SAMPLE PREPARATION INSTRUCTIONS FOR SUCCESSFUL SMRTbell LIBRARIES
(modified from guidelines provided by Pacific Biosciences)

The Pacific Biosciences library preparation process does not utilize amplification techniques and the resulting library molecules are directly used as templates for the sequencing process. As such, 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., interstrand crosslinks, etc.) or sample contaminants can result in impaired performance in the system. High-quality, clean and high-molecular-weight genomic DNA is imperative for obtaining long read lengths and optimal sequencing performance.
Please be warned that if any of these requirements are not met, the GTF cannot guarantee that sequencing of your samples will be successful. In the case of poor sequencing performance due to template-related problems, we cannot reimburse you for either the library prep or the sequencing costs.

I. DNA/RNA quantity

We will require a variable amount of good quality DNA depending on the size of your insert for sequencing and type of project. Please be aware that excess DNA is needed since initial fragmentation and AMPure bead concentration/clean up steps can be subject to unavoidable template loss. If the template is very limited/low, contact us to find the best approach.

Library type	**Minimum amount needed per sample (measured by Qubit)**
WGS	3ug
Bacteria multiplexing	250ng normalized in 15ul, **no EDTA,** PCR plate filled in column
Ultra low input	1-50ng, max 65 ul
Kinnex 16S	100ng (pool)
Kinnex RNA-seq	300ng RIN >8, max 7 ul
Kinnex single cell	15-75ng cDNA single-cell, max 15ul
Full length 16S rRNA	200ng (pool)
Amplicons	200ng-500ng (pool), depending on the size
PCR-free targeted seq. e.g Pure Target	4ug
Targeted seq with panel	500ng
Fiber-seq	3ug

Mandatory before giving gDNA samples to the GTF:

- **gDNA samples have to be RNAse treated; RNAse has to be preferentially removed during the extraction procedure. The final purification step has to be discussed if nanodrop ratios are low.**
- **ensure that the genomic DNA is in an appropriate buffer (e.g.,Qiagen Elution Buffer (EB), 10 mM Tris-Cl pH 8.5, no or low EDTA)**
- **Submission form including quality metrics (section V)**

II. Important measures impacting DNA quality

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

- is double-stranded; single-stranded DNA cannot be used to generate the sequencing template.
- has not undergone multiple freeze-thaw cycles as they can lead to DNA damage.
- has not been exposed to high temperatures (e.g. > 65 C for 1 hour) or pH extremes (< 6 or > 9).
- has an OD260/OD280 ratio of 1.8 to 2.0.
- has 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, but 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 (e.g., heme, humic acid, polyphenols, etc.)
- The majority of the DNA must be equal or greater than 40 kbp.

III. DNA extraction

These are general recommendations to help obtain high molecular weight DNA.

Options for DNA Extraction

Kit	Method	Sample Amount	Typical Yields
QIAGEN MagAttract HMW DNA Kit	Magnetic bead	• Blood: 200 µL • Bacterial cells: 2 x 10^9 • Tissue: up to 25 mg	• Blood: 4-8 µg • Gram-negative bacteria: up to 14 µg • Gram-positive bacteria: up to 3.5 µg • Tissue: 0.5-2.8 µg / mg tissue
QIAGEN PAXgene Blood DNA Kit	Precipitation	• Blood: 8.5 mL	• 150-500 µg depending on the number of nucleated cells
QIAGEN Gentra Puregene Kit	Precipitation	• Cells: up to 6.7 x 10^9 • Tissue: up to 100 mg	• 7 µg per 1 million cells • Tissue: 5-100 µg
QIAGEN Genomic-tip 20/G Kit	Anion exchange column	• Blood: 1 mL • Cells: 5 x 10^6 • Yeast: 1.5 x 10^9 • Bacteria: 4.5 x 10^9 • Tissue: up to 20 mg	• 1-20 µg
Circulomics Nanobind CBB Kit	Nanobind disc	• Blood: 200 µL • Cells: 1 x 10^6 • Bacteria: 5 x 10^8 - 5 x 10^9	• 5-34 µg depending on sample type and input amount

