Bionano Prep Frozen Blood Protocol

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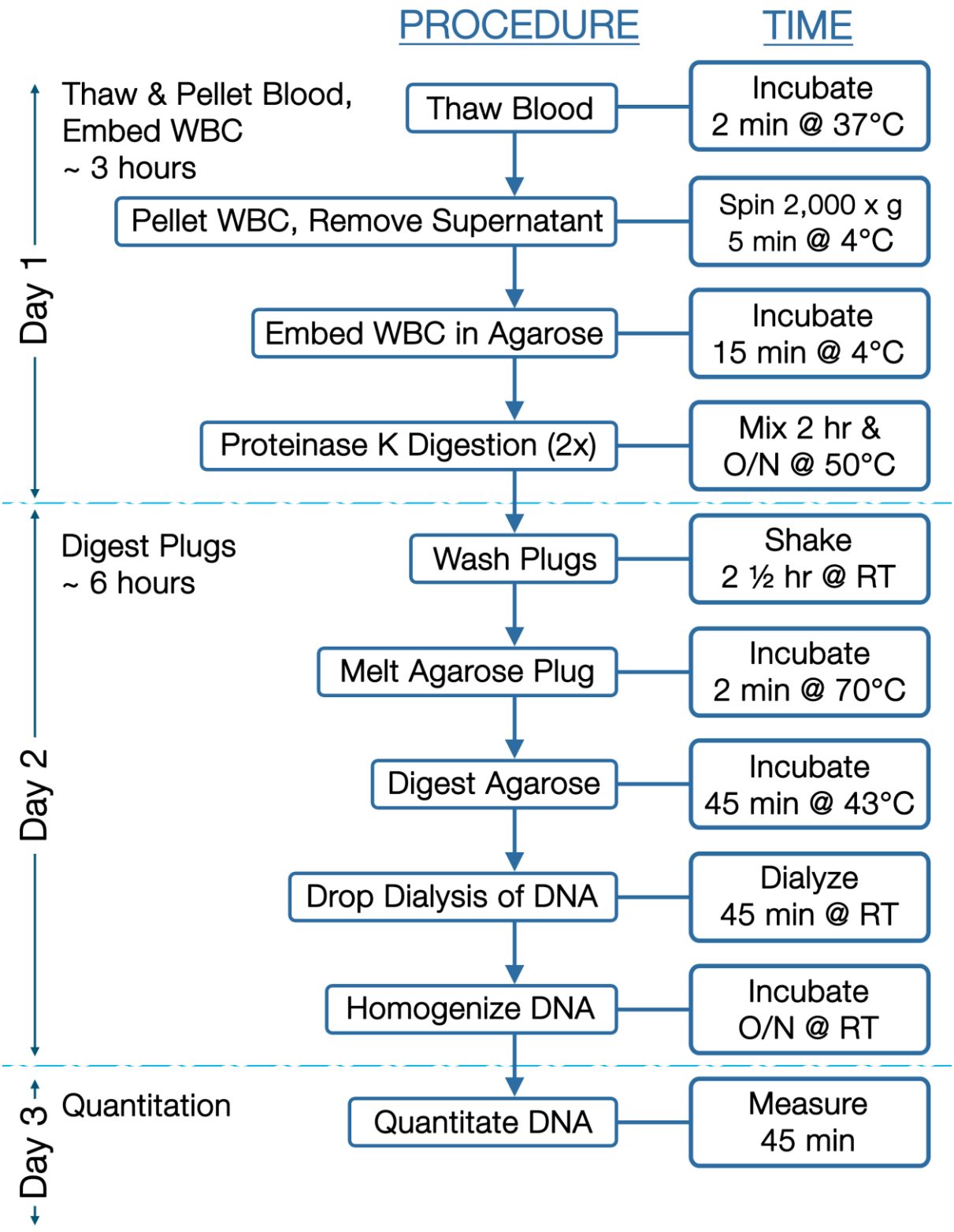
Workflow Overview – 3 Days



Introduction

The Bionano Prep Frozen Blood Protocol targets recovery of white blood cells (WBCs), taking advantage of differential red blood cell lysis occurring as a result of the freeze-thaw process. The resulting WBCs are pelleted, immobilized in agarose, and subjected to plug lysis treatment for megabase genomic DNA (gDNA) recovery.

