a single index pair can be used for all

samples. Dispense 24.25 uL of the proxy PCR mix, one well per specimen, into your preferred qPCR vessel (whether plate or strip). Add 0.75 uL of your template cDNA, pipetting into the dispensed master mix. Seal and spin down to mix.

Set up a qPCR programme to run as follows:

1. 98 C / 2 minutes
2. 98 C / 10 seconds
3. 68 C / 90 seconds (measure fluorescence at end of cycle)
4. GOTO step 2 for a total of 35 cycles

Your qPCR instrument should be set up to read in the SYBR Green fluorescence channel, with no background dye. A melting curve is optional (and default programmes usually work for this).

Working from the raw fluorescence data, for each well, you should see a clearly sigmoid growth curve. Locate the cycle number closest to the inflection point of the curve (the point at which the rate of increase slows). An annotated example screenshot is shown below.

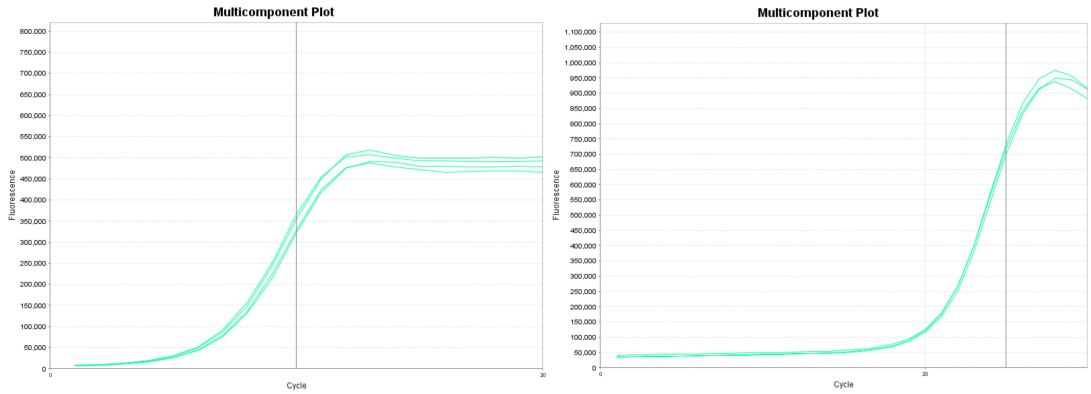

Figure 1 - Two example amplification plots, showing the inflection-associated cycle (10 and 25, for left and right panels respectively), marked with a vertical line.

For each sample, record X, the cycle number associated to this inflection point. When in doubt, choose the lower of the two potential values.

9 PCR amplification of cDNA libraries

Prepare a master mix on ice, vortexing and spinning down before use:

cDNA library PCR Master Mix (cDNA-PCR, final volume 50 uL per reaction)

- 25 uL 2X Terra Direct PCR buffer
- 1 uL Terra Direct PCR enzyme
- 7 uL MB-grade water

Dispense 33 uL of the cDNA-PCR master mix per specimen to your PCR vessel of choice. To each, add:

- 2.5 uL of a NxtR2_i5_[idxnm] indexing primer, 10 uM
- 2.5 uL of a NxtR1_i7_[idxnm] indexing primer, 10 uM
- 12 uL purified cDNA template

Cap/vortex/spin down to mix.

The PCRs should be conducted in a thermal cycler programmed as such, with a flush fit heated lid:

1. 98 C / 2 minutes
2. 98 C / 10 seconds
3. 68 C / 10 minutes
4. GOTO step 2 for a total of Y cycles

Where Y = X - 3, X being the inflection-point associated cycle from the Proxy PCR. This will differ from sample to sample - plan to batch samples requiring the same cycle number together. In my experience samples rarely require more than 20 cycles of amplification, more typically in the 12-18 range.

Note

The rationale here is that the cDNA present in the 0.75 uL used in the proxy PCR, after four cycles of 100% efficient doubling, will equal that found in the 12 uL used for full library amplification ($0.75 \times 2^4 = 12$). Therefore, if we were amplifying in a 25 uL volume as with the proxy, we would use 4 fewer cycles. However, because we amplify the full library in 50 uL volume, with a greater reserve of primers/dNTPs, we can afford one extra cycle. Therefore, we subtract only 3 cycles. In this way we sacrifice only ~6% of the cDNA to amplify the remaining 94% using a precise estimate of the cycles needed.

10 Cleanup and quality control of amplified cDNA

Collect a 1 mL aliquot of ProNex cleanup beads from 4 C storage and let it come to room temperature before use.

