Q&A for "DNA Quality Requirements for Single Molecule, Real-Time (SMRT®) Sequencing"

1. **Have you sequenced FFPE DNA samples using SMRT Sequencing?**

   Answer: Yes – FFPE samples have been sequenced using SMRT Sequencing. For further details please see the following poster that was presented at AGBT 2015: http://www.pacb.com/wp-content/uploads/SMRT-Sequencing-Formalin-Fixed-and-Paraffin-Embedded-Tissues-Covaris.pdf

   Poster abstract is shown below:

   SMRT Sequencing of DNA and RNA samples extracted from formalin-fixed and paraffin-embedded tissues using adaptive focused acoustics by Covaris.

   Author(s): Baybayan, Primo and Weiand, Michael and Eng, Kevin and Durin, Guillaume and Packard, Brian and Sethuraman, Anand and Hon, Lawrence and Kujawa, Steve

   Recent advances in next-generation sequencing have led to an increased use of formalin-fixed and paraffin-embedded (FFPE) tissues for medical samples in disease and scientific research. Single Molecule, Real-Time (SMRT®) Sequencing offers a unique advantage for direct analysis of FFPE samples without amplification. However, obtaining ample long-read information from FFPE samples has been a challenge due to the quality and quantity of the extracted DNA. FFPE samples often contain damaged sites, including breaks in the backbone and missing or altered nucleotide bases, which directly impact sequencing and target enrichment. Additionally, the quality and quantity of the recovered DNA vary depending on the extraction methods used. We have evaluated the Covaris® Adaptive Focused Acoustics (AFA) system as a method for obtaining high molecular weight DNA suitable for SMRTbell™ template preparation and subsequent PacBio® RS II sequencing. To test the Covaris system, we extracted DNA from normal kidney FFPE scrolls acquired from the Cooperative Human Tissue Network (CHTN), University of Pennsylvania. Damaged sites in the extracted DNA were repaired using a DNA Damage Repair step, and the treated DNA was constructed into SMRTbell libraries for sequencing on the PacBio System. Using the same repaired DNA, we also tested the efficiency of PCR in amplifying targets of up to 10 kb. The resulting amplicons were also constructed into SMRTbell templates for full-length sequencing on the PacBio System. We found the Adaptive Focused Acoustics (AFA) system by Covaris to be effective. This system is easy and simple to use, and the resulting DNA is compatible with SMRTbell library preparation for targeted and whole genome SMRT Sequencing. The data presented here demonstrates feasibility of SMRT Sequencing with FFPE samples.
   Organization: Pacific Biosciences
   Year: 2015

2. **Do you have any recommendations for gene-specific PCR library preparation, such as immunoglobulin repertoires, and how to maximize the diversity of sequences?**

   Answer: Takara LA Taq DNA Polymerase, Hot-Start Version is a high-performance enzyme from Clontech that is optimized for long-range PCR. For further information, please see the Clontech website: http://www.clontech.com/FO/Products/PCR/Long_PCR/LA_Taq_Hot-Start_Version

3. **Any suggestions for DNA extraction of algae?**

   Answer: For detailed information on algea DNA extraction please click the link below for the 'DNA extraction of *Chlamydomonas* using CTAB' protocol (http://www.pacb.com/wp-content/uploads/2015/09/DNA-extraction-chlamy-CTAB-JGI.pdf)

4. **Any experience with gelase-treated DNA?**

   Answer: Yes, but it is not a good one. The remnants of agarose, as well as the gelase itself must be thoroughly removed. We have seen some users to try dialysis, with mixed results.

5. **I do a lot of work on mollusks that are slimy: lots of long-chain sulfonated mucopolysaccharides that tend to follow DNA around. Any suggestions for dealing with these?**

   Answer: It may be helpful to consider performing a phenol-chloroform cleanup of the extracted genomic DNA prior to SMRTbell library prep by following the steps outlined in PacBio's 'Guidelines for Using a Salt:Chloroform Wash to Clean Up gDNA' document: http://www.pacb.com/wp-content/uploads/2015/09/Shared-Protocol-Guidelines-for-Using-a-Salt-Chloroform-Wash-to-Clean-Up-gDNA.pdf

6. **You recommended the washing of bacterial cells prior to DNA extraction. Can you indicate with what (e.g. culture media)? What is the purpose of this washing?**

   Answer: Before DNA extraction, bacterial cells grown in liquid culture can be washed with a buffer such as 1x PBS to help reduce levels of accumulated extracellular compounds and/or growth culture medium components that could interfere with downstream SMRTbell library prepration and/or SMRT Sequencing.