This qualified protocol has been tested on a number of frozen normal human blood samples that were drawn into EDTA tubes and frozen within three days of blood draw. While we believe it to work for a variety of other genomes, we have not tested non-human species with this protocol, nor are we able to guarantee success for all types of frozen blood. Contact Customer Support for guidelines on non-human species.

Procedure

Frozen blood is quickly thawed and then spun at low speed to pellet intact WBCs. The resulting supernatant is removed and the resuspended pellet is embedded into low-melting point agarose plugs. Megabase-long gDNA is recovered from overnight proteinase K treated plugs following extensive washing followed by melting and agarase treatment. DNA is further cleaned by drop dialysis and subjected to a homogeneity mixing step prior to quantitation.

Note: A majority of normal, healthy blood donors will yield 4.5 - 11 x 10^6 WBC's/mL before freezing. WBC recovery after thawing frozen aliquots ranges from 30 - 70% leading to a range of 1.4 - 7.7 x 10^6 WBC's/mL (~8.4 - 44.4 µg gDNA/mL). We recommend embedding one plug each with 200 µL and 400 µL starting volumes of blood to obtain the desired concentration range of gDNA after drop dialysis (45-150 ng/µL).

Blood Specifications

Repeated freeze-thaw cycles can dramatically reduce the number of intact WBCs and should be avoided. Therefore, immediately upon receiving fresh blood samples, we recommend preparing several 650 µL aliquots and then freezing and storing at -80°C. The freezing speed does not appear relevant and so it is suggested to freeze the freshly isolated blood aliquots directly at -80°C. Larger aliquots can be frozen for increased DNA yield if needed. The fresh blood should be mixed for at least 5 minutes on a platform rocker before aliquotting and freezing to guarantee uniform sampling. It is highly recommended to prepare frozen blood aliquots within 3 days of the blood draw date. Resulting gDNA using this protocol is highly dependent on quality, isolation, and storage of initial blood.

DNA Quantitation

DNA quantitation is used to measure DNA concentration and serves as a gauge of DNA homogeneity. A homogenous DNA solution is mandatory for efficient labeling. Qubit DNA quantitation is preferred over other quantitation methods since it can also be used for measuring DNA concentration of the labeling reactions and is less prone to inaccuracies introduced by contaminants in absorbance-based measurements. The Qubit Broad Range (BR) dsDNA Assay measures DNA concentration following plug lysis, while the High Sensitivity (HS) dsDNA Assay measures labeled DNA concentration.

Bionano Prep Blood DNA Isolation Kit Contents 80004 (10 preps)

Item	Volume	Part Number
Cell Buffer	100 mL	20340
Lysis Buffer	30 mL (2x)	20255
5X Wash Buffer	90 mL (2x)	20256