After each cDNA library has been PCR amplified, to each add:

2 uL Proteinase K, Molecular Biology Grade

Cap/vortex/spin down to mix, and incubate at 55 C for at least 15 minutes. As in the reverse transcription reaction, without this step, ProNex beads become adherent and difficult to resuspend after cleanup, presumably due to denatured or aggregate proteins/nucleoproteins.

After digestion, to each reaction add 70 uL ProNex cleanup beads, resuspended by vortexing before use. Pipette thoroughly (at least 10x) to mix. Follow the same cleanup procedure outlined

in **Step 7**, however eluting in 20 uL DNA elution buffer. Eluted cDNA libraries may be stored at 4 C for up to one week or at -80 C for longer; minimize freeze-thaw cycles.

Take 1 uL of the purified product for quantification with a Qubit dsDNA High Sensitivity Assay. Typically libraries will have concentration in a 5-50 ng/uL range.

Take a further 1 uL forward into your favorite fragment size distribution assay; I typically use a D5000 TapeStation; High Sensitivity D5000 Tapes or Bioanalyzer High Sensitivity DNA assays are also suitable, but these may require dilution as per the manufacturer's indications prior to loading to achieve accurate sizing.

Libraries made from undegraded RNA should have a mean size of 1-3 kb (for most small invertebrates, 1-1.5 kb is typical), with a relatively smooth bell-curve like distribution around the mean and very few cDNAs shorter than 250 bp or longer than 5 kb. A few examples of expected cDNA distributions are given below for reference:

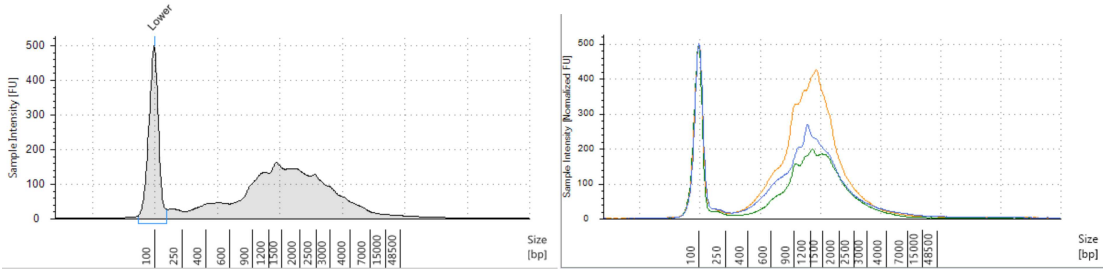

Figure 2 - Examples of successful cDNA libraries analyzed on an Agilent TapeStation D5000 assay, showing approximately bell-shaped distributions with a mean 1-1.5 kb size.

11 Multiplexing of cDNA libraries (optional)

Using the sizes inferred from the "Add Region" function of the Tapestation D5000 assay (with upper and lower thresholds set to fully encompass the library), and using a Qubit dsDNA HS assay to measure the molecular weight concentration of your cDNA libraries, calculate the molar concentration of each library, using a tool such as:

https://www.bioline.com/media/calculator/01_07.html

This should be in broad agreement with the nmol/L calculation given by the TapeStation, but is generally slightly more accurate.

For each library to be pooled, verify that unique index combinations have been assigned, then aim to dilute each sample in DNA EB to the concentration of the least concentrated library, if forming an equimolar pool. For pipetting accuracy, try not to work in final dilution volumes of less than 10 uL.

For ONT sequencing, we are aiming to achieve a final pooled volume of 30 uL; whereas for

PacBio, we are aiming for 46 uL. If required, a pool can be cleaned up in 1.35X ProNex beads to concentrate the sample to the desired pool concentration. For PacBio Iso-Seq library prep, ensure that the final pool contains between 160-500 ng amplified cDNA per 46 uL. For Oxford Nanopore LSK114 library prep, aim for a pool of 100 fmol final cDNA in 30 uL (3.33 nM).

12 Preparation of Iso-Seq libraries using the PacBio SMRTBell prep kit 3.0 (option 1)

For this protocol step, we will follow "Preparing Iso-Seq® libraries using SMRTbell® prep kit 3.0", PN 102-396-000 REV02 APR2022: https://www.pacb.com/wp-content/uploads/Procedure-checklist-Preparing-Iso-Seq-libraries-using-SMRTbell-prep-kit-3.0.pdf

As we have already synthesized and amplified our cDNA, begin at step 4, "Repair and A-Tailing". In all other respects, the manufacturer's protocol may be followed as indicated, to Sequel sequencing as the final step.

13 Preparation of nanopore cDNA libraries using the LSK114 kit (option 2)

For this protocol step, we follow the ONT Ligation sequencing DNA V14 (SQK-LSK114) kit protocol, treating the cDNA inserts as if they were genomic DNA.

