Bionano Prep DLS-G2 Protocol

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Legal Notice

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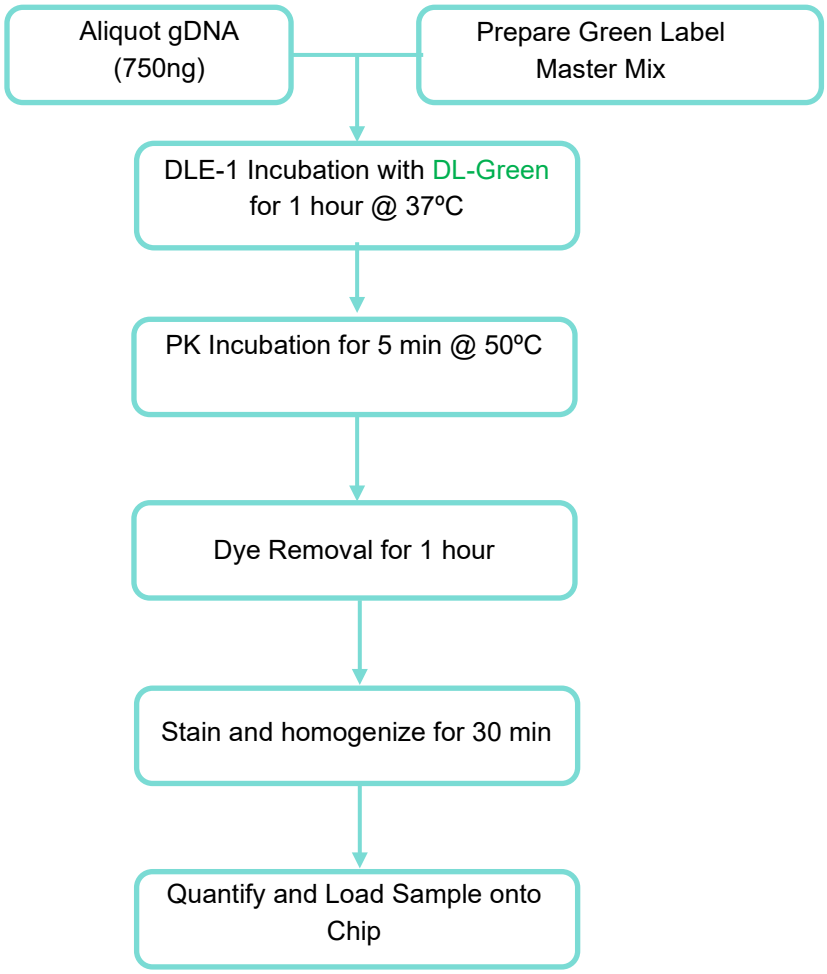
Revision History

REVISION	NOTES
A	Initial release
B	Updated verbiage in steps 9 and 10 for clarification.
C	Removed Troubleshooting Guide/FAQ sections. Now contained in standalone document: CG-30608. Removed time estimates in protocol steps.

Bionano Prep DLS-G2 Overview

The Bionano Prep DLS-G2 (Direct Label and Stain-G2) Labeling Kit and Protocol is a sequence-specific labeling kit and protocol for labeling ultra-high molecular weight (UHMW) genomic DNA (gDNA) for use on the Bionano Optical Genome Mapping (OGM) platform using a Direct Labeling Enzyme (e.g., DLE-1).

WORKFLOW



Bionano Prep DLS-G2 Labeling Kit and User-Supplied Materials

Table 1: Bionano Prep DLS-G2 Labeling Kit Contents (P/N # 80046)

Component	Part #	Quantity	Storage	Handling Considerations
10x DLE-1	20430	44 µl	-25°C to -15°C	Flick tube three times to mix, and centrifuge briefly. Keep in -20°C enzyme cooler until use.
20x DL-Green	20429	22 µl	-25°C to -15°C	Thaw at room temperature (RT). Vortex and centrifuge briefly. Keep on pre-chilled aluminum block until use.
5x DLE-1 Buffer	20428	175 µl	-25°C to -15°C	Thaw at room temperature. Vortex and centrifuge briefly. Keep at room temperature until use.
DNA Stain	20356	65 µl	-25°C to -15°C	Thaw at room temperature. Vortex and centrifuge briefly. Keep at room temperature until use; DMSO in DNA Stain will crystalize on ice.
10x DTT	20432	90 µl	-25°C to -15°C	Thaw at room temperature. Vortex and centrifuge briefly. Keep at room temperature until use.
Proteinase K	20434	60 µl	2°C to 8°C	
4x Flow Buffer	20431	225 µl	2°C to 8°C	Vortex and centrifuge briefly. Keep at room temperature until use.
Ultra Pure Water	20355	900 µl	2°C to 30°C	May keep at room temperature.
DLS 24 Well Plate	20357	One plate	15°C to 30°C	Keep covered to avoid dust.
DLS Membranes	20358	25 ea	15°C to 30°C	Avoid excess moisture.
DLS Tape Sheets	20433	12 ea	15°C to 30°C	
DLS Amber Tubes	20437	14 ea	15°C to 30°C	

