Bionano Prep™ Labeling - NLRS Protocol

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Contents

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Bionano Prep™ Labeling-NLRS (300/600/900 ng)

Sequence-specific labeling of megabase gDNA for Bionano mapping by **N**icking, **L**abeling, **R**epairing, and **S**taining (NLRS)

Workflow Overview – 2 days

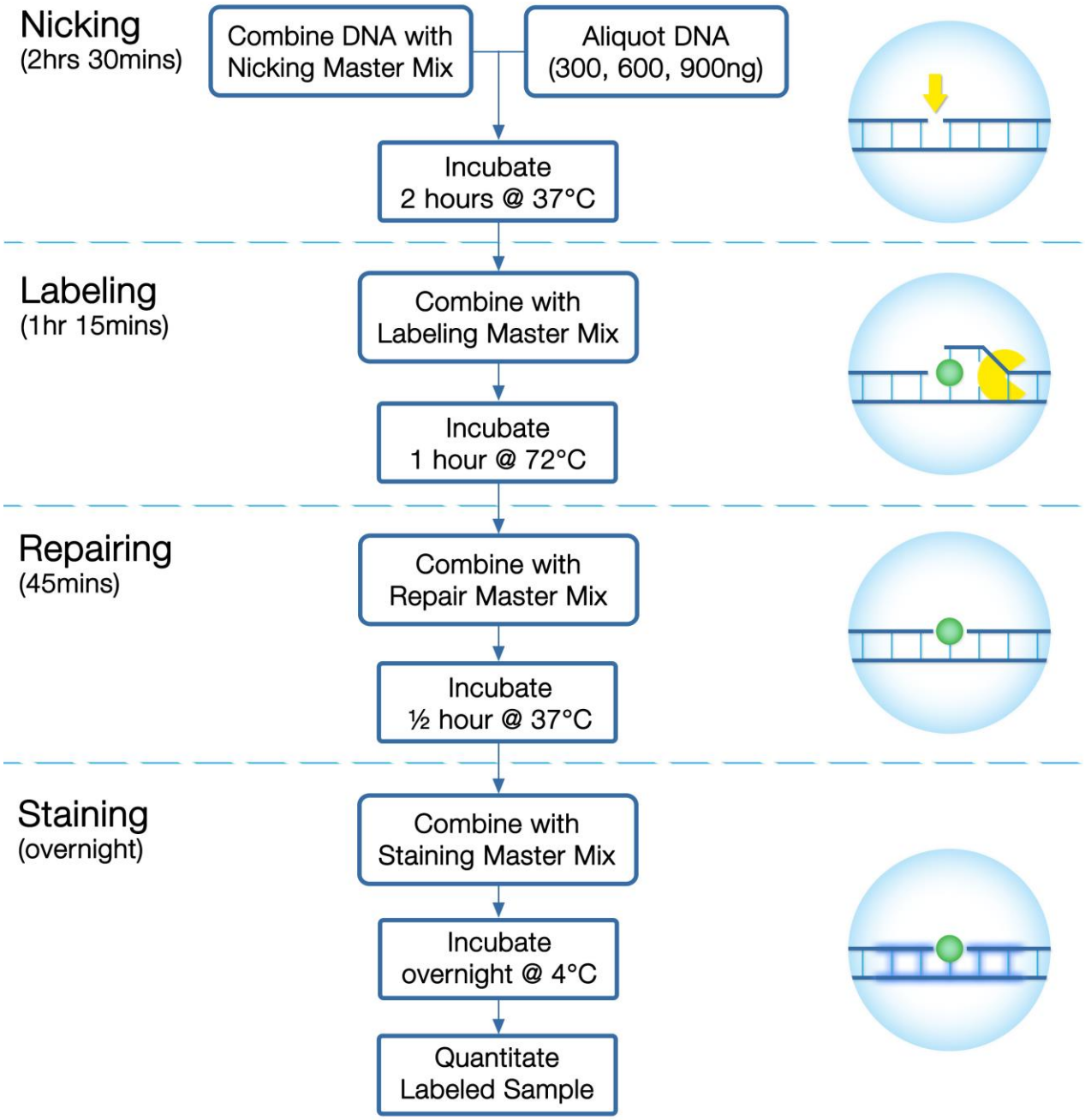

Table 1: NLRS Kit Contents (Part # RE-012-10)

