GENOMIC DNA SAMPLE QC

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Author(s): Mansi Chovatia, Aditi Sharma
Reviewer(s): Juying Yan, Kathleen Lail, Timothy Williams, Alba Gutierrez, Chia-Lin Wei

SUMMARY

Before shipping your DNA sample(s), please be sure to follow the JGI sample preparation and sample submission guidelines available at http://jgi.doe.gov/collaborate-with-jgi/pmo-overview/project-materials-submission-overview/

This protocol describes how to perform quality control of DNA samples to evaluate the quantity (using Qubit Fluorometer), quality (using standard agarose gel electrophoresis), and purity (with NanoDrop Spectrophotometer). We recommend all DNA samples to be evaluated with this protocol prior to shipping to JGI for sequencing.

MATERIALS

Materials	Vendor	Part Number
Disposables		
Pipette tips	Many available	n/a
Qubit assay tubes	Life Technologies	Q32856
Microcentrifuge tubes (nuclease-free)	Many available	n/a
Kimwipes	Many available	n/a
Reagents		
70% Ethanol	Many available	n/a
Qubit dsDNA BR Assay Kit	Life Technologies	Q32853
Qubit dsDNA HS Assay Kit	Life Technologies	Q32854
GenePure LE Agarose	ISC BioExpress	E-3120-500
50X TAE Buffer	GrowCells	MRGF-4210
SYBRSafe DNA gel stain (10,000X concentrate in DMSO)	Life Technologies	S33102
TE Buffer, pH 8.0	Life Technologies	AM9849
DNA Molecular Weight Marker II (0.12-23.1kbp)	Roche	10 236 250 001
Gel Loading Dye, Blue (6X)	New England Biolabs	B7021S
Lambda DNA	Thermo Scientific	SD0011
Nuclease-free water	GrowCells	NUPW-0500
Equipment		
Pipettes	Many available	n/a
Qubit Fluorometer 2.0	Life Technologies	Q32866
Microcentrifuge	Many available	n/a
Vortex	Many available	n/a

12x14cm Horizontal Gel Electrophoresis Device	CLP	72.1214
12x14cm Horizontal Device Comb (25 well, 1.5mm)	CLP	72.1214-MT-25D
Molecular Imager Gel Doc XR System w/Image Lab Software	Bio-Rad	170-8170
NanoDrop Spectrophotometer	Thermo Scientific	ND-1000

SAFETY INFORMATION

Safety glasses, lab coat, and nitrile gloves should be worn at all times while performing this protocol. Thermal gloves are recommended when handling warm liquids during gel preparation. For more information, please contact the manufacturer and consult the appropriate MSDS.

PROCEDURE

1. Collect gDNA samples for QC. Until ready to begin lab work, store DNA in a -80°C freezer.
2. Prepare lab bench for use with DNA samples. Wipe lab bench, tube racks, pipettes, and other equipment with 70% Ethanol.
3. After removing DNA samples from the -80°C freezer, immediately transfer to ice. Thaw all DNA samples on ice.
4. Briefly centrifuge tubes to collect droplets from the tube wall and lid.
5. Pipette one DNA sample at a time until the exact volume is determined. Record the volumes.

SAMPLE QUANTITATION

Note:

1. *JGI uses a microplate reader and the Promega QuantiFluor™ dsDNA System kit for quantitation. A three point standard curve along with a blank is used for the assay. If a microplate reader is not available for determining concentration, then Qubit assay for quantitation is recommended.*
2. *If the quantitation by Qubit is performed, then it is recommended to keep Qubit kit reagents bundled in their original lot; i.e. dye, buffer and standards should ideally be of the same lot when evaluating a particular sample/ set of samples.*

1. Gently vortex DNA samples (~4 seconds), centrifuge briefly to collect the sample to the bottom of the tube.
2. Set up the required number of Qubit assay tubes for standards and samples. Label the tube lids appropriately.
3. Make sufficient Qubit working solution for the total number of reactions (standards and samples), by combining 1µl Qubit dsDNA reagent to 199µl Qubit dsDNA buffer for each reaction. For example, for 10 reactions, add:

# reactions	1X	10X
dsDNA Buffer	199	1990
dsDNA Reagent	1	10
Total (µl)	200	2000

4. Add 190µl of Qubit working solution to the assay tubes for the standards. Add 10µl of each Qubit standard to the appropriate tube, and mix by vortexing ~4 seconds. If there are bubbles, briefly centrifuge.
5. Add 198µl Qubit working solution to the assay tubes for samples. Add **2µ**l DNA sample and mix by vortexing ~ 4 seconds. If there are bubbles, briefly centrifuge.
6. Incubate all tubes at room temperature for 2 minutes.