We skip not only fragmentation but also end-repair, so long as the eluted cDNA has been stored at 4 C for no longer than one week. This is because the Terra polymerase we use for amplification is A-tailing, and the indexing primers we used for PCR were 5' phosphorylated - further end-manipulation is therefore redundant.

We therefore start from "Adapter ligation and clean-up", performing the reaction at 1/2 volume:

30 uL size-selected DNA (50-100 fmol)
12.5 uL Ligation Buffer (LNB)
2.5 uL Ligation Adapter (LA)
5 uL NEBNext Quick T4 DNA Ligase

Mix the viscous solution thoroughly by pipette (10-20x) and ligate 10 minutes at RT as per protocol.

Clean the adapter-ligated library up with 20 uL AMPure XP beads (0.4X) or 41 uL ProNex beads (0.82X), washing the bead pellet twice in 125 uL Short Fragment Buffer (SFB). Elute in 15 uL EB and Qubit-quantify before proceeding with sequencing, as per protocol, loading 10-20 fmol finished library per flow cell (diluting in further EB if required). Store the library at 4 C for up to one week, or -80 C for longer, minimizing freeze-thaw cycles required prior to sequencing.

14 Genomic DNA purification

Set a refrigerated benchtop microcentrifuge to cool to 4 C, and chill a freshly made tube of 75% EtOH on ice.

Return to the previously set aside 400 uL lysate containing the genomic DNAs and carrier tRNA.

To each, add:

- 130 uL NaCl aqueous solution, 5 M
- 800 uL Ethanol, 100%

Cap and invert to mix - the meniscus should be close to but below the internal seal. Salt precipitates may initially form but the solution should clarify after mixing to homogeneity. With highly concentrated genomic DNA, you may be able to see threadlike webs of DNA precipitate out of solution and coalesce into a clot. With small samples, typically no visible precipitate will form.

Place the tubes at -20 C or below for at least 30 minutes to allow to come to temperature. Ethanol-precipitated DNA is extremely stable and can be cryo-stored in this form, in principle, for decades. Precipitated HMW DNA is also a preferred option for courier shipping (these 60% solutions fall into IATA Packing Group III), to minimize the risk of freeze-thaw or shear damage.

Keeping tubes on ice in between handling, set them up in the pre-cooled centrifuge in a balanced configuration with the hinges facing out, and spin down for 15 minutes at max speed (at least 10,000 rcf).

Inspect the tubes after this initial centrifugation - you should see (wiping condensation away) a small pale grey to yellow smear at the base of the tube - this is the pellet containing (mostly) carrier tRNA and your genomic DNAs.

> **Note**
>
> If you do not see a pellet, do not proceed. This implies that the starting sample volume had too high a water content. It may be possible to recover by transferring the contents to a 2 mL tube and titrating additions (e.g. in 100 uL volumes) of 100% ethanol, re-centrifuging and inspecting for a pellet in increments. Beyond a point, however, salt will also precipitate.

Pipette up and discard the entire supernatant, without disrupting the pellet (it should be fairly adherent). Add 1 mL chilled 75% EtOH to the tube wall. Cap and return to the centrifuge for a further 5 minute precipitation at max speed.

As before, remove and discard the supernatant, and give the pellet a final wash in 500 uL 75% Ethanol, re-spinning 5 minutes as before. Remove the wash ethanol as completely as possible without disrupting the pellet.

Add to the pellet 30 uL of DNA elution buffer, if necessary using a wide-bore tip to wash any residual pellet down from the tube wall using these 30 uL. Let tubes rest uncapped for 15 minutes at 37 C for any residual ethanol to evaporate and for the pellet to fully rehydrate. Alternatively, the DNA can be let to rehydrate (capped) at 4 C overnight.

Note

This "salting out" DNA extraction protocol has been tested and shown to give high efficiency extraction of relatively pure HMW DNA from a variety of organisms. However, for some mucilaginous invertebrates (in my experience, planarians and enteropneust hemichordates), contaminating polysaccharides can also precipitate, leading to a highly viscous and/or pigmented DNA extraction and potentially complicating downstream analysis. In such cases, alternative approaches such as pre-treatment with N-Acetyl Cysteine or the MoLSC protocol (doi: 10.1111/1755-0998.12616) may be attempted.

15 Genomic DNA aliquoting & quality control (optional)

For numerous downstream purposes, at this stage it can be useful to take a 3 uL aliquot (10%) from the concentrated HMW DNA. For small meiofauna (<1 mm), it may be sensible to omit this step in favor of preserving library diversity.

Example applications:

DNA quantification/QC with a Qubit dsDNA HS assay, and if the concentration allows, DNA sizing using agarose gel electrophoresis, a Genomic DNA Tapestation, or the FEMTO Pulse Genomic DNA 165 kb Kit. Before attempting, however, it is advisable to treat the extraction with RNAse A, as the high concentration of tRNAs can mislead fluorescence-based assays.