Component	Part #	Volume	Storage	Handling Considerations
10x Buffer 3	20251	15 µl	-20°C	Thaw at room temperature (RT). Vortex and centrifuge briefly. Keep on ice until use.
10x Buffer 2	20258	15 µl	-20°C	
10x Labeling Mix	20167	23 µl	-20°C	
50x Repair Mix	20250	6 µl	-20°C	
5x DTT	20166	180 µl	-20°C	
DNA Stain	20162	75 µl	-20°C	Thaw at RT. Vortex and centrifuge briefly. Keep at RT until use as DMSO will crystallize on ice.
10x Labeling Buffer	20168	23 µl	4°C	Vortex and centrifuge briefly before use. Keep on ice.
4x Flow Buffer	20161	225 µl	4°C	Vortex and centrifuge briefly. Keep at RT until use.
Stop Solution	20164	15 µl	RT	
Ultrapure Water (Nuclease Free)	20165	300 µl	RT	N/A

Table 2: Reagents Provided by User

Component	Source	Part #	Volume	Storage	Handling Considerations
Nb.BssSI (20 U/µl)	NEB	R0681S	50 µl	-20°C	Invert 3 times and centrifuge briefly before use. Keep on ice or in freezer block until use.
Nt.BspQI (10 U/µl)	NEB	R0644S	100 µl	-20°C	
Nb.BbvCI (10 U/µl)	NEB	R0631S	100 µl	-20°C	
Taq DNA Polymerase (5 U/µl)	NEB	M0267S	80 µl	-20°C	
Taq DNA Ligase (40 U/µl)	NEB	M0208S	50 µl	-20°C	
10x ThermoPol Buffer	NEB	B9004S	1.5 ml	-20°C	Thaw at RT. Vortex and centrifuge briefly. Keep on ice until use.
10x NEB 3.1 Buffer	NEB	B7203S	1.25 ml	-20°C	
50 mM NAD+	NEB	B9007S	200 µl	-80°C	After thaw store 5 µl aliquots at - 80°C.

Table 3: User-Supplied Consumables

Item	Description
PCR Tubes, 0.2 ml, Thin-Walled, Frosted Lid, Nuclease-Free	Thermo Fisher, Catalog # AM12225 or Equivalent
Amber Microcentrifuge Tube, 0.5 ml, Nuclease-Free	USA Scientific, Catalog # 1605-0007 or Equivalent
UltraPure, Nuclease Free Water	Thermo Fisher, Catalog # 10977-015 or Equivalent
Qubit® HS (High Sensitivity) dsDNA Assay Kit (recommended)	Thermo Fisher, Catalog # 10977-015
Qubit® Assay Tubes (recommended)	Thermo Fisher or Axygen Catalog # Q32856 or 10011-830
Pipette Tips, Aerosol-Resistant, 2, 10, 20 and 200 µl	General Supplier
Pipette Tips Wide-Bore, Filter, Aerosol, 200µl	VWR, Catalog # 46620-642 or Equivalent
Pipette Tips, 200 µL, for Xplorer Pipette (optional)	Eppendorf, Catalog # 0030077555
Pipette Tips, 10 µL, C-10 for Positive Displacement (optional)	Rainin, Catalog # 17008604 or Equivalent

Table 4: User-Supplied Equipment

Item	Description
Bath Sonicator (recommended)	Branson, Catalog # CPX 952-119R or Equivalent
Pipettes (2, 10, 20 and 200 µl)	General Lab Supplier
Ice Bucket and Ice, or 4°C Tube Block	General Lab Supplier
Vortexer	General Lab Supplier
Microcentrifuge for 0.2, 0.5, and 1.5ml Tubes	General Lab Supplier
Thermal Cycler with Heated Lid	General Lab Supplier
Fluorometer, Qubit 3.0 *(optional for Saphyr System)*	Thermo Fisher, Catalog # Q33216 or Equivalent
Xplorer Plus Automated Pipette (optional)	Eppendorf, Catalog # 4861000716
Positive-Displacement Pipette MR-10 (optional)	Rainin, Catalog # 17008575 or Equivalent