7. On the Qubit 2.0 fluorometer, select your assay (dsDNA Broad Range or dsDNA High Sensitivity), press YES to run a new calibration, and then insert the tube containing Standard #1. Close the lid, and press READ.
8. Insert the tube containing Standard #2, close the lid, and press READ. Calibration of Qubit is now complete.
9. Insert the tube containing DNA sample, close the lid, and press READ. Select calculate concentration, select the volume (2µl), select the measurement units as ng/µl, and record your concentration. Repeat for all DNA samples.
    a. If the sample concentration is too low, then use dsDNA HS Assay kit.
    b. If the sample concentration is too high, then set up serial dilutions of your samples and repeat the assay using dsDNA BR Assay kit.

Important!
We recommend using 2**µ**l of Standard #2 to be treated as a sample to verify the Qubit was calibrated correctly. If the concentration of this standard is >10% of the expected concentration, then please recalibrate the Qubit.

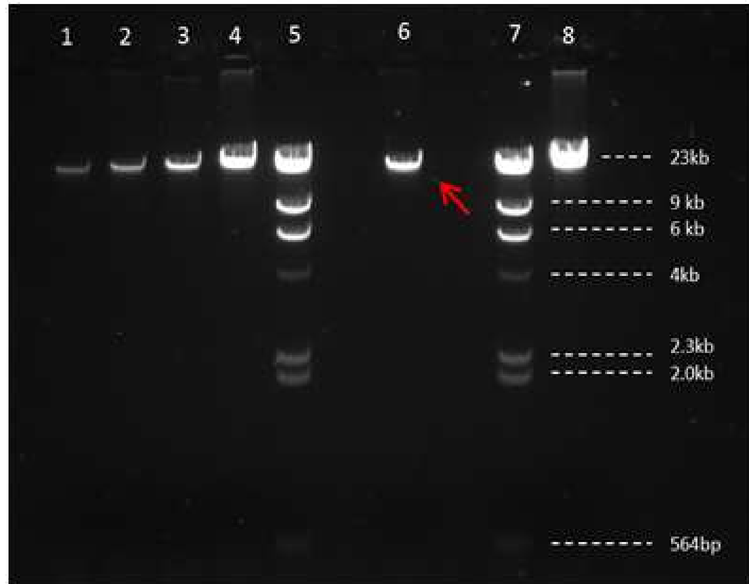
Important!
Review JGI Sample requirements. Please concentrate samples if they are too dilute.

SAMPLE QUALITY

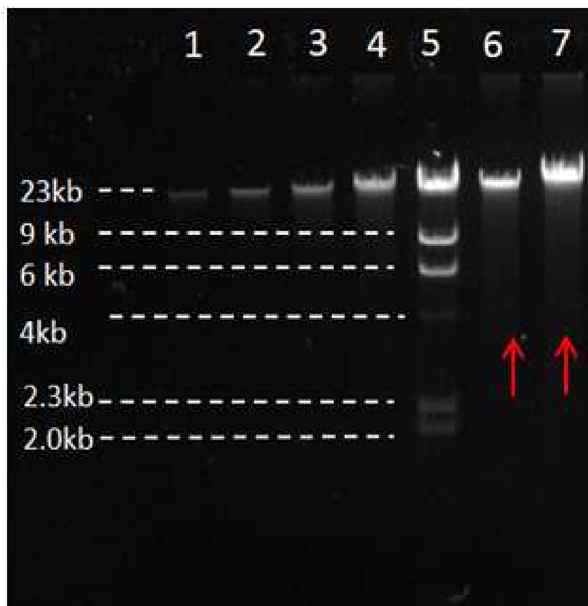
Important!
Sample quality is crucial for large insert libraries.

Note:
1. JGI uses the BioRad Imager amd Image Lab 3.0 to assess DNA quality on agarose gel.