For example prepare the following 1:5 dilution:
0.5 uL RNAse A 10 mg/mL
3.5 uL H2O
1 uL genomic DNA

And incubate at 55 C for 15 minutes to digest the tRNA carrier.

Linked read library preparation e.g. using TELL-seq, stLFR, Haplotagging, or other methods. Previous experience with 10x linked-reads (no longer available) showed such data to have use in scaffolding and assembly polishing.

Whole genome isothermal amplification with e.g. the Qiagen RepliG-sc or the BioSkryb ResolveDNA kit. In principle these amplification modalities may have different but complimentary biases to long-PCR library amplification, leading to a better final assembly using combined data.

16 Genomic DNA shearing

Equipment	
Megaruptor® 3	NAME
Diagenode	BRAND
B06010003	SKU
https://www.diagenode.com/en/p/megaruptor-3	LINK

We seek to predictably shear our DNA to a tight distribution around a mean size in excess of 10 kb but under 20 kb, at which point PCR library amplification becomes unworkable. The optimal tool for this is the Megaruptor 3 (Diagenode).

Using molecular grade water (not DNA EB), dilute your DNA extract to 150 uL (e.g. adding 123 uL if 27 uL remain), and mix and transfer with a wide-bore pipette to a labelled Megaruptor 3 Hydrotube. Ensure no bubbles are visible within the liquid. Assemble a Hydropore-syringe to fit snugly onto the Hydrotube. Do not "test" the assembled complex.

Set up the Megaruptor 3 in a balanced configuration as per manual, to run at a volume of 150 uL with 001 ng/uL (adjust if you have a Qubit estimate). In practice I have found that the mean DNA sizes achievable for a given shear speed setting given in the Megaruptor 3 Manual are overestimates. As a rough guide:

Speed 36: 8-10 kb
Speed 33: 10-12 kb
Speed 30: 12-16 kb

The optimum degree of shearing to aim for may differ from sample to sample. In general, the smaller the organism & the genome, the greater the shearing should be to maximize library diversity.

When the shearing has finished (normally 30-45 mins), collect all tubes, and manually express any residual DNA from the hydropore into the collection tube. It is worthwhile to attempt to disassemble the Hydropore-syringe adapter and extract residual DNA with a P10 tip - up to 15 uL can be left behind.

Note

If you do not have access to a Megaruptor 3, it is possible, though less desirable, to adapt to other shearing methods. Covaris g-TUBEs are an option: the related PacBio ULI protocol recommends shearing 50 uL of 5-20 ng DNA with a speed of 1677 g for a 10 kb shear. However, the shears achievable with a g-TUBE are concentration dependent and the distribution is broader than what is achievable via Megaruptor. 150 uL DNA can also be sheared within a microcentrifuge tube using a 0.5ml 29G 12mm needle BD Micro-fine Syringe. Aim for 15-20 passes through the needle, and adust the syringe handle slowly to avoid cavitation/frothing. This can achieve similar results to a Megaruptor 3 but the degree of shearing is harder to control, and cavitation can result in production of <2 kb DNA.

17 Concentration of sheared genomic DNA

We begin library preparation with a 23 uL sheared DNA volume, but necessarily sheared at a higher volume. Two options to concentrate are available:

SpeedVac Set up the DNA in the Megaruptor Hydrotubes or 1.5 mL Eppendorf tubes to spin uncapped at no more than 45 C. The aim is to concentrate the DNA volume to under 23 uL while strictly avoiding letting it dessicate entirely (which can be fatal to an experiment). The best approach is empirical: stop the SpeedVac to check the progress of the concentration every 15 minutes, or even more often when the tubes begin to approach 23 uL volume. It can be helpful to keep aside a sealed calibration tube with the target volume to compare to visually. The total time required may be between 45-90 minutes.

If the volume falls substantially below 23 uL, using a P10 or P20 tip, estimate the final sample volume. A negative pipetting technique can be helpful (i.e. set the micropipette to 20 uL, aspirate the entire sample, and then adjust downward the volume setting on the micropipettor until the sample touches the pipette tip). Then, transfer to a 0.2 mL PCR tube or strip, and adjust the final volume to 23 uL with MB-grade water.

SPRI cleanup This is not a preferred method for very small meiofauna, as it involves some (albeit minor) sample loss, but larger input samples can be concentrated using a 1.2X volume ProNex cleanup as per the manufacturer's protocol. Elute in 23 uL DNA EB and leave the tubes on a 37 C heat block uncapped for 15 minutes to allow elution to complete and any residual ethanol to evaporate.