Table 2: User Supplied Materials

Item	Description	Catalog #
HulaMixer Sample Mixer	Thermo Fisher	15920D
Thermocycler with heated lid	General lab supplier	
PCR tubes, 0.2 ml, thin-walled, flat cap, nuclease-free	Thermo Fisher or equivalent	AM12225
Microcentrifuge tubes, 0.5 ml, Amber, nuclease-free	USA Scientific or equivalent	1605-0007
Pipet tips, unfiltered, 200 µl	USA Scientific or equivalent	1111-1810
Pipet tips, wide bore, filtered, 200 µl	VWR or Rainin equivalent	46620-642
Pipet tips, standard, filtered; 2, 10, 20 and 200 µl	General lab supplier	
-20°C benchtop enzyme cooler	VWR or equivalent	414004-286
4°C aluminum cooling tube block	Sigma Aldrich or equivalent	Z740270
Forceps, pointed and curved	Electron Microscopy Sciences or	78141-01
Pipets (2, 10, 20 and 200 µl)	General lab supplier	
Ice bucket and Ice	General lab supplier	
Vortexer	VWR or equivalent	10153-838

Item	Description	Catalog #
Microcentrifuge for 0.2 ml, 0.5 ml, and 1.5 ml Tubes	General lab supplier	
Qubit Fluorometer	Thermo Fisher	Q33238
Qubit® Assay Tubes	Thermo Fisher	Q32856
Qubit® HS (High Sensitivity) dsDNA Assay Kit	Thermo Fisher	Q32851
Bath sonicator (recommended)	Branson or equivalent	CPX 952-119R
Positive-displacement pipet MR-10 (optional)	Rainin or equivalent	17008575
Pipet tips, 10 µl, C-10 for pos. displacement (optional)	Rainin or equivalent	17008604

Introduction and Important Notes

INTRODUCTION

This protocol describes an enzymatic labeling approach for direct fluorescent labeling of UHMW gDNA (hundreds of kilobase pairs to mega base pairs in length) at a specific sequence motif by the Direct Labeling Enzyme (DLE-1). This direct labeling does not introduce nicks into DNA and allows users to generate highly contiguous genome maps, with N50's of 20-100 Mbp, depending on the genome and sample quality.

The Bionano Prep DLS-G2 kit provides reagents for sequence-specific labeling of UHMW gDNA for Bionano Optical Genome Mapping (OGM) on the Saphyr® system. After sequence-specific labeling with DLE-1, the labeled DNA is stained for backbone visualization. DL-Green fluorophores are seen as green labels on a blue molecule when imaged on the Saphyr® instrument.

DLE-1 Reaction Size

This protocol yields 60µl of labeled DNA. This is sufficient to load on a single flow cell of a Saphyr Chip® consumable, with enough sample remaining for one additional flow cell in cases of low throughput or other failure. Starting material should be at least in the hundreds of kilobases in length. If necessary, the size can be determined via pulsed field gel electrophoresis (PFGE). Labeling metrics are determined on the Saphyr instrument and are measured in labels/100 kilobase pairs (kbp). Additional labeling metrics can be determined by supplying a reference and monitoring map rate, positive label variance (PLV), and negative label variance (NLV). See the "Important Notes" section below for additional details.

Details on expected metrics can be found in the Saphyr Molecule Quality Report Guidelines document (P/N 30223).

IMPORTANT NOTES

General Considerations

- Use an aluminum tube cooling block pre-chilled on ice to hold thawed reaction components and to assemble labeling reactions is recommended.