7. **What are your thoughts on how to ensure DNA fragments above 20 kb for eukaryotes?**

   Answer: For isolating high-molecular weight genomic DNA from eukaryotes, one may consider using the following kit options:

   - Qiagen Genomic-tip kit (50–100 kb) Product Information
   - Qiagen Gentra® Puregene® kit (100–200 kb) Product Information

   An example protocol from the University of Washington for using the Gentra Puregene Kit for isolating human genomic DNA can be found here: http://www.pacb.com/wp-content/uploads/2015/09/Gentra-Puregene-Qiagen-DNA-Isolation.pdf

The following open-access articles also contains a number of examples of DNA extraction methods that were successfully employed to prepare yeast, plant, fungal and insect samples for SMRT Sequencing: http://www.nature.com/articles/sdata201445#methods, Long-read, whole-genome shotgun sequence data for five model organisms, *Scientific Data*, 1:140045 (2014)

8. **Regarding lyophilized maize leaf tissue, I have not been able to recover high-molecular weight DNA. Is it always sheared?**

   Answer: For isolating high-molecular genomic material from plant leaf tissue, it is best to start with fresh leaf material. The following example protocols may be helpful for isolating gDNA from plant leaf samples:

   Switchgrass (*Panicum virgatum*) DNA isolation (http://www.pacb.com/wp-content/uploads/2015/09/Switchgrass-DNA-isolation.pdf)
   Preparing *Arabidopsis* Genomic DNA for Size-Selected ~20 kb SMRTbell™ Libraries (http://www.pacb.com/wp-content/uploads/2015/09/Shared-Protocol-Preparing-Arabidopsis-DNA-for-20-kb-SMRTbell-Libraries.pdf)

9. **We have eluted DNA in a high volume of TE (1:10) (because we have low yield and many replicates) that we dry on speed vac. We think that there might be a high quantitity of salt in the samples. Is this a contaminant? If so, what can we do?**

   Answer: The presence of excess residual salt contaminants can have a negative downstream impact on SMRT Sequencing. In order to remove excess residual salt contaminants from the starting genomic DNA material (prior to shearing), one may consider performing a 1X AMPure bead cleanup of the gDNA or a standard ethanol precipitation.

10. **a) Why use a spin-filter for post clean up? Spin-filters are notorious for breaking DNA into ~40 kb pieces. This then makes fragmenting the DNA down to 20 kb or larger much more difficult.**
    b) The two post-extraction cleanup kits you recommended are both column-based. Any solution-based cleanup methods?

    Answer: As an alternative to the MoBio PowerClean Kit and Zymo Research Genomic DNA Clean & Concentration Kit, you could consider performing a 1X AMPure bead cleanup or a high-salt phenol chloroform wash post gDNA extraction (http://www.pacb.com/wp-content/uploads/2015/09/Shared-Protocol-Guidelines-for-Using-a-Salt-Chloroform-Wash-to-Clean-Up-gDNA.pdf). However if the gDNA material still shows one of the following characteristics then you might not have an option.

    - The gDNA concentration is less than 200 ng/µL.
    - The OD 260/280 is less than 1.8 or greater than 2.0.
    - The gDNA concentration determined by a Qubit fluorometer is significantly less than the concentration determined by a NanoDrop spectrophotometer. This may indicate RNA contamination.

11. **In our laboratory, we always do some tissue maceration with magnetic bead extraction. DNA integrity is never higher than 10 kb. Do you think that is a result of mechanical grinding or selective binding by magnetic bead?**

    Answer: It is highly possible that the DNA integrity is being negatively impacted by the mechanical grinding effects. PacBio does not have extensive data to evaluate the effectiveness of magnetic bead-based DNA extraction methods, but a few external customers have reported success using QIAgen's MagAttract HMW DNA Kit (https://www.qiagen.com/ca/shop/sample-technologies/dna-sample-technologies/genomic-dna/magattract-hmw-dna-kit-48/#productdetails) for isolating high-molecular weight DNA from various sources.