User-Supplied Materials

Item	Description
Preparing Plugs - Day 1	
Conical centrifuge tubes, 50 mL	VWR, Catalog # 21008-951 or equivalent
Microcentrifuge tubes, 1.7 mL	VWR, Catalog # 87003-294 or equivalent
Microcentrifuge with temperature control (4°C)	General lab supplier
2 Heat blocks for 1.7 mL tubes, or 2 water baths	General lab supplier
CleanCut Agarose, 2%	Bio-Rad, Catalog # 1703594
Plug Molds, Disposable (5 x 50 plugs)	Bio-Rad, Catalog # 1703713
Green Screened Caps for 50 mL Conicals	Bio-Rad, Catalog # 1703711
Proteinase K Enzyme, Puregene 5 mL	Qiagen, Catalog # 158920
Thermomixer	Eppendorf, Catalog # 5382000023 or equivalent
50 mL Thermoblock	Eppendorf, Catalog # 5365000010 or equivalent
Microwave (for initial agarose preparation)	General lab supplier
UltraPure™ DNase/RNase-Free Distilled Water	Thermo Fisher, Catalog # 10977-015
Ice Bucket and Ice	General lab supplier
Tube Rocker	VWR, Catalog # 10159-756 or equivalent
DNA Recovery - Day 2	
Agarase (0.5 unit/µL)	Thermo Fisher, Catalog # EO0461
TE Buffer, pH 8.0	Thermo Fisher, Catalog # AM9849
Orbital Platform Shaker	Cole-Parmer, Catalog # EW-51820-30 or equivalent
Dialysis Membrane Filter, MF, 0.1 µm	Millipore, Catalog # VCWP04700 or equivalent
Petri Dishes, sterile, 60 x 15 mm	VWR, Catalog # 28384-092
Metal Spatula	VWR, Catalog # 82027-530
Pipette Tips, 200 µL, Wide-Bore	VWR, Catalog # 46620-642
Pipette Tips, 200 µl, Nonfiltered and beveled	USA Scientific, Catalog # 1111-1810
DNA Quantitation - Day 3	
Bath Sonicator	General lab supplier
Vortexer	General lab supplier
Fluorometer, Qubit 3.0	Thermo Fisher, Catalog # Q33216 or equivalent
Qubit BR (Broad Range) dsDNA Assay Kit	Thermo Fisher, Catalog # Q32850 or equivalent
Qubit Assay Tubes	Thermo Fisher, Catalog # Q32856 or equivalent
Positive-Displacement Pipette MR-10 (optional)	Rainin, Catalog # 17008575 or equivalent
Pipette Tips, 10 µL, C-10 for Positive Displacement (optional)	Rainin, Catalog # 17008604 or equivalent

Frozen Blood Protocol

Day 1: Embedding WBCs in Agarose, Proteinase K Digestion (3 hours)

Isolation of Megabase DNA from Frozen Blood

Before First Use

1. Dilute 5x Wash Buffer (Bionano) to 1x using nuclease free water.
2. For first-time use of 2% Agarose (Bio-Rad), melt entire bottle in microwave-boiled water for 15 minutes. Then aliquot melted agarose into 500 µL portions in microfuge tubes that can be stored at 4°C. Use one or more 500 µL aliquots for each new procedure.

Set Up

3. Gather materials (see 'User Supplied Material' section above).
4. Set up heat blocks/water baths. Fill wells with water and verify temperature with thermometer:
    a. Set a heat block or water bath to 70°C.
    b. Set another heat block or water bath to 43°C.
5. Melt 2% Agarose (Bio-Rad) at 70°C for 5 minutes and equilibrate at 43°C until ready to prepare WBC plugs.
6. Pre-chill the Cell Buffer (Bionano) and plug mold/plunger (Bio-Rad), and set centrifuge to 4°C.
7. Equilibrate a Thermomixer fitted with 50 ml adapter to 50°C for Proteinase K digestion.

Protocol Start:

Blood Samples Starting Amount

Starting with a 650 µL aliquot of frozen blood, we recommend using 200 µL and 400 µL per plug. Larger aliquots can be subdivided and processed for increased DNA yield if needed. Depending on the health and WBC count of the donor, more or less blood may be required to reach the optimal range. However, using more than 600 µL or less than 100 µL per plug is not recommended.

The following protocol is for casting 2 agarose plugs from a 650 µL aliquot. This can be scaled up. *Process as many blood samples as you feel comfortable handling with the understanding that a dedicated Thermomixer is required for every four blood samples.*

WBC Preparation / Agarose Plugs

1. Thaw 650 µL frozen blood at 37°C for 2 minutes. Invert several times to ensure a homogenous WBC suspension before removing the aliquots for processing. Proceed quickly to step 2.
2. Pipette 200 µL and 400 µL of thawed and mixed blood into two separate 1.7 mL tubes. Centrifuge the two tubes for 5 minutes at 2,000 *x g* at 4°C to pellet intact WBCs.
3. Pipette out the supernatant containing plasma and lysed RBCs, leaving up to 40 µL per 200-400 µL starting frozen blood. For example, remove ~160 µL of supernatant from 200 µL aliquot.

