Ramaciotti Centre for Genomics
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Sample Requirements: Long and Linked Read Sequencing Services

UNSW SYDNEY
Australia's Global University

Contents

1 INTRODUCTION

Obtaining long or linked sequencing reads requires long undamaged DNA fragments, as the quality of the DNA starting material will be directly reflected in the sequencing results. Any irreversible DNA damage present in the input material (e.g. inter-strand crosslinks, etc.) will result in impaired performance. High quality, high molecular weight genomic DNA is imperative for obtaining long read lengths or linked read information and ensuring optimal sequencing performance.

2 GENERAL GUIDELINES FOR HANDLING HIGH MOLECULAR WEIGHT DNA

In general, the following precautions should be taken when handling genomic DNA:

- Avoid over drying of genomic DNA. Allow DNA to air dry and do not use heat. Do not use heat if drying in a vacuum dryer.
- When resuspending DNA be gentle. Either carefully invert the tube several times after adding buffer and/or tap the tube gently. Alternatively, allow the DNA to stand in buffer overnight at room temperature (20-22°C) to resuspend.
- Avoid vortexing or harsh pipetting as it can shear genomic DNA.
- DNA should be eluted in neutral, buffered solution (e.g. 10 mM Tris Acetate or Tris-HCl, pH 8).
- To prevent enzymatic inhibition during downstream sample library preparation avoid buffers containing EDTA (e.g. TE).
- If gel purification is required avoid using ethidium/UV based visualization methods. An alternative method is SYBR® Safe (Thermo/Invitrogen) and visualization with blue light.
- If RNAase treatment is required follow vendor instructions. However, it is best to avoid heat inactivation where possible as overheating can introduce DNA damage. An alternative method is AMPure® XP bead purification. It is essential that XP beads must be washed prior to use. Refer to section 8 for further details.
- DNA should be stored at 4°C (short-term) or –20°C / –80°C (long-term).
- Repeated freezing and thawing of genomic DNA should be avoided as this will lead to DNA shearing.
- For amplicon sequencing PCR products should be clean and single band product, i.e. no non-specific products or multiple bands visible on an agarose gel.

3 FACTORS THAT IMPACT DNA QUALITY

To maximize read length and quality, it is ***essential*** that the DNA sample:

- Is double-stranded.
- Has not undergone multiple freeze-thaw cycles as this can lead to DNA damage.
- Has not been exposed to high temperatures (e.g. > 65°C for 15 min) or pH extremes (< 6 or > 9).
- Has an OD260/OD280 ratio of 1.8 to 2.0 and an OD260/OD230 ratio of ~2.0.
- Does not contain insoluble material.
- Does not contain RNA contamination.
- Has not been exposed to intercalating fluorescent dyes or ultraviolet radiation. SYBR dyes are not DNA damaging. Avoid ethidium bromide.
- Does not contain denaturants (e.g., guanidinium salts or phenol) or detergents (e.g. SDS or Triton X100).
- Does not contain carryover contamination from the original organism/tissue (haem, humic acid, polyphenols, etc.)

4 QUALITY ASSESSMENT

The following checks are required steps to ascertain DNA integrity, purity and concentration before submitting samples for sequencing:

1) **Gel images of DNA sample:** Genomic DNA integrity should be assessed by pulse field gel electrophoresis. Regular agarose gels do not have a sufficient resolution to determine integrity of high molecular weight DNA. If you do not have access to a pulse field system a 0.8%-1.2% agarose gel showing one predominant band of high molecular weight DNA with no degradation will act as a guide. Pulse field gel analysis is included in the Centre's sample QC workflow.

2) **Purity of your DNA sample:** DNA purity should be determined by using the NanoDrop® instrument or equivalent spectrophotometer. Readings of both A260:A280 and A260:A230 ratios need to be obtained. Low ratios indicate the presence of contaminants. Additional sample purification may be required.

3) **Concentration of your DNA sample:** Qubit® or Picogreen® must be used to specifically measure double stranded DNA. Please note that NanoDrop® is not specific for dsDNA. A large discrepancy between Nanodrop and Qubit concentrations indicates the presence of contaminants. Additional sample purification may be required.

5 QUANTITY OF DNA REQUIRED

The table below summarises the quantity and concentration of DNA required for PacBio or 10x Genomics library preparation and sequencing:

Library Insert Size	Minimum Quantity*	Minimum Concentration**
PacBio Amplicon pools 1-6kb	2ug	50ng/ul
PacBio Microbial Multiplexing (no size selection)* **	3.5ug	75ng/ul
PacBio Microbial Multiplexing (size selected)* **	3.5ug	75ng/ul
PacBio 10kb (AMPure kit)	6ug	250ng/ul
PacBio 10-20kb (Blue Pippin kit)	21ug	250ng/ul
PacBio 30-40kb (Blue Pippin kit)	27ug	250ng/ul
10x Genomics -Chromium (BluePippin >40kb size selection)	5ug	120ng/ul
10x Genomics - Chromium (no size selection)** **	400ng	15ng/ul

***** Recommended amounts for submission represent DNA quantities required for one library prep and additional QC.

****** Please note that purely spectrophotometric based methods will overestimate concentration.

*** Conditions apply. Refer to Ramaciotti staff for guidance. Minimum multiplexing number is four genomes.

**** DNA must be fully intact and >50kb, as assessed by pulsed field gel. An image of the gel must be provided on submission. Please note that regular agarose gels do not have a high enough resolution to determine if DNA is >50kb.