12. **A visiting MoBio rep recently told me they are discontinuing the PowerClean kit. Any experience with the PowerClean Pro kit that appears to be the replacement? The rep was unable to answer my questions about whether size and yield were better with the Pro version.**

    Answer: We have no experience with it.

13. **What percentage of mercapto do you recommend for extraction of plant nuclear DNA? I have seen percentages of up tp 7%.**

    Answer: We are not performing the extractions ourselves, but have seen successful user protocols with 2%. One may need to determine the optimal concentration experimentally.

14. **What are your recommended lysis times?**

    Answer: Those depend greatly upon the organism that you are working with. I would suggest a pilot experiment with a time curve to determine which timing gives the best DNA yield.

15. **What DNA quality control would you recommend for FFPE DNA extraction, e.g. measurement/QC?**

    Answer: We are only aware of the Tape Station (Agilent Technologies) that estimates DIN (DNA Integrity Index).

16. **Are measurments with the Qubit instrument more reliable than the results of the cDrop?**

    Answer: PacBio is currently in the process of evaluating the DropSense96 analytical tool.

17. **Do you recommend separating DNA on a pulse-field gel to determine both the integrity (e.g., molecular weight) and purity?**

    Answer: To ensure the most accurate characterization of the size distribution of unsheared and sheared genomic DNA samples, the size and integrity of your gDNA should be assessed using a pulsed-field gel electrophoresis system before beginning SMRTbell library preparation. For the construction of large-insert libraries (>30 kb), PacBio highly recommends using the Bio-Rad CHEF Mapper XA Pulsed Field Electrophoresis system to achieve resolution of DNA fragments 50 kb and higher. (Other PFGE systems

may not provide sufficient resolution above 50 kb.) Genomic DNA suitable for preparing >30 kb libraries will migrate almost exclusively above the ~50 kb size marker.

To assess the purity of gDNA samples, PacBio recommends that an analysis of spectrophotometric absorbance ratios be performed using the NanoDrop tool or equivalent system. In particular, A260/A280 and A260/A230 ratios should be measured. Use the following general guidelines to perform QC analysis of the absorbance ratios:

- The absorbance maxima for nucleic acids is at 260 nm while the absorbance maxima for proteins is at 280 nm
- A260/A280 ratio of ~1.8 is generally accepted as "pure" for DNA.
- A260/A230 ratio of 1.8-2.2 is generally accepted as "pure" for nucleic acid.
- A low 260/280 ratio may be the result of a contaminant such as protein or a reagent such as phenol, that is absorbing at 280 nm or less. In some cases, this can also be due to issues with measurement – therefore, Nanodrop QC readings should be repeated to rule out any potential issue with measurement as the cause of this. High 260/280 purity ratios are generally not indicative of an issue with the sample.
- A low A260/A230 ratio may be the result of a contaminant absorbing at 230 nm or less. Such contaminants include carbohydrates, residual phenol, residual guanidine and/or glycogen. On the other hand, a high A260/A230 ratio may be the result of either making a blank measurement on a dirty pedestal or using a blank solution that is not of a similar ionic strength as the sample solution

For additional information regarding assessment of DNA purity using spectrophotometric absorbance ratio measurements, please refer to the following NanoDrop Technical Bulletin: T042-TECHNICAL BULLETIN NanoDrop Spectrophotometers (http://www.nanodrop.com/Library/T042-NanoDrop-Spectrophotometers-Nucleic-Acid-Purity-Ratios.pdf)

18. Is there a minimun concentration of DNA needed for PacBio sequencing, and which is the best method to measure it?

Answer: The minimum amount of starting input DNA material needed for PacBio sequencing depends on the desired insert size of the SMRTbell library. The Table below shows suggested quantities of starting DNA that should be submitted to a PacBio Service Provider to enable QC assessment and generation of SMRTbell libraries of different target insert sizes. In addition, the recommended minimum quantities of input DNA required for carrying out each type of SMRTbell Library Prep Protocol are also shown. Generally, DNA samples submitted to PacBio Service Providers for SMRTbell library prep and sequencing should have a minimum concentration of ~50 ng/µl (ideal range is ~150 - 200 ng/µl or higher; for 20 kb or larger libraries, the samples should have a concentration of ~ 200-300 ng/µl or higher).