Note: Experienced users may leave behind as little volume as possible, improving purity at the risk of lower yield. If pellet is visible, carefully remove all of the plasma and lysed RBCs without disturbing pellet, to maximize purity.

4. Add Cell Buffer (Bionano) to a final volume of exactly 54 µL. Gently resuspend the WBC pellet by mixing up and down with a 200 µL pipette. Keep on ice until ready to proceed.
   Note: The 54 µL volume is critical. Double-check the final volume with pipette set to 54 µL.
5. Equilibrate WBC-Cell Buffer suspension at 43°C for 2 minutes. During equilibration, have pipettes and Plug Molds (on ice or chill plate) ready near 43°C water bath or heat block.
   Note: If processing more than one sample, process one at fa time starting at this step, to prevent prolonged incubation at 43°C. It is important not to let cells remain at 43°C for longer than 2 minutes before adding the agarose.
6. Add 36 µL pre-warmed 2% agarose (Bio-Rad) to WBC-Cell Buffer suspension and quickly mix 3 times with pipette set to 90% solution volume, avoiding bubble formation.
7. Avoiding bubbles, immediately fill first well of the plug mold by pipetting the WBC-Agarose mixture into pre-chilled plug mold. If there is extra volume, do not overfill mold.
   Note: If the volume of the WBC-Agarose Mixture is insufficient to fill the plug mold, add the entire volume and then proceed to the next step.
8. Repeat steps 5-7 for all samples.
9. Place plug mold at 4°C for 15 minutes to solidify agarose.

Digest with Proteinase K (2 hours)

Up to five plugs can be processed simultaneously per 50 ml conical tube if each plug is from the same blood sample. Ensure all plugs are fully submerged in Proteinase K Lysis Solution throughout processing. For maximum workflow flexibility, two Proteinase K digestion options may be employed: 2 hours of Proteinase K digestion followed by overnight digestion with a fresh Proteinase K Lysis Solution, or overnight Proteinase K digestion followed by 2 hours digest with a fresh Proteinase K Lysis Solution the following day.

10. Prepare fresh Proteinase K Lysis Solution by mixing 167 µL of Qiagen Proteinase K Enzyme with 2.5 mL of Bionano Prep® Lysis Buffer (Bionano) per 1-5 plugs to be processed in each 50 mL conical tube.
11. Transfer plugs to conical tube containing approximately 2.7 mL of Proteinase K Lysis Solution, by first removing tape from bottom of plug mold followed by dislodging plugs into conical tube with Plug Mold Plunger (Bio-Rad kit). Make sure all plugs are fully submerged. Use spatula or blunt edged instrument to submerge plugs if they become stuck to conical tube walls.
12. Cap each conical and incubate in Thermomixer for 2 hours or overnight at 50°C with intermittent mixing (mixing cycle: 10 seconds at 450 rpm followed by 10 minutes at 0 rpm).
13. Near the end of the incubation, prepare fresh Proteinase K Lysis Solution by mixing 167 µL of Proteinase K Enzyme with 2.5 mL of Bionano Prep® Lysis Buffer per 1-5 plugs to be processed per tube.

14. Remove each tube from the Thermomixer and equilibrate to room temperature for 5 minutes. Remove cap and attach a Green Screened Cap (Bio-Rad). Drain the Proteinase K Lysis Solution through the screened cap, and tap the bottom of the tube on the bench surface several times with strong repetitive force to localize the plugs at the bottom of tube.

15. Remove the screened cap, and add freshly mixed Proteinase K Lysis Solution. Account for all plugs and ensure that they are completely submerged in the Proteinase K Lysis Solution. Tightly cap each tube with its original cap. Incubate in Thermomixer with intermittent mixing as before for 2 hours or overnight, depending on which Proteinase K lysis workflow was chosen.