- Enzymes and buffers should be accurately pipetted out, with no droplets hanging on the outside of the pipet tip. The enzyme should be completely delivered into the reaction tube, and bubble formation should be carefully avoided to ensure reproducible reactions. This is best achieved by holding reagent tubes at eye level when aspirating or dispensing, to visualize the process.
- Slow and thorough pipet mixing of DLE-1 master mix with gDNA is a critical step and promotes DNA homogeneity and enzyme accessibility for efficient labeling of highly viscous DNA.
- This protocol involves the handling of light-sensitive fluorescent molecules. It is important to minimize the exposure to light while working. Protect both the reactions and light-sensitive reagents from light during storage.
- Labeled DNA concentration is measured on Day 1 or 2, after labeling, cleanup, homogenization, and staining, and prior to loading. DNA homogeneity is assessed by quantitating in duplicate (Coefficient of Variation (CV) < 0.30). Homogenous, labeled DNA allows for an accurate estimate of concentration and more uniform loading of DNA onto the chip. The labeled DNA concentration should be between 4 and 16 ng/µl.

Batch Size

- Up to twelve samples can be processed at a time.
    - Each Bionano Prep DLS-G2 Kit contains reagents sufficient for twelve samples.

Requirements for Starting DNA

- The sample should contain mega base-length gDNA, typically determined by high viscosity and/or PFGE.
- gDNA concentration should be between 39 and 150 ng/µl.
    - gDNA samples > 150 ng/µl should be diluted with TE (pH 8.0) to 50 – 150 ng/µl, mixed five times with a wide bore tip, and allowed to relax overnight at room temperature. Verify final DNA concentration and homogeneity before labeling.
    - For gDNA samples < 39 ng/µl, contact Technical Support at Support@bionanogenomics.com.

Determining Enzyme

- For non-human samples, before starting the DLS-G2 protocol, import the sequence data for the desired sample into either the In Silico Digestion feature of the Bionano Access® software, or the standalone Label Density Calculator software to ensure that DLS-G2 labeling is an appropriate choice for the sample. Actual label density should be within ± 2 labels of the predicted label density. Contact Technical Support at Support@bionanogenomics.com for guidance if uncertain.

- For non-human samples, current downstream analysis tools are most successful with genomes that have DLS-G2 label densities between 9 and 25 labels per 100 kbp.

MANAGING GENOMIC DNA

General

- This protocol involves the handling of viscous gDNA, which is difficult to pipet accurately. It is critical to follow all steps in the protocol to ensure accurate sampling of DNA to achieve proper enzyme-to-DNA and DNA-to-Stain ratio, and to minimize unnecessary handling of the gDNA, which can result in molecules of insufficient size for analysis.

Adding gDNA to Labeling Reaction

- To ensure accurate sampling from the viscous gDNA stock, first maximize stock DNA homogeneity by gently pipet mixing the room temperature, equilibrated DNA solution with a wide-bore tip five times and follow the guidelines below for proper pipetting into and out of a standard pipet tip, or positive displacement pipet, for complete delivery.
- Before drawing viscous gDNA into a standard tip, pipet an identical volume of water and mark the solution level on the tip with a fine tipped marker to serve as a guide when pipetting gDNA. Save the marked tip as a guide and use a new one for DNA retrieval. Alternatively, the use of a positive displacement pipet can improve consistency when pipetting viscous gDNA.
- To draw viscous gDNA into a standard tip, hold the stock DNA tube for close-up visualization, depress the pipet plunger until the first stop, submerge the pipet tip toward the middle of the viscous solution, and carefully release the plunger, as **slowly** as possible while moving the tip in a circular motion, to draw the viscous DNA into the tip, carefully monitoring DNA uptake in the process. Keep the tip submerged even after the viscous DNA solution stops moving upward and levels off (use the marked tip as rough guide to see if viscous solution levels off at the appropriate mark). Viscous DNA can take up to 30 seconds to fill the tip to the appropriate level. Releasing the plunger too quickly can produce a bubble in the tip, resulting in under-sampling (**NOTE**: user must start over if this occurs). After the solution in the pipet tip has leveled off and while the tip is still submerged in the DNA solution, scrape the tip against the bottom of the tube five times using a circular motion. Remove the tip from the DNA solution and visually inspect to confirm that it is filled to the appropriate level, by comparing to the marked tip. Removing the pipet tip from the gDNA solution too early, or improperly scraping the tip on the bottom of the tube, can produce a bubble at the end of the pipet tip, indicating under-sampling (**NOTE**: user must start over if this occurs). **Accurate pipetting of viscous gDNA is possible with practice and patience.**
- To deposit the entire volume of viscous gDNA into a tube or master mix, manually hold the reaction tube for close-up visualization and deliver the DNA by inserting the pipet tip in the solution and gently pressing the plunger until the first stop, then to the second stop, while monitoring DNA release, until all the DNA has been

delivered from the tip. Immediately remove the tip as soon as the last of the DNA has left the pipet tip while maintaining constant pressure to avoid uptake of fluid or introduction of air bubbles. Visually inspect the tip after removing from solution to confirm it is empty.