Introduction

The Bionano Prep™ Nick Label Repair Stain (NLRS) assay provides sequence-specific labeling of mega-base containing gDNA for Bionano mapping at the single molecule level. Sequence specificity is provided by the **N**ickase. **L**abeling is carried out by a limited-drive nick translation process in the presence of a fluorophore-labeled nucleotide. The labeled nicks are **R**epaired to restore strand integrity. The labeled DNA is **S**tained for backbone visualization. Labeled nicks are seen as dots on a string when run on the Bionano Instrument.

NLRS Reaction Sizes (300, 600, 900 ng)

The 300 ng protocol produces approximately 60 µl of labeled gDNA sample and is designed for tackling genomes that can be interrogated on a single Bionano Chip or for monitoring DNA quality and establishing labeling conditions when processing novel sample types. The 600 ng or 900 ng reactions (direct scale up of the 300 ng reaction) produce approximately 120 and 180 µl of labeled gDNA sample, respectively, and are essential for data collection from genomes where more than one Bionano Chip is required, once megabase content and DNA quality are established. Megabase length gDNA content is determined by Pulse Field Gel Electrophoresis (PFGE). DNA and labeling quality is determined on the Bionano System by monitoring labeling error rate. See the 'Important Notes' section below.

To determine the size of reaction needed, calculate the number of Bionano Chips estimated below and determine the amount of labeled sample that will be necessary. Using the example below for a single nickase, you would need 19 µl per flowcell for Saphyr Chip (FC-030-01) which would correspond to a 300 ng reaction size.

Estimating Chip Needs

To estimate the number of Bionano Chips for a genome using one nickase, use the following formula:

Expected # Chips = [Genome Size (Gbp) x Filtered Coverage (x-fold)] / Chip Throughput (Gbp)

- **Genome Size** is either the known, or estimated, size of the genome in Gbp.
- **Filtered Coverage** generally targets 100x of filtered (>150 kbp) data that maps to the genome.
- **Chip Throughput** is the filtered (>150 kbp) throughput collected for a particular Bionano Chip type. For example, Saphyr Chip (FC-030-01) is guaranteed 640 Gbp/Chip for human blood or cells (> 150 kbp), while IrysChip (FC-020-01) is expected to produce 50-100Gbp/Chip (>20 kbp).

Human Example using Saphyr Chip (FC-030-01) with a Single Nickase:

Expected # Chips = [3.2 Gbp x 100x] / 640 Gbp → 0.5 Chips (or, 1 FlowCell on 1 Chip)

For additional information, please refer to **Data Collection Guidelines** document on the **Support** page: https://bionanogenomics.com/support-page/bionano-access/

Important Notes:

Requirement for Starting DNA

- The sample should contain megabase DNA typically determined by high viscosity and/or PFGE of the sample.
- DNA concentration should be between 45 and 200 ng/µl.

Determining Nickase

- Before starting the NLRS protocol, import the sequence data for your sample into either Bionano Access or the Label Density Calculator software to determine the optimal Nickase for your sample. Contact Technical Support at Support@bionanogenomics.com for guidance on Nickase selection or Nickase amount.
- Label Density Calculator Software available at: http://bnxinstall.com/labeldensitycalculator/LabelDensityCalculator.htm
- Optimal labeling density should be between 8 and 15 labels per 100 kbp.