Target Library Insert Size	Recommended DNA Quantity for Submission to PacBio Service Provider for QC and Library Prep*	Min Input DNA Amount Required for SMRTbell Library Prep Protocol (Post-Shearing)**
250 bp	600 ng	250 ng
500 bp	600 ng	250 ng
1 kb	1.2 µg	500 ng
2 kb	1.2 µg	500 ng
5 kb	2.4 µg	1 µg
10 kb	2.4 µg	1 µg

20 kb (BluePippin™ device)	15 µg	5 µg
30 kb (BluePippin™ device)	20 µg	5 µg

* DNA input amounts recommended for submission represent quantities needed for one (1) SMRTbell library prep and includes extra quantity needed for any additional QC (with conservative excess).

** Refer to the appropriate PacBio SMRTbell Template Procedure & Checklist document at http://www.pacb.com/support/documentation/ for further details regarding minimum DNA amounts shown.

PacBio highly recommends that DNA concentration measurements be performed using a double-stranded DNA (dsDNA)-specific fluorometric-based assay such as a Qubit Fluorometer system. Compared to UV spectrophotometric methods, fluorescence-based quantitation is generally more sensitive and is often specific for the nucleic acid of interest. The Qubit dsDNA assay employs a fluorescent DNA-binding dye that enables sensitive and specific quantitation of small amounts of dsDNA in solution. The dye shows minimal binding to single-stranded DNA (ssDNA) and RNA. [In contrast, UV absorbance measurements are not selective and cannot distinguish DNA, RNA, or protein. Values are easily affected by other contaminants (e.g., free nucleotides, salts, and organic compounds) and variations in base composition. In addition, the sensitivity of spectrophotometry is often inadequate, prohibiting quantitation of DNA and RNA at low concentrations.] Ensure the following practices are followed when performing a fluorometric Qubit analysis:

- Ensure bubbles are not introduced into the sample at the time of the reading as this can affect the results. Slight tapping on the tube wall or brief centrifugation will often help dissipate bubbles.
- If you get a concentration value as “too high” or “too low” it means that your sample is out of range. As needed, use a sample that is more concentrated or use a lower dilution.
- The assay should be performed at room temperature and the assay tubes must be at room temperature at the time the reading is taken. Do not hold assay tubes in your hand for too long while trying to read the samples

19. **Do you have any experience where the Qubit and Nanodrop readings are very different, and then Dropsense analysis groups it under nucleic acid but not dsDNA. Could this be caused by contaminants or is DNA single stranded due to the way it was extracted?**

Answer: In cases where the Qubit reading and Nanodrop readings are very different, it is advisable to perform additional clean-up steps since this often indicates that the sample contains contaminants and the DNA purity is low. In these types of situations it may be useful to consider performing a 1X AMPure bead cleanup of the starting DNA material or a high-salt phenol chloroform wash (http://www.pacb.com/wp-content/uploads/2015/09/Shared-Protocol-Guidelines-for-Using-a-Salt-Chloroform-Wash-to-Clean-Up-gDNA.pdf). The MoBio PowerClean Kit and Zymo Research Genomic DNA Clean & Concentration Kit discussed in the webinar may also be considered.

PacBio is currently in the process of evaluating the DropSense96 analytical tool.

20. **With the Nanodrop device, samples are measured in a broad spectrometric range. Did you investigate if wavelength values (or ratio's) other than the 230, 260 and 280 can be used to determine specific contaminants or components in the sample (as with the Triean system)? The Nanodrop device has some nice technical application notes for contamination detection at different wavelengths.**

Answer: No other length values have been investigated at this point. The effect of different contaminant absorptions in relation to sequencing performance are summarized in the following posters: http://www.pacb.com/wp-content/uploads/2015/09/Sample-Quality-and-Contamination.pdf, http://www.pacb.com/wp-content/uploads/2015/09/Importance-of-Sample-QC.pdf.

For additional information regarding assessment of DNA purity using spectrophotometric absorbance ratio measurements, please refer to the following NanoDrop Technical Bulletin: T042-TECHNICAL BULLETIN NanoDrop Spectrophotometers (http://www.nanodrop.com/Library/T042-NanoDrop-Spectrophotometers-Nucleic-Acid-Purity-Ratios.pdf).