18 DNA damage repair, end-polishing, and amplification adapter ligation

For this step I use the NEB Ultra II DNA library prep protocol, summarized for convenience here, at 1/2 volume as a cost saving measure. Before beginning, set a 1 mL aliquot of ProNex cleanup reagent to come to room temperature.

Working on ice, and using freshly thawed and mixed reagents, set up the DNA repair reaction, using a master mix if needed:

- 23 uL sheared & concentrated template DNA
- 3.5 uL FFPE DNA Repair Buffer v2
- 1 uL NEBNextFFPE DNA Repair Mix v2

Mix thoroughly with wide-bore pipette tips. Incubate at 37 C with a heated lid set to 50 C for at least 15 minutes.

To digest the DNA repair enzymes, including uracil deglycosylase which can digest the NEB hairpin adaptors, add 1 uL Thermolabile Proteinase K, and mix again with WB tips. Incubate in a thermal cycler with a heated lid set to 75 C, using the following program:

1. 37 C / 15 minutes
2. 65 C / 5 minutes
3. 4 C / hold

After this, add 1.5 uL NEBNext Ultra II End Prep EnzymeMix to each tube, and mix via WB pipette, spinning down after.

Set the end-repair reaction up in a thermal cycler with a heated lid set to 75 C:

1. 20 C / 30 minutes
2. 65 C / 30 minutes
3. 4 C / hold

Next, add 1.25 uL of the the NEB hairpin adaptor. This should be pre-diluted from the stock concentration (15 uM) using DNA EB. For most meiofauna the recommended working concentration to add is 0.6 uM (giving a final concentration in ligation of ~16 nM). For larger specimens (with 25-100 ng input DNA), one can use 1.5 uM.

Then, add the remaining ligation reagents (a master mix can be made immediate before - vortex thoroughly as the liquid is viscous):

15 uL NEBNextUltra II Ligation Master Mix
0.5 uL NEBNext Ligation Enhancer

Mix the viscous reagents together thoroughly (>10x) with a WB tip. Set reaction to ligate at 20 C without use of a heated lid for 15 minutes.

Add 1.5 uL NEB USER enzyme, and mix as above with a WB tip. Incubate in a thermal cycler with a 50 C heated lid at 37 C for 15 further minutes.

19 DNA library cleanup and preamplification

Add 1 uL yeast tRNA carrier (10 mg/mL) to each sample to maximize cleanup efficiency. Then add 48 uL ProNex beads (~1X), and mix thoroughly with a WB tip, cleaning up as previously described.

For small meiofauna (inputs <10 ng), elute in 9.5 uL DNA EB, waiting up to 10 minutes at 37 C for elution to complete.
For larger specimens, instead elute in 40 uL (and do not carry forward to preamplification).

With small animals, we first perform a "pre-amplification" step, with the goal of amplifying enough to create redundancy against further cleanup loss, but not enough to risk approaching overamplification. This also allow us to perform two SPRI cleanups to completely remove any adapter dimer, which can mislead proxy-PCR.

Creating a master mix on ice (*PreAMP, final volume 25 uL per reaction*):

12.5 uL 2X Terra Direct PCR buffer
0.5 uL Terra Direct PCR enzyme
1.25 uL TruSeqR1_i7_[idxnm] indexing primer, 10 uM
1.25 uL TruSeqR2_i5_[idxnm] indexing primer, 10 uM
9.5 uL template DNA library

First add 1.25 uL each indexing primer to the 9.5 uL DNA (separated in a fresh tube from residual beads, which inhibit Terra amplification), then adding 13 uL Terra Buffer/Enzyme to each tube. Mix via WB pipette then spin down.

Set up to run in a thermal cycler programmed as such:

1. 98 C / 2 min
2. 98 C / 10 sec
3. 68 C / 20 min (this can be decreased to 15 min for samples sheared at speed 36)
4. GOTO step 2 for a total of 8 cycles

Add 1 uL Proteinase K, and cap/invert/spin down to mix. Incubate at 55 C for 15 minutes.

Add 25 uL ProNex cleanup beads (1.0X) and mix thoroughly via WB pipette, cleaning up as before and eluting in 40 uL DNA EB.

20 Stringent cleanup before proxy PCR

Proxy PCR will track the amplification of any DNA, including both template and contaminating adapter-dimers, which despite adapter dilution can be substantial. Additionally, samples may contain substantial levels of <5 kb DNAs which amplify with greater efficiency than longer templates during PCR. To ensure that the proxy PCR step only tracks amplification of large template molecules, we first perform a second round of stringent size-selection.