Kit	Method	Sample Amount	Typical Yields
Circulomics Nanobind Tissue Big DNA Kit	Nanobind disc	• Depends on tissue and preservation method; typically around 25 mg	• 5-100 µg
Circulomics Nanobind Plant Nuclei Big DNA Kit	Nanobind disc	• Up to 10 g	• 5-20 µg
Lucigen MasterPure Kit	Precipitation	• Cells: 1 x 10^6 • Blood: 200 mL • Bacteria: 3.5 x 10^6	• Cells: 3-12 µg • Blood: 3-9 µg • Bacteria: 1.3-1.6 µg
NEB Monarch Genomic DNA Purification Kit	Anion exchange column	• Blood: 100 µL • Bacteria: 2 x 10^9 • Cells: 5 x 10^6 • Tissue: 10 mg	• Blood: 2.5-4 µg • Gram-negative bacteria: 6-10 µg • Gram-positive bacteria: 6-9 µg • Mammalian cells: 7-9 µg • Tissue: 5-30 µg
Macherey-Nagel NucleoBond HMW DNA Kit	Anion exchange column	• Blood: 2 mL • Plant: 1.5 g of leaves • Bacteria: up to 100 mg • Cells: 10^7 • Animal tissue: up to 300 mg	• Dependent on sample type and input amount
QIAGEN DNeasy PowerMax Soil Kit	Bead-beating; Anion exchange	• Soil: up to 10 g	• Sample dependent
QIAGEN QIAamp PowerFecal DNA Kit	Bead-beating; Anion exchange	• Stool or biosolids: 250 mg	• Sample dependent

Bacteria growth

Before DNA extraction:

a. Avoid incubation in complex or rich media.

b. No growth on plates but in liquid media

c. Harvest from several replicate cultures rather than a single, high-density culture during **early- to mid-logarithmic growth phase**.

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

Plants and filamentous fungi

DNA extraction from plants and micromycetes possesses multiple challenges due to high levels of polysaccharides, phenolic compounds and a variety of secondary metabolites in the starting material. It is therefore recommended to use high-salt, 1% CTAB clean-up of such samples. You can either use CTAB-containing extraction buffer or use an additional CTAB clean-up of the DNA precipitate. CTAB removes the majority of the polysaccharides and some lipids, however the CTAB itself must be carefully removed from the prep, since it can inhibit the sequencing process.

If phenol-chloroform DNA extraction was performed, make sure phenol has been completely removed. Phenol has an absorption spectrum between 270-275 nm, which is very close to that of DNA. Phenol contamination can artificially inflate spectrophotometer readings and mimic both higher purity and concentration of the sample.

The QIAGEN Genomic-tip 500/G or MagAttract kits are highly recommended even for plant and fungal material. An additional CTAB clean-up is highly recommended as well. Make sure not to overload the columns with start material.

IV. General guidelines for handling high-molecular-weight DNA

In general, the following precautions need to be taken when handling DNA:

- **Use wide bore tips if possible**
- Avoid overdrying of genomic DNA. Allow the DNA to air dry. Do not heat when drying in a speed-vac.
- DNA should be eluted in neutral, buffered solution (e.g., 10 mM Tris Acetate or Tris-HCl, pH 8). Avoid eluting in RNAse-free H_2O or unbuffered solutions.
- PCR products should be clean amplicons, without non-specific products or multiple bands.
- If gel purification is required, avoid using ethidium/UV based visualization methods. One alternative is to use SYBR® Safe (Invitrogen) and visualize with blue light.
- To help resuspend the DNA, 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 25°C to resuspend.
- **Overheating can introduce DNA damage. If inactivate DNAse is recommended by the vendor kit, it is best to avoid heat inactivation when possible. An alternative is AMPure® purification.**
- **Avoid vortexing** genomic DNA when possible as vortexing can cause shearing of the DNA.
- DNA storage conditions: 4°C (short-term); –20°C (long-term).
- Repeated freezing and thawing of genomic DNA should be avoided as this will lead to DNA shearing.