Day 2: DNA Stabilization Washes, DNA Recovery, Drop Dialysis (7 hours)

Set Up

1. Gather materials (see 'User Supplied Materials' section above).
2. Set up heat blocks/water baths. Fill wells with water and verify temperature with thermometer:
    a. Set a heat block or water bath to 70°C for melting of plugs.
    b. Set another heat block or water bath to 43°C for Agarase treatment of plugs.

Washing Plugs to Stabilize DNA (1 hour 30 minutes)

3. Prepare 70 mL of 1x Wash Buffer for each 50 mL conical tube, using the Bionano Prep® 5x Wash Buffer and nuclease-free water. Mix thoroughly and store at room temperature until use.
4. After the second Proteinase K digestion, replace original cap with screened cap, drain the Proteinase K solution, and tap the bottom of each tube on the bench surface several times with a strong repetitive force to localize plugs at bottom of the tube.
5. Rinse each tube 3 times by:
    a. Adding 10 mL of 1x Wash Buffer through the screened cap.
    b. Swirling tube gently for 10 seconds.
    c. Discarding wash solution through the screened cap.
    d. Tapping plugs to bottom of tube before next rinse.
6. Wash each tube 4 times by:
    a. Adding 10 mL of 1x Wash Buffer through screened cap and capping tube.
    b. Gently shaking tube for 15 minutes on an orbital platform shaker with continuous mixing at 180 rpm.
    c. Discarding wash solution through the screened cap.
    d. Tapping plugs to bottom of tube before next wash. Do not discard the last wash.

***Note**:* Plugs can be stored in 1x Wash Buffer for up to 2 weeks at 4°C without significant degradation of DNA quality.

DNA Recovery (2 hours)

7. Discard final 1x Wash Buffer through the screened cap and tap plugs to the bottom of the tube.
8. TE Wash plugs 5 times by:
    a. Adding 10 mL of TE Buffer (pH 8.0) per wash through the screened cap and capping tube.
    b. Continuous shaking for 15 minutes on an orbital platform shaker at 180 rpm.
    c. Discarding the wash through the screened cap.
    d. Tapping plugs to bottom of tube before adding the next wash.
9. Discard the last TE wash through the screened cap and tap plugs to bottom of tube.
10. Scoop one plug at a time from conical tube with a metal spatula and drain excess liquid from plug by touching the bottom of the spatula to a clean KimWipe, being careful to not make contact with the gel plug. Place each semi-dried plug into a 1.7 mL microcentrifuge tube.
11. Pulse spin each microcentrifuge tube briefly to collect the plug at the bottom of the tube.
12. Melt each agarose plug in a water bath or heat block set at 70°C for 2 minutes.
13. Immediately transfer each tube to a water bath or heat block set at 43°C, incubate 5 minutes.
14. Process one tube at a time: add 2 µL of 0.5 U/µL Agarase (Thermo Fisher) enzyme to tube and stir mixture gently with a pipette tip for 10 seconds.
15. Incubate tubes at exactly 43°C for 45 minutes.
    Note: A temperature difference of +/-3°C can inactivate the Agarase enzyme.

Drop Dialysis to Clean DNA (1 hour)

16. Place 15 mL of TE Buffer into 6 cm Petri Dish (Thermo Fisher/VWR) per each DNA sample.
17. Float a 0.1 µm Dialysis Membrane (Millipore) on the surface of the TE Buffer. Place a cover on the Petri dish and let the membrane hydrate for 10 minutes.

    Note: Use forceps to grip the membrane by the edge and gently float it on the surface of the TE Buffer horizontally to prevent dipping or sinking during application. According to the manufacturer, both sides perform equivalently; either side can face up.
18. Pipette mix the entire volume of DNA solution 2 times with a wide bore tip and add as a single drop on the center surface of the dialysis membrane.