Nick-Label-Repair-Stain (NLRS)

- NLRS protocol strives for nicking and labeling every nickase site on every DNA molecule. All sites can be labeled (no bias), but not on every molecule, permitting accurate reconstruction of the original nick pattern. Error rate is monitored by determining the labels per 100 kbp and/or by assessing the negative label variance (NLV) and positive label variance (PLV) rates following reference mapping.
- Nicking is the most sensitive step of the NLRS that best gauges DNA quality. Follow best practices below to maximize labeling, once DNA quality is established. Consult Bionano Support for more information and tips for getting the highest quality DNA from non-validated biological sample types.
- Nicking, labeling, and repair reactions should be carried out sequentially during the course of a single day with minimal holding at 4°C between steps to be followed immediately by overnight staining of the DNA (NLRS). While we have found that NLRS DNA from plug lysis of *E. coli*, cultured human cell lines and blood from healthy human donors is stable for at least one month when properly stored in amber tubes at 4°C, we recommend running samples within two weeks.
- Master mixes should be utilized for nicking, labeling, repairing and staining reactions to avoid pipetting small volumes (< 1 µl) of enzymes/reagents and to minimize excess handling of the gDNA. For this reason, the tables indicate a minimum number of reaction volumes for each master mix. When preparing master mixes, an additional half-reaction should be added to account for sample loss.

- Enzymes and buffer should be accurately pipetted out, with no droplets hanging on the outside of the pipette tip, and completely delivered with no bubble formation to ensure reproducible reactions. This is best achieved by holding reagent tubes to eye level when aspirating or dispensing to visualize the process.

- Controlled pipette mixing after every enzymatic step (nicking, labeling and repair) promotes DNA homogeneity and enzyme accessibility for efficient labeling of highly viscous gDNA.

- This protocol involves the handling of light sensitive fluorescent molecules. For all steps beginning with labeling, it is important to minimize the exposure to light while working and to protect both the reactions and reagents from light during storage.

- A homogenous DNA concentration after NLRS (CV < 0.25) defines a critical parameter for selecting the proper waveform for loading DNA onto IrysChip. NLRS DNA concentration is determined by sampling the NLRS reaction from top, middle and bottom, due to sample viscosity, to determine a concentration average and CV.

Handling Genomic DNA

General:

- This protocol involves the handling of viscous genomic DNA (gDNA), which is difficult to accurately pipette. It is critical to follow all steps in the protocol to ensure accurate sampling of DNA for (1) proper enzyme to DNA ratio, to prevent under- and over-nicking, (2) proper DNA to DNA stain ratio, to ensure adequate staining for effective molecule visualization, and (3) to minimize handling of the DNA, which can cause shearing resulting in insufficiently long molecules for analysis.

Adding gDNA to Nicking Master Mix:

- To ensure accurate sampling from the viscous gDNA stock, first maximize stock DNA homogeneity by gently pipette mixing the room temperature, equilibrated DNA solution with a wide-bore tip 5 times and follow guidelines below for proper pipetting into and out of a standard pipette tip for complete delivery to nicking master mix.

- Before drawing viscous DNA 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 the viscous DNA. Save the marked tip as the guide and use a new one for DNA retrieval. Alternatively, the use of a positive displacement pipette 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 pipette plunger until the first stop, submerge the pipette 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 while carefully monitoring DNA uptake. Keep the tip submerged even after the viscous DNA solution stops moving upward and levels off (can use the marked tip as rough guide to see if viscous solution levels off at the appropriate level). Viscous DNA can take ~30 seconds to fill the tip to the appropriate level. Releasing the plunger too fast can produce a bubble in the tip leading under-sampling (start over if this occurs). After the solution in the pipette tip has leveled off and while the tip is still submerged in the DNA solution, scrape the tip against the bottom of the tube 5 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, or better yet, by comparing to anther pipette tip set to the same volume but containing non-viscous water. Removing the pipette tip from the gDNA solution too early, or improperly scraping the tip to break continuity between the viscous solution inside the tip and on its outside, can produce a bubble at the tip of the pipette tip indicating under-sampling (start over if this happens). **Accurate pipetting of viscous gDNA is possible with some practice and patience.**

- To deposit the entire volume of viscous gDNA into the tube containing the nicking master mix, hold the reaction tube for close-up visualization and deliver the DNA by inserting the pipette tip in the nicking master mix and gently pressing the plunger until the first stop, then to the second stop, while monitoring DNA release, until the last bit of DNA has left the tip. Immediately remove the tip as soon as the last bit of DNA has left the pipette tip while maintaining a constant pressure to avoid uptake of fluid or introduction of air bubble. Visually inspect the tip after removing from solution to confirm that it is empty.