V. DNA sample quality assessment

A thorough DNA quality check is required prior to submitting DNA for PacBio sequencing. The following steps to ascertain DNA integrity, purity, and concentration are recommended:

1) **Gel images of DNA sample (pulse field):** Genomic DNA integrity can be assessed by agarose gel electrophoresis. For best results, DNA samples must show little/no signs of degradation, which is evidenced by smeared DNA bands. The presence of one predominant band showing high MW DNA with no degradation is optimal. A good practice is to indicate relevant marker sizes, and the amount of sample loaded in the agarose gel. For amplicon or cDNA samples, a Bioanalyzer trace can be used as an alternative.

   If gel purification of your DNA sample is required, we recommend using SYBR Gold or SYBR Safe coupled with blue light for visualization. Do not use ethidium bromide and/or UV light since they can induce DNA damage. The SYBR stains can be easily removed from nucleic acids during the gel-extraction process, e.g., using Qiagen gel-extraction kits.

2) **Purity of your DNA sample:** DNA purity can be determined by using the NanoDrop instrument or other spectrophotometers. Readings of both A260:A280 and A260:A230 ratios need to be obtained:

260/280 ratio:

The ratio of absorbance at 260 nm and 280 nm is used to assess the purity of DNA. A ratio of ~1.8 is generally accepted as “pure” for DNA, but is dependent on the nucleotide composition of the submitted sample.

A low A260/A280 ratio may indicate the presence of protein, phenol, or other contaminants that absorb strongly at or near 280 nm. Sometimes it may be caused by a very low concentration of nucleic acid.

High 260/280 ratios are normally not problematic.

Ensure that DNA measurements are conducted in a buffered environment such as (TE or Tris HCl, pH8). Measurements are sensitive to small changes in the pH of the solution which will cause the 260/280 ratio to vary. Acidic solutions will skew the 260/280 ratio lower, while basic solutions will skew the ratio higher.

260/230:

The 260/230 ratio provides a secondary measurement of DNA purity to make inferences about the quality of sample extraction. Values are normally higher than the respective 260/280 values. Expected 260/230 values are commonly in the range of 2.0-2.2. Abnormal 260/230 values may indicate a problem with the sample extraction procedure.

A low A260/A230 ratio may be the result of:

- Carbohydrate carryover (often a problem with plants).
- Residual phenol from nucleic acid extraction.
- Residual guanidine (often used in column-based kits).
- Glycogen used for precipitation.

A high A260/A230 ratio may be the result of:

- Making a blank measurement on a dirty pedestal of a Nanodrop instrument.
- Using an inappropriate solution for the blank measurement.

The blank solution should be the same pH and of a similar ionic strength as the sample solution.

3) **Concentration of your DNA sample:** Accurate quantitation of DNA concentration is critical for the PacBio template preparation procedures. Traditional spectrophotometric assays cannot accurately determine DNA concentrations <15 ng/µl. More importantly, almost all spectrophotometric assays do not distinguish between different types of nucleotides (e.g., double-stranded DNA, RNA, dNTPs, and single-stranded DNA). While the presence of single-stranded DNA will not impair library preparation, this will result in inaccurate yield quantitation.

For PacBio library preparation, it is critical to determine the concentration of the double-stranded DNA, since only double-stranded DNA will be converted into sequencing templates. RNA, dNTPs, and single-stranded DNA included in the concentration measurement will skew the concentration reading. Therefore, **it is highly recommended to use the PicoGreen assay or a Qubit fluorimeter for quantitation purposes.**

Please closely follow the recommended guidelines provided by the respective vendors when carrying out quantitation assays. In particular, the following steps should be observed: 1) periodically have the instrument calibrated (preferably by the vendor); 2) conduct a standard curve alongside samples when using the Picogreen or Qubit assays; 3) perform replicate readings of concentration and use the average of replicates as the final

concentration whenever possible. Accurate, consistent pipetting skill is needed to obtain reliable quantitation information.

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