Bionano Prep DLS-G2 Protocol

SETUP

1. Thaw 20x DL-Green. Vortex well, pulse spin, and store on ice in a 4°C aluminum block.
2. Thaw 5x DLE-1 Buffer**.** Vortex well, pulse spin. Store at room temperature for later use.
3. Flick 10x DLE-1 enzyme three times and pulse spin. Store on bench in a -20°C enzyme block.
4. Remove Ultra Pure water tube from 4°C (if necessary) and keep at room temperature.

DLE-1 LABELING

Dilute gDNA and combine with Labeling Mix

5. If gDNA quantitation was performed according to Bionano Prep SP Protocol instructions immediately prior to labeling, then proceed to Step 6. If not, pulse spin for two seconds and repeat quantitation prior to proceeding to Step 6.
6. In a thin-walled PCR tube, add 750 ng gDNA (a) to Ultra Pure water (b) to a total volume of 19.5 µl. Use **Table 3** to record the volumes of gDNA and water for each sample.
    a. 750 ng/ [gDNA concentration (ng/µl)] = **µl of gDNA**
    b. 19.5 µl – (**µl of gDNA**) = µl of Ultra-Pure water.

Table 3: gDNA Quantity Calculation

gDNA Sample ID	gDNA Concentration (ng/µl)	Volume of Ultra Pure Water (µl)	Volume of gDNA (µl)

7. Prepare a Labeling Master Mix in a 0.5 ml amber tube. Add the components in the order outlined in **Table 4**. Mix by pipetting the entire volume up and down with a standard pipet tip, five times, taking care not to generate bubbles. Pulse spin for two seconds and keep in an aluminum block on ice until use. Use as soon as possible after mixing the components.

Table 4: Labeling Master Mix Calculation Table

Labeling Reaction	Volume for One Sample	# of Samples	Master Mix Excess	Master Mix Total
5x DLE-1 Buffer	6.0 µl		× 1.2	µl
20x DL-Green	1.5 µl		× 1.2	µl
10x DLE-1	3.0 µl		× 1.2	µl
Total Master Mix Volume	**10.5 µl**			**µl**

8. Using a standard pipet tip, add 10.5 µl Master Mix on top of the 19.5 µl (gDNA + Ultra-Pure water). Adjust the pipet to 28 µl, mix the sample slowly up and down five times (one up + one down = one mix). Pulse-spin the tube for two seconds. **WARNING: Protect the samples from light.** ⚠

   NOTE: The video entitled DLS Master Mix mixing at https://bionanogenomics.com/support-page/dna-labeling-kit-dls/ should prove helpful.

   NOTE: A carefully and thoroughly mixed sample is necessary to efficiently label all molecules. Draw the sample from the bottom and dispense near the top (without touching the pipette tip to the tube) to maximize mixing.

Labeling Reaction

9. Incubate in a thermocycler with the heated lid set to 47°C, or "On" if no temperature choice is available:
   a. 1 hour at 37°C (thermocycler temperature)
   b. Hold at 4°C until next step. **WARNING: Protect the samples from light.** ⚠

   NOTE: After placing the samples into the thermocycler, prepare the microplate for DL-Green cleanup (Step 12) while the labeling reaction is incubating.

PROTEINASE K DIGESTION

10. Dispense 5 µl Proteinase K directly into the central bulk of the sample contained in the polymerase chain reaction (PCR) tube. To avoid inadvertently removing DNA that may adhere to the tip, do not mix.
11. Incubate in a thermocycler with the heated lid set to 60°C, or "On" if no temperature choice is available:
    a. 5 minutes at 50°C (thermocycler temperature)

b. Hold at 4°C until next step. After removing from the thermocycler, proceed quickly to the next step. Pulse spin briefly. **WARNING: Protect the samples from light.** ⚠

DL-GREEN CLEANUP

Membrane Adsorption in Microplate

NOTE: Membranes can be wetted immediately after setting up the labeling reaction. Make sure to seal the plate with tape until time of use.

NOTE: For Steps 12-13, please see the video entitled DLS Membrane Demo at the DLS Labeling Kit support site.