To the 40 uL eluted DNA (whether pre-amplified or not), add exactly 35 uL (0.88X) room-temperature ProNex cleanup reagent with a non-wide-bore tip (accurate volume addition is important here), and mix thoroughly. Let rest at room temperature 10 minutes, and clean up as per manufacturer's protocol. Elute in 25.5 uL DNA elution buffer at 37 C with lids uncapped for 15 minutes to allow the library to fully elute & ethanol to evaporate. Store at 4 C for up to one week.

21 Proxy PCR for genomic DNA library amplification cycle number estimation

Set up a proxy real-time PCR, as described in Step 8, with the following alterations:

Proxy PCR Master Mix (PPCR, final volume 25 uL per reaction)

- 12.5 uL 2X Terra Direct PCR buffer
- 0.5 uL Terra Direct PCR enzyme
- 1.25 uL EvaGreen (20X in water)
- 1.25 uL TruSeqR2_i5_[idxnm] indexing primer, 10 uM
- 1.25 uL TruSeqR1_i7_[idxnm] indexing primer, 10 uM
- 6.75 uL MB-grade water

The indices you choose for the master mix are arbitrary, and a single index pair can be used for all samples. Dispense 23.5 uL of the proxy PCR mix, one well per specimen, into your preferred qPCR vessel (whether plate or strip). Add 1.5 uL of your template genomic DNA, pipetting into the dispensed master mix. Seal and spin down to mix.

Set up a qPCR programme to run as follows. This step can take over 12 hours to complete and is best set up to run overnight.

1. 98 C / 2 minutes
2. 98 C / 10 seconds
3. 68 C / 20 minutes (this can be decreased to 15 min for samples sheared at speed 36; measure fluorescence at end of cycle)
4. GOTO step 2 for a total of 35 cycles

As in step 8, for each sample, inspect the raw fluorescence signal for X, the cycle number associated to the inflection point of each amplification curve.

22 Genomic DNA library long-range PCR amplification

Working on ice, set up a master mix:

gDNA PCR Master Mix (gDNA-PCR, final volume 100 uL per reaction)

- 50 uL 2X Terra Direct PCR buffer
- 2 uL Terra Direct PCR enzyme
- 5 uL TruSeqR2_i5_[idxnm] indexing primer, 10 uM*
- 5 uL TruSeqR1_i7_[idxnm] indexing primer, 10 uM*
- 14 uL MB-grade water
- 25 uL genomic library template*

Choose & note the index combinations that best suit your multiplexing intentions. The reagents marked with * should be separately added to each reaction vessel and mixed thoroughly (10x or more) by WB tip with the 66 uL remaining master mix volume, then *split into two separate PCR tubes/strips containing 50 uL each*. Reaction splitting can be avoided if your thermal cyclers are set up with deep-well blocks (e.g. as offered by Bio-Rad C1000 cyclers).

Set up the PCRs to amplify with the following programme:

1. 98 C / 2 minutes
2. 98 C / 10 seconds
3. 68 C / 20 minutes (this can be decreased to 15 min for samples sheared at speed 36)
4. GOTO step 2 for a total of Y cycles

Where Y = X - 2, e.g. if the proxy inflection cycle was determined to be 13 we would perform 11 cycles. The rationale here is as explained in step 8; however, as we are amplifying in 100 uL rather than 50 uL, we can afford to add one more cycle and therefore subtract 2 instead of 3 from X.

23 Amplified gDNA library cleanup and QC

After amplification, repool samples to the 100 uL volume, and add 2 uL Proteinase K, mixing thoroughly. Incubate at least 15 minutes at 55 C to digest.

Then add 100 uL room temperature ProNex cleanup reagent, mixing thoroughly, and cleaning up per manufacturer's protocol. Elute in 32 uL DNA elution buffer, waiting up to 10 minutes at 37 C to fully elute.

Take 1 uL forward for Qubit dsDNA HS quantification. Typical yields will be between 10-50 ng/uL, depending on insert size.

Take another 1 uL forward for TapeStation Genomic DNA ScreenTape analysis; if the library is in excess of 100 ng/uL concentration, dilution is required for accurate sizing. Typically libraries should consist of a main peak in excess of 10 kb which makes up >70% of the DNA mass; a smear or minor peak of shorter fragments <10 kb may also be visible. A FEMTO Pulse Genomic DNA fragment analysis may instead be used if desired.