    Note: In order to avoid submersion of the membrane and loss of sample, it is critical to place the entire volume of DNA in the center of the membrane and avoid excessive downward force when applying or withdrawing DNA from membrane surface.
19. Place cover on the Petri dish and let the sample dialyze for 45 minutes at room temperature.
20. Transfer DNA to a 1.7 mL microcentrifuge tube with a wide bore tip.

    Note: Do not pipette mix the DNA while on the membrane.

Homogenization of DNA Solution (10 minutes)

21. Test DNA viscosity by slowly withdrawing the entire DNA volume into a non-filtered 200 µL tip while slowly releasing pipette plunger. If the DNA is not viscous, do not pipette mix. If the DNA is extremely viscous, pipette mix up to 9 strokes (stroke = 1 up stroke + 1 down stroke) until entire DNA sample is taken up in a continuous flow. The DNA should aspirate evenly without lag.

    Note: If DNA uptake stalls due to high viscosity, it may be necessary to stir gently while slowly releasing the plunger to withdraw the DNA. Viscous samples should get easier to pipette mix as the number of strokes increases.

22. Allow the DNA to equilibrate overnight at room temperature (25°C) for better homogeneity.

Day 3: Quantitation

Qubit Quantitation - BR dsDNA Assay

1. If gDNA was stored at 4°C, equilibrate to room temperature before use.
2. Equilibrate Qubit BR Assay kit standards to room temperature from 4°C.
3. Prepare 3 separate 0.5 ml Qubit Assay tubes per labeled sample, each containing 18 µl of Qubit BR Buffer.
4. Using a 200 µl pipette with a wide bore tip, gently mix the entire sample volume by pipetting up and down 5 times, being careful not to generate bubbles.
5. Follow the methods described in the "Pipetting Viscous Genomic DNA" section, to ensure for accurate pipetting of viscous DNA for quantitation. Using a standard bore pipette tip or positive displacement pipette, and a different tip for each draw:

   Remove 2 µl aliquots from the top, middle, and bottom of each sample and dispense into buffer of corresponding Qubit Assay tube, rinsing tip. Place finished Qubit tubes in a floating rack and sonicate in a bath sonicator for 10 minutes. Proceed to steps 6 & 7 during sonication.

   a. If a bath sonicator is not available, vortex for at least 30 seconds at maximum speed, then spin down briefly for 2 seconds.
6. Refer to the Qubit dsDNA BR Assay Kit user manual for full details, but briefly:

   I. Prepare Working Solution by diluting the Dye Assay Reagent into BR Dilution Buffer (1:200):

      i. Prepare 200 µl Working Solution for each of the two standards (400 µl total).

      ii. Prepare 200 µl Working Solution for each sample aliquot (600 µl for each sample).
7. For the Qubit DNA standards, add 10 µl of Standards 1 and 2 to separate Qubit Assay Tubes and add 10 µl of Qubit BR Buffer to each.
8. Once sonication is complete, retrieve assay tubes and spin down tubes briefly for 2 seconds. Vortex tubes for 5 seconds at maximum speed, then spin down tubes again for 2 seconds.
9. Add 180 µl of Working Solution (prepared in step 6) to each tube of sonicated labeled DNA and Qubit DNA Standard plus BR Buffer. Vortex for 5 seconds, and centrifuge briefly to collect solution at the bottom of tubes.
10. Incubate samples in the dark for at least 5 minutes, then proceed with quantitation on the Qubit Fluorometer.
11. Coefficient of variation (CV) from the three separate readings should be < 0.25.
    (CV = standard deviation/mean).

    ***Note*:** If CV > 0.25, gently pipette-mix the entire volume of DNA with one additional stroke (1 stroke = 1 up stroke + 1 down stroke) using a non-filtered 200 µL tip (i.e. USA Scientific, #1111-1810 or equivalent), pipette mix the entire volume of DNA 5 times with wide bore tip and let the DNA rest overnight at room temperature. Repeat quantitation of DNA the next day.

Labeling

DNA is ready for labeling and data collection. See “Kits and Consumables” section at https://bionanogenomics.com/support/ for applicable kits and protocols.

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