12. For each sample, wet the underside of 1 DLS Membrane with 1x DLE-1 Buffer n the Bionano-supplied microplate:
    a. For each sample, prepare 30 µl of 1x DLE-1 Buffer (6 µl 5x DLE-1 Buffer + 24 µl Ultra Pure water). Vortex to mix. Pulse spin for two seconds.
    b. Dispense 25 µl of 1x DLE-1 Buffer into the center of one well of the DLS Microplate.
    c. Use blunt end forceps to place a DLS Membrane on top of the buffer.
    d. Seal wells immediately with a DLS Tape Sheet to prevent evaporation until ready to proceed.

NOTE: **Ensure that membranes are completely wetted after three minutes**. Wetted membranes will have a uniform, translucent blue appearance across the membrane. If the membranes are not wetted after three minutes, please discard the membrane, and wet a new one from the membrane pack. Contact support@bionanogenomics.com with any questions or concerns.

13. Perform DL-Green cleanup by dispensing labeled DNA sample onto the center of the wetted membrane:
    a. Using a 200 µl standard pipet tip with the pipette set to 37 µl, dispense entire volume (~35 µl) of labeled DNA onto the middle of the DLS Membrane.
    b. Seal membrane wells with DLS Tape Sheets. While holding the microplate, apply pressure to secure the Sealing Strip to the top rim of the wells to prevent evaporation.
    c. **WARNING: Protect the microplate from light (cover)** ⚠ and incubate at room temperature for one hour. Ensure that the plate stays undisturbed and there is no inadvertent movement of the plate during incubation.
14. During the one hour incubation period, bring 10x DTT, 4x Flow Buffer, and DNA Stain to room temperature. Once thawed, vortex all tubes well, and pulse-spin briefly to collect contents. Keep all tubes at room temperature until ready to use.
15. After one hour, hold the plate securely, and carefully remove the DLS Tape Sheet.
16. Using a 200 µl standard pipette tip, with the pipet set to 35 µl, slowly aspirate the entire labeled sample while

making contact perpendicularly with the membrane and move the tip across the DNA area while aspirating to collect the DNA. Transfer into a new PCR tube or an amber 0.5 ml microfuge tube. Pulse spin for two seconds. **WARNING: Protect tubes from light (cover).** ⚠

17. Using a 200 µl pipet, dispense 20 µl of the labeled sample from the PCR or 0.5 ml amber tube into the DLS Round Bottom Amber Tube (2 ml) and proceed to the next step (DNA Staining and Homogenization).
    a. If sample volume recovered is < 20 µl, bring the volume up to a total of 20 µl using 1x DLE-1 Buffer.

DNA STAINING AND HOMOGENIZATION

18. Prepare Staining Master Mix according to **Table 5**.

Table 5: Staining Master Mix Calculation Table

Staining Reaction	1 Sample	# of Samples	Master Mix Excess	Master Mix Total
4x Flow Buffer	15 µl		× 1.25	µl
10x DTT	6 µl		× 1.25	µl
DNA Stain	3.5 µl		× 1.25	µl
Ultra Pure Water	15.5 µl		× 1.25	µl
Total Staining Reaction Mix Volume	**40 µl**			**µl**

NOTE: Flow Buffer is viscous, so pipet solutions containing it slowly to increase accuracy.

19. For each labeled DNA, add 40 µl Staining Master mix on top of the labeled sample (20 µl) contained in the DLS Round Bottom Amber tube (2 ml). Do not mix.

    NOTE: Master mix is dispensed on top of solution to avoid inadvertently drawing out DNA that may stick to the pipet tip.

20. Place DLS Round Bottom Amber Tubes containing samples into a HulaMixer (Thermo Fisher) with the speed set to 5 rotations per minute (rpm). The tube holder surface should be flat and parallel to the work surface. Mix for thirty minutes at room temperature with all options other than rotation turned off.

21. After thirty minutes, remove samples from the HulaMixer. Pulse spin for two seconds.

    NOTE: Do not allow the rotation to proceed for longer than thirty minutes, as this may decrease molecule N50.

22. If data collection is desired on the same day, proceed with Quantitation immediately before loading samples on the Saphyr Chip consumable. Otherwise, store the samples at 4°C, **protected from light.** ⚠

(Protocol continues below; potential end of Day One, if desired)

NOTE: See list of User Supplied Consumables and Equipment to ensure all is available.