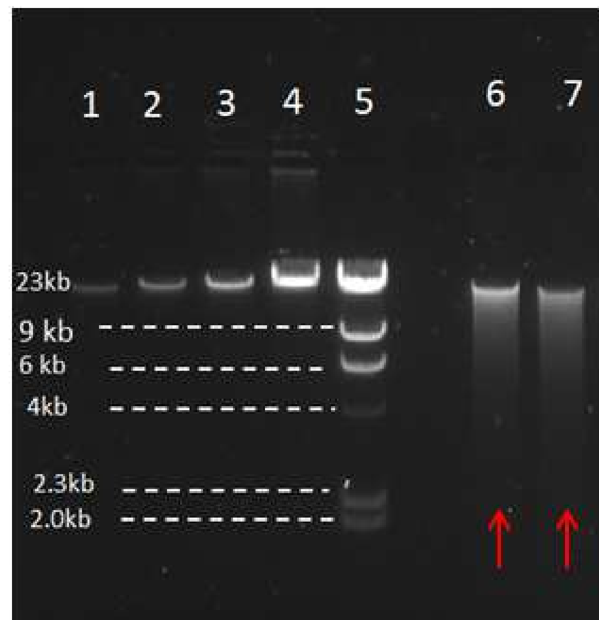
1. Prepare ~100ml 0.7% agarose gel with 1X TAE buffer and SYBRSafe DNA gel stain.
2. Based on the concentrations from the Qubit fluorometer, transfer approximately **30-60ng** of genomic DNA per sample into new microcentrifuge tube. Bring the volume up to 5µl with 1X TE buffer, pH 8.0.
    2.1 If the sample is higher than 100ng/µl, then dilute the sample to ~20ng/µl and transfer 3-5µl.
3. Add 1µl of 6X loading dye. The amount of loading dye should remain consistent among all samples to maintain even DNA migration on the gel. Gently vortex, and briefly centrifuge.
4. Load 6µl of each of the DNA sample(s) and 5µl of Marker II.
5. Optional: Load 5µl of each of the Lambda Mass Standards.
   Note: Refer to Appendix B for preparation of mass standards.
6. Run the gel for 90 minutes at 90V in 1X TAE buffer. If different electrophoresis equipment is being used, ensure the genomic DNA bands have ran ≥2 cm below from the well and the separation of the bands in the ladder is apparent.
7. Image the gel to evaluate the quality of the sample:
    7.1 High molecular weight genomic DNA should appear to be a tight band at ~23kb.
    7.2 Any sign of smearing below 23kb is fragmented/degraded DNA.
    7.3 Any sample stuck in the well of the gel could mean that the sample possibly contains high molecular weight DNA or high molecular weight contaminants.
   Note: A Nanodrop check and the evaluation of the A260/280 and A260/230 can provide insight into whether this could be high molecular weight contaminants.



A. Lane 6 – High molecular weight DNA at 23kb



B. Lane 6 & 7 – Partially degraded DNA (smearing visible)



C. Lane 6 & 7 – Completely degraded DNA

Figure	Lane	Sample
A	1	7.8ng Lambda mass standard #6
A	2	15.6ng Lambda mass standard #5
A	3	31.3ng Lambda mass standard #4
A	4	62.5ng Lambda mass standard #3
A	5	Marker II
A	6	Intact high molecular weight DNA band seen at 23kb.
A	7	Marker II
A	8	125ng Lambda mass standard #2
B	1	7.8ng Lambda mass standard #6
B	2	15.6ng Lambda mass standard #5
B	3	31.3ng Lambda mass standard #4
B	4	62.5ng Lambda mass standard #3
B	5	Marker II
B	6	Partially degraded sample with smearing seen below 23kb
B	7	Partially degraded sample with smearing seen below 23kb
C	1	7.8ng Lambda mass standard #6
C	2	15.6ng Lambda mass standard #5
C	3	31.3ng Lambda mass standard #4
C	4	62.5ng Lambda mass standard #3
C	5	Marker II
C	6	Completely degraded sample with no intact band seen at 23kb
C	7	Completely degraded sample with no intact band seen at 23kb

Important!
We highly recommend sending intact high molecular weight DNA for sequencing [Figure A]. Partially degraded DNA can affect library construction and sequencing quality[Figure B]. Degraded DNA will not be accepted by JGI for large insert library preparation[Figure C].

SAMPLE PURITY

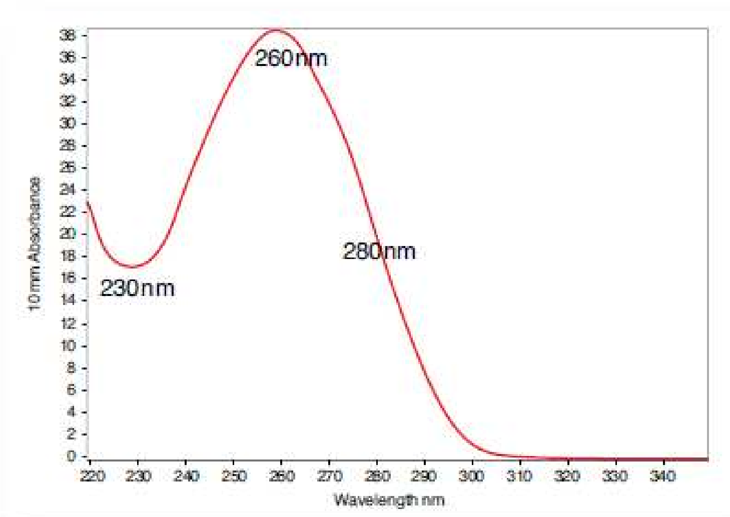
Note:

1. *JGI uses Nanodrop ND-1000 to determine sample purity only. We do not recommend using Nanodrop to determine sample concentration.*
2. *We recommend submitting purified DNA samples for sequencing. Data from Nanodrop helps in troubleshooting whether the contaminant present in the sample has an effect on the quality.*

Optional:

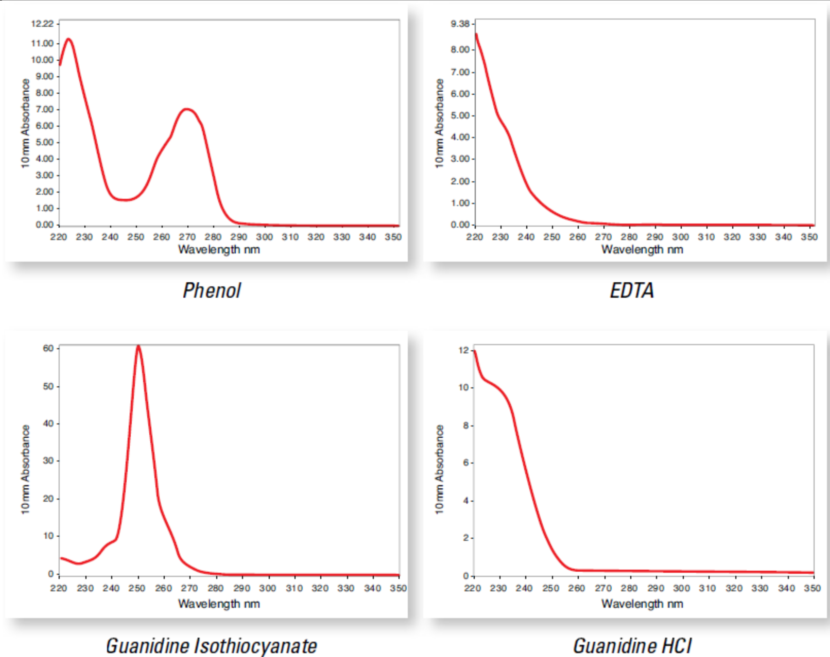
1. Clean pedestal and sampling arm with nuclease-free water and Kimwipes.
2. Pipette 1.6µl of nuclease-free water directly onto the pedestal.
3. Lower the sampling arm and select Initialize from the NanoDrop software. Surface tension is used to hold samples in place between two optical fibers.
4. When the measurement is complete, raise the sampling arm and wipe the water from the pedestal and arm.
5. Select the Nucleic Acid application, and the appropriate Sample Type.

6. Pipette 1.6µl of 1X TE buffer, pH 8.0 or appropriate buffer (if sample was not eluted in 1X TE buffer, pH 8.0) directly onto the measurement pedestal.
7. Lower the sampling arm and select Blank.
8. When the measurement is complete, raise the sampling arm and wipe the buffer from the pedestal and arm.
9. Pipette 1.6µl of the DNA sample onto the pedestal, and close the sampling arm. Select Measure, and when complete record and save the A260/A280 and A260/A230 readings.
10. Between and after all sample measurements, clean the pedestal and arm with nuclease-free water and Kimwipes.



Typical Nucleic Acid Spectrum

11. Review the spectral image and the absorbance ratios to assess the purity of the sample. Use the following guidelines to perform analysis of the spectrum and absorbance ratios:
    - The absorbance maxima for nucleic acids is at 260nm while the absorbance maxima for proteins is at 280nm
    - 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. It could also be due to issues with measurement . Nanodrop QC readings should be repeated to rule out issue with measurement as the cause of this. High 260/280 purity ratios are generally not indicative of an issue.
    - A low A260/A230 ratio may be the result of 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
    - Wavelength of the trough in sample spectrum– this should be at ~230 nm and wavelength of the peak in sample spectrum– this should be at 260 nm.



APPENDIX A : TROUBLESHOOTING

Troubleshooting Qubit readings: Ensure the following practices are followed when performing 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.
- Ethanol precipitation of speed-vac can be used to concentrate samples. We recommended that the concentration for these samples is checked after the samples have been concentrated.
- 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.