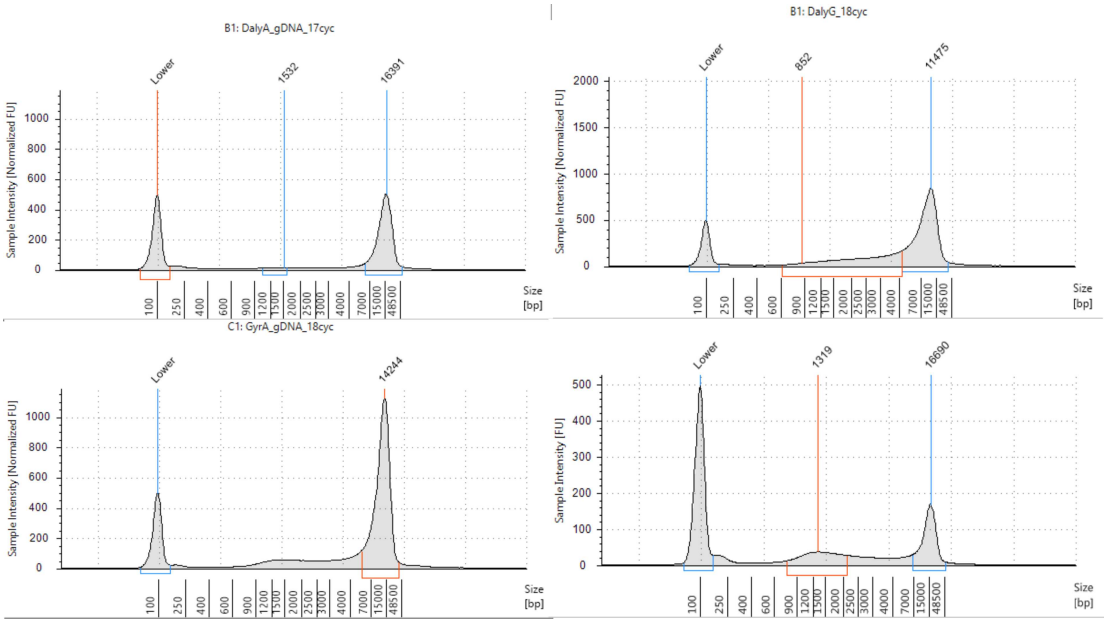

Figure 3 - Example fragment size distributions of successfully amplified genomic DNA libraries, visualized on an Agilent TapeStation Genomic DNA Screen Tape assay.

24 Genomic DNA Multiplexing (optional)

If preparing to multiplex, select a pool of samples which have passed QC, and which have distinct index combinations.

Using the "add regions" tool of the TapeStation analysis software, or the "Smear Analysis" tool in ProSize if using a FEMTO pulse, estimate the molarity of each sample from a fixed lower size threshold to the maximum size of the sample. The threshold to select should depend on the lowest mean insert size of the pool. As a rule of thumb, it is advisable to select a threshold at least 3 kb below the mean size if using a TapeStation, and at least 2 kb if using a FEMTO pulse. If performing a bead-based size selection (see Step 22), choose 5 kb as the lower threshold value.

Noting the concentrations within the selected size range in nM, prepare to dilute all libraries to the lowest concentration sample. For PacBio library preparation we are aiming to make a pool of volume not in excess of 46 uL; calculate dilution volumes accordingly (e.g. if splitting 3-ways aim for 16 uL/sample). For Oxford Nanopore library preparation we are aiming for a final volume of 30 uL. Aim for a total mass well in excess of 500 ng, ideally above 1 ug (to a maximum of 5 ug). In either case, if required to acheive this mass threshold, a 1.0X ProNex cleanup can be used to concentrate a greater volume.

25 PacBio SMRTBell Express 3.0 library preparation and size selection (option 1)

When aiming to sequence via the PacBio platform, it is advisable to perform library preparation first and a size-selection step on the purified & nuclease-treated SMRTbell library.

The SMRTBell Express 3.0 library prep kit (PN 102-182-700) protocol may be followed, starting

from Step 2 (Repair & A-tailing) - no further shearing is advisable.

If planning to use a *BluePippin size selection* (which is the preferred option, but requires >800 ng DNA), at Step 6, perform a 1X AmpurePB cleanup, eluting in 31 uL DNA EB. Take 1 uL for a Qubit dsDNA HS assay to confirm in excess of 800 ng DNA total remain after library prep before proceeding.

For BluePippin size selection, use an in-date BLF7510 Marker S1 casette and reagents, or if using a PippinHT, a HPE7510 casette. To operate, follow the BluePippin High-Pass DNA Size Selection Guide, using S1 as the external marker, and selecting as your cassette definition: "0.75% DF Marker S1 high-pass 6-10kb vs3", or if using a PippinHT with HPE7510, using casette definition "0.75% Agarose, 6-10kb High Pass, 75E". Input the lower size cutoff you selected during fragment analysis as "BP START", and select 50000 as "BP END". After the program has finished, collect the sample into a fresh LoBind tube, performing as well the optional rinse of the elution well with TE+0.1% Tween 20, and pooling these together to make ~80 uL of eluted, size selected library. Cleanup with 80 uL (1X) ProNex beads, eluting in a final volume of 15 uL EB from the SMRTbell Express 3.0 kit. NB that for PippinHT casettes, elution volumes are 30 uL; therefore in place of 80 uL, expect a 60 uL final elution volume and use 60 uL ProNex beads for cleanup.