6 DNA Extraction Guidelines

6.1 Before DNA extraction:

a. Avoid incubation in complex or rich media.

b. Harvesting from several replicate cultures rather than a single, high-density culture during early to mid-logarithmic growth phase is preferred.

c. Extraction of small volumes is preferred over large volumes to avoid accumulating high concentrations of potentially inhibiting secondary components.

6.2 Options for DNA Extraction:

a. QIAGEN® MagAttract® HMW kit (100-200 kb)

b. QIAGEN® Genomic-tip kit (50-100 kb)

c. QIAGEN® Gentra® Puregene® kit (100-200 kb)

d. Phenol-chloroform extraction (protocol available for download on PacBio's website).

Important Note! Ensure phenol is fresh and not oxidized; use within three months of opening of reagent bottle.

6.3 Additional DNA Clean Up

If additional sample clean up is required we recommend the following methods. If required the Ramaciotti Centre can perform AMPure® bead clean up for an additional charge.

a. Purification of DNA with the AMPure® XP kit. Please note that if using AMPure® XP beads, the beads must be washed before use (refer to section 8). AMPure® PB beads can be used without pre-washing.

b. The Mo Bio PowerClean® kit is recommended for highly contaminated samples. Multiple samples can be prepared simultaneously, however, some modifications to the protocol are suggested below in order to maintain high molecular weight DNA and minimise damage:

i. After adding Buffer 2 quickly vortex and add Buffer 3. Minimise time exposure of DNA in Buffer 2 to prevent damage.

ii. After adding Buffer 3 and vortexing add 1 µl of glycogen and vortex before incubation on ice.

ii. During the final elution step elute at 50 µl, pulse spin for 1 second in a microcentrifuge, and incubate for 1 min at room temp. Proceed as stated in the protocol by adding 50 µl of eluent to the membrane for a 1 minute incubation and final spin of 2 minutes.

Important Note! DNA recovery is low following this procedure: 10 µg of genomic DNA per column results in ~30-50% recovery. It is important that you do not overload columns, lower sample input will result in higher recovery, whereas a high sample input will result in lower recovery.

Please note that listed third party products are not officially endorsed by the Ramaciotti Centre and are only provided as possible options.

7 SHIPPING GUIDELINES

- Please ship via the fastest method available (same day/overnight if available).
- Ensure all our contact details are correct and that you have forwarded on the tracking details to the contact person at the Ramaciotti Centre.
- For interstate and overseas shipping, schedule your shipments to go out at the start of the week (Monday or Tuesday).
- If you are shipping from overseas the Ramaciotti Centre can provide an import permit.
- DNA stored at 4°C, should be shipped on enough 4°C cold packs to maintain temperature throughout the journey.
- DNA that has been stored at -20°C should be shipped on enough frozen ice packs to maintain temperature throughout the journey.

8 AMPURE® XP BEAD PRE-WASH PROTOCOL

Important Note! Follow the protocol below before using AMPure® XP beads to clean up DNA samples.

AMPure® XP Bead Pre-Wash

Before preparing your SMRTbell™ library templates using AMPure XP beads, you must perform a bead pre-wash step. There is a contaminant that elutes from the beads (simultaneously with DNA) and interferes with polymerase binding, particularly when the DNA is in low amounts.

To wash the beads:

1. Thoroughly resuspend your AMPure XP beads.
2. Pipette 500ul of AMPure XP beads into a 1.5mL microtube.
3. Spin down 1 minute at 16K rpm (or maximum speed) in a bench top microcentrifuge.
4. Place the tube on a magnet rack.
5. Remove and **save** supernatant. It is important to save the supernatant.
6. Add 1mL Molecular Biology Grade water to the beads, and vortex to resuspend beads completely.
7. Spin down 1 minute at 16K rpm (or maximum speed) in a bench top microcentrifuge.
8. Place tube on a magnet rack.
9. Remove and discard water wash.
10. Repeat water wash 4 more times (steps 6 to 8).
11. Add 1ml 10mM TrisHCl, pH 8.5 (Qiagen® buffer EB can be used) to beads, and vortex to resuspend beads completely.
12. Spin down 1 minute at 16K rpm (or maximum speed) in a bench top microcentrifuge.
13. Place tube on a magnet rack.
14. Remove and discard Tris wash.
15. Resuspend beads in original AMPure XP supernatant reserved in step 5. Note that you may need to re-vortex the beads to resuspend.

The beads are now ready for use in preparing your samples. Note that the washed beads can be stored at 4ºC for at least 3 months.