QUANTITATION OF LABELED AND STAINED DNA

Determine the final concentration of the labeled and stained DNA prior to loading on the Saphyr chip consumable. Best results will be obtained if the DNA concentration (average of two measurements) is between 4 and 16 ng/µl. Variation in the final concentration is due to the difficulties in accurately sampling the viscous starting gDNA and variation in gDNA recovery from the DL-Green removal step. If the sample concentration does not fall within this range, see the Troubleshooting Guide (CG-30608) for recommendations.

Qubit dsDNA HS (High Sensitivity) Assay Kit & Qubit Fluorometer:

NOTE: The standard Qubit dsDNA HS Assay protocol will not provide accurate measurements of concentration due to the extremely long lengths of the labeled DNA. The Qubit protocol has been modified to include a sonication step to fragment an aliquot of the labeled DNA to ensure accurate concentration measurements. Refer to the Qubit dsDNA HS Assay Kit user manual for kit details.

1. Using a wide bore tip on a 200 µl pipet set at 50 µl, mix labeled and stained DNA five times. Pulse-spin.
2. Let Qubit HS Standards and labeled DNA come to room temperature for at least thirty minutes.
3. Prepare 0.5 ml Qubit Assay Tubes:
    a. Two separate Assay Tubes for the HS Standard measurement, each containing 10 µl of Qubit HS Buffer.
    b. Two separate Assay Tubes per labeled sample, each containing 18 µl of Qubit HS Buffer.
4. Using a standard pipet tip or positive displacement pipet, remove two separate 2 µl aliquots from each sample and dispense into 18 µl HS Qubit buffer in a Qubit Assay tube, rinsing the tip. Place Qubit tubes in a floating rack and sonicate in a bath sonicator for ten minutes. During sonication, prepare the Working Solution as described in Step 5.

    NOTE: If a long string of DNA is attached to the tip when removing the tip from the tube, dispense the sample back into the tube and repeat aliquot removal with a new tip.

    a. If a bath sonicator is not available, vortex for at least thirty seconds at maximum speed, then spin down for two seconds.
5. Prepare Working Solution by diluting the Dye Assay Reagent into HS Dilution Buffer (1:200):
    a. Prepare 200 µl Working Solution for each of the two standards (400 µl total).

b. Prepare 200 µl Working Solution for each sample aliquot (400 µl for each sample).

6. For the Qubit DNA standards, add 10 µl of Standards 1 and 2 to separate, labeled Qubit Assay Tubes containing 10 µl of Qubit HS Buffer from Step 3a.

7. Once sonication is complete, retrieve assay tubes and centrifuge briefly to collect solution at the bottom of the tubes. Vortex tubes for five seconds at maximum speed, then spin down tubes for two seconds.

8. Add 180 µl of Working Solution (prepared in step 5) to each tube of sonicated labeled DNA and Qubit DNA Standard plus HS Buffer. Vortex for five seconds, and centrifuge briefly to collect solution at the bottom of the tubes.

9. Incubate samples in the dark for two minutes before quantitation on the Qubit Fluorometer.

   NOTE: The labeled DNA concentration should ideally fall between 4 - 16 ng/µl with a CV (standard deviation ÷ mean) between the measurements < 0.30. If both measurements are outside of 4 - 16 ng/µl, see the Troubleshooting Guide (CG-30608). If one measurement is between 4 - 16 ng/µl and the other is outside of this range:

   - If one measurement is between 4 - 16 ng/µl and the other is above 16 ng/µl, proceed to load the chip.
   - If one measurement is between 4 - 16 ng/µl and the other is below 4 ng/µl, repeat HulaMixer mixing for thirty minutes and repeat the quantitation.

10. Record Qubit measurements in **Table 6**.

11. If the samples will not be run immediately, store in the dark at 4°C until use.

Table 6: gDNA Qubit Measurements

Sample ID	Measurement 1 (ng/µl)	Measurement 2 (ng/µl)	Average (ng/µl)	CV (SD/mean)

LOADING SAPHYR CHIP

Refer to the **Saphyr System User Guide** (for Saphyr P/N 60325 or 60239) for complete instructions on chip loading and instrument operation.

NOTE: When aspirating DLS-labeled sample for chip loading, draw from the middle of the tube.

Technical Assistance

For technical assistance, contact Bionano Genomics Technical Support.

You can retrieve documentation on Bionano products, SDS's, certificates of analysis, frequently asked questions, and other related documents from the Support website or by request through e-mail and telephone.

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