Troubleshooting might be required to confirm the quality of certain samples once the QC gel has been analyzed. Few examples where troubleshooting maybe needed include:

- If majority of the sample is stuck in the well of the QC gel then it could either be due to presence of high molecular weight impurities or due to the fact that the sample contains very high molecular weight DNA. A Nanodrop QC in such cases is recommended to determine where there may be presence of high molecular weight impurities such as proteins, carbohydrates present in the sample (A260/280 ratio <1.8). It is possible in such cases diluting an aliquot of the sample and running this aliquot on a new QC gel might help the sample move through the QC with more ease during electrophoresis.
- If you notice additional bands in the gel image then it should be determined whether it is expected to see these additional bands or not in the DNA sample. Additional bands could indicate presence of contamination.

Troubleshooting the purity of the sample determined by Nanodrop:

- Dirty sample pedestal can lead to erroneous absorbance readings. Ensure that sample surface have been completely cleaned before starting the readings and also between samples.

Refer to Appendix C for further details from the references.

APPENDIX B

Preparation of 0.7% Agarose Gel:

1. Prepare 0.7% Agarose Gel (for example – dissolve 0.7gms of Agarose in 100ml of 1X TAE).
   Note: Use thermal gloves and be aware of surroundings when handling hot liquid agarose.
   Note: Prepare 100 mL agarose 0.7% solution for analysis of up to 16 samples. Alternatively for a large gel prepare 200 mL of agarose 0.7% gel solution
2. To the 100 mL agarose solution pipet 10 µL SYBR Safe 10000:1 stock (20 µL for large gel). Swirl gently to mix, avoid introducing bubbles to the solution.
3. Ensure the gel tray is tightly fit into the casting tray prior to pouring the gel into the gel tray .
4. Gently pour the gel solution into the gel tray. Avoid introducing bubbles if possible.
5. Place the suitable gel comb into the gel tray/
6. Pout the prepared 0.7% Agarose gel solution slowly into the casting tray.
7. Allow at least 30 minutes for the gel to set.

Lambda Mass Standards

For Lambda mass std #1 (50ng/µl):

Lambda DNA (300ng/µl stock)	250
1X TE Buffer, pH 8.0	1000
6X Loading Dye	250
Total (µl)	1500

For Lambda mass std #2 (25ng/µl):

Lambda mass std #1 (50ng/µl)	750
1X TE Buffer, pH 8.0	625
6X Loading Dye	125
Total (µl)	1500

For Lambda mas std #3 (12.5ng/µl):

Lambda mass std #2 (25ng/µl)	750
1X TE Buffer, pH 8.0	625
6X Loading Dye	125
Total (µl)	1500

For Lambda mass std #4 (6.3ng/µl):

Lambda mass std #3 (12.5ng/µl)	750
1X TE Buffer, pH 8.0	625
6X Loading Dye	125
Total (µl)	1500

For Lambda mas std #5 (3.1ng/µl):

Lambda mass std #4 (6.3ng/µl)	750
1X TE Buffer, pH 8.0	625
6X Loading Dye	125
Total (µl)	1500

For Lambda mass std #6 (1.6ng/µl):

Lambda mass std #5 (3.1ng/µl)	750
1X TE Buffer, pH 8.0	625
6X Loading Dye	125
Total (µl)	1500

1X TAE Buffer
40ml 50X TAE Buffer
1960 mL Milli-Q ddH2O

APPENDIX C REFERENCES

1. Qubit:
    - (https://tools.lifetechnologies.com/content/sfs/manuals/mp32850.pdf) and Qubit dsDNA HS Assay
    - (http://tools.lifetechnologies.com/content/sfs/manuals/mp32851.pdf) to become familiar with the protocol before setting up the assay.

2. Nanodrop :
    - T042 Technical Bulletin – Assessment of Nucleic Acid Purity http://www.nanodrop.com/Library/T042-NanoDrop-Spectrophotometers-Nucleic-Acid-Purity-Ratios.pdf
    - User Guide and further troubleshooting http://www.nanodrop.com/library/nd-1000-v3.7-users-manual-8.5x11.pdf

3. Promega Quantifluor dsDNA Assay:
    - https://www.promega.com/~/media/files/resources/protocols/technical%20manuals/101/quantifluor%20dsdna%20system%20protocol.pdf

4. Image Lab : http://www.bio-rad.com/en-us/product/image-lab-software?source_wt=imagelabsoftware_surl