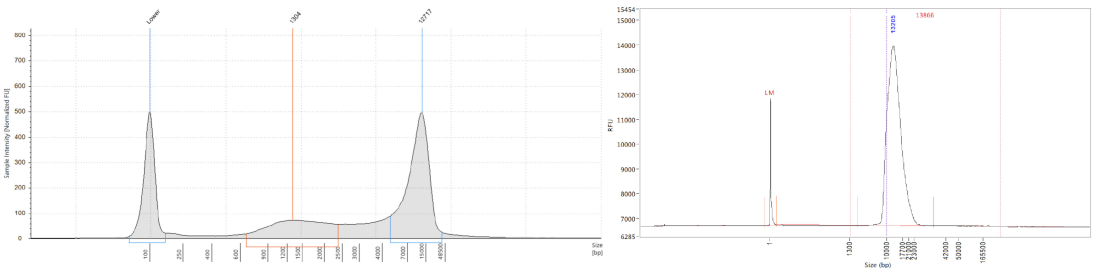

Figure 4 - Example of a genomic DNA library BluePippin size-selection at 8 kb using a BLF7510 cassette, visualized before and after size-selection on a Genomic DNA ScreenTape and FEMTO Pulse Genomic DNA 165 kb assay, respectively.

If instead planning to use a *bead-based size selection* (depleting <5 kb fragments), perform the cleanup with 35% v/v diluted AmpurePB beads as directed in Step 6 of the SMRTBell express prep kit 3.0 manual.

Regardless of size-selection method, it is advisable to perform one final QC of the size-selected library using a Qubit dsDNA HS assay and a FEMTO pulse or other fragment analysis instrument, prior to proceeding to "Anneal and Bind SMRTbell Templates" and from there to Sequel IIe loading. The total sample molarity should be at least 2 nM to proceed to sequencing. Store eluted libraries for no more than 7 days at 4 C, or indefinitely at -80 C, and minimize freeze-thaw cycles prior to sequencing.

26 Size selection and Oxford Nanopore LSK114 library preparation (option 2)

Because the ONT nucleoprotein adapter contains an enzyme which can be denatured, it is best to perform size-selection prior to nanopore library preparation.

If planning to use a *BluePippin size selection* (the preferred option), follow the BLF7510 procedure outlined in Step 22, eluting from the post-size selection ProNex cleanup in 32 uL DNA EB.

If planning to use a *bead-based size selection* (depleting <5 kb fragments), dilute the DNA to exactly 100 uL in DNA EB, and add 82 uL (0.82X) ProNex beads, and cleaning up as standard. A second round of cleanup with the same parameters will yield a more stringently size-selected library and higher read lengths, at the cost of a reduced yield. Typical recoveries are 50-80% per cleanup. Elute finally in 32 uL DNA EB.

Prior to library prep, confirm size-selection and sufficient yield by performing one more QC of the size-selected library using a Qubit dsDNA HS assay and a Genomic Tape, FEMTO pulse or other fragment analysis instrument. Confirm that between 50-100 fmol of size-selected library is present in 30 uL (1.66-3.33 nM) - concentrations below this threshold may not have sufficient yield to achieve high pore occupancy, and those above this threshold may show a high proportion of blunt-end ligated chimeric inserts.

For nanopore library prep, following the "Ligation sequencing DNA V14 (SQK-LSK114)" protocol, we may forgo not only fragmentation but also end-repair, so long as the eluted DNA has been stored at 4 C for no longer than one week. This is because the Terra polymerase we use for amplification is A-tailing, and the indexing primers we used for PCR were 5' phosphorylated - further end-manipulation is therefore redundant.

We therefore start from "Adapter ligation and clean-up", performing the reaction at 1/2 volume:

30 uL size-selected DNA (50-100 fmol)
12.5 uL Ligation Buffer (LNB)
2.5 uL Ligation Adapter (LA)
5 uL NEBNext Quick T4 DNA Ligase

Mix the viscous solution thoroughly by pipette (10-20x) and ligate 10 minutes at RT as per protocol.

Clean the adapter-ligated library up with 20 uL AMPure XP beads (0.4X) or 41 uL ProNex beads (0.82X), washing the bead pellet twice in 125 uL Large Fragment Buffer (LFB). Elute in 15 uL EB and quantify before proceeding with sequencing, as per protocol, loading 10-20 fmol finished library per flow cell (diluting in further EB if required). Store the library at 4 C for up to one week, or -80 C for longer, minimizing freeze-thaw cycles required prior to sequencing.