Protocol: NLRS 300 ng/600 ng/900 ng

See Single Chip versus Multiple Chip Project section to determine the appropriate protocol to follow.
See Important Notes section to better understand the critical steps of NLRS.
See Kit Contents and Reagents sections for proper handling and storage of reagents.
See Determining Nickase section to select a nickase for your particular genome.

Nicking (10 µl/20 µl/30 µl Reaction, 2 hours 30 minutes)

Prepare Nicking Master Mix (Nb.BssSI, Nt.BspQI or Nb.BbvCI)

1. Remove gDNA from 4°C storage and allow it to equilibrate to room temperature for 30 minutes. It is essential that starting DNA concentration be between 45 ng/µl and 200 ng/µl (see Important Notes). Determine the volume of DNA required for the **300 ng/600 ng/900 ng** reaction and record in the appropriate table to calculate the correct amount of water to yield **10 µl/20 µl/30 µl** final reaction volume.

2. On ice, prepare the ***Nicking Master Mix*** for the desired number of reactions, scaling up master mix to avoid pipetting less than 1 µl of the enzyme (see tables below). Always add the enzyme as the last reagent. Follow proper pipetting guidelines in Important Notes section. After the enzyme is added, mix the Nicking Master Mix by pipetting up and down 5 times using a pipette set to 80% of the Master mix volume and pulse-spin briefly for 2 seconds.

Note: When preparing Nicking Master Mix for more than the minimum reaction volumes, ensure that an additional ½ reaction is added to account for pipetting loss.

Nb.BssSI Calculation Table-

Nickase: Nb.BssSI				*Nicking Reaction (add extra ½ reaction)*
Per reaction	300 ng	600 ng	900 ng	
DNA	____ µl	____ µl	____ µl	µl
10x NEB 3.1	1.0 µl	2.0 µl	3.0 µl	µl
Nb.BssSI* [20 U/µl]	**2.0 µl**	**4.0 µl**	**6.0 µl**	µl
Ultrapure Water	to final volume	to final volume	to final volume	µl
Final volume	10 µl	20 µl	30 µl	µl

** For plug lysis DNA from all sample types, Nb.BssSI starts at 2.0 µl. Titrate up or down if undernicking or overnicking, respectively, is observed.*

Nt.BspQI Calculation Table-

Nickase: Nt.BspQI				*Nicking Reaction (add extra ½ reaction)*
Per reaction	300 ng	600 ng	900 ng	
DNA	____ µl	____ µl	____ µl	µl
10x Buffer 3	1.0 µl	2.0 µl	3.0 µl	µl
Nt.BspQI* [10 U/µl]	**1.0 µl**	**2.0 µl**	**3.0 µl**	**µl**
Ultrapure Water	to final volume	to final volume	to final volume	µl
Final volume	10 µl	20 µl	30 µl	µl

** For plug lysis DNA from cultured human cell lines, human blood or E coli, start Nt.BspQI at 0.8 µl. Titrate down to 0.7 µl if overnicking is observed.*

** For plug lysis DNA from non-validated samples, start Nt.BspQI at 1.0 µl. Titrate down to 0.7 µl if overnicking is observed.*

Nb.BbvCI Calculation Table-

Nickase: Nb.BbvCI				*Nicking Reaction (add extra ½ reaction)*
Per reaction	300 ng	600 ng	900 ng	
DNA	____ µl	____ µl	____ µl	µl
10X Buffer 2	1.0 µl	2.0 µl	3.0 µl	µl
Nb.BbvCI* [10 U/µl]	**0.2 µl**	**0.4 µl**	**0.6 µl**	**µl**
Ultrapure Water	to final volume	to final volume	to final volume	µl
Final volume	10 µl	20 µl	30 µl	µl

** Not recommended as a first enzyme selection for plant samples.*

** For plug lysis DNA from most sources, Nb.BbvCI starts at 0.2 µl. Titrate up to 0.6 µl if undernicking is observed.*

3. On ice, add the appropriate volume of ***Nicking Master Mix*** for each reaction (H_2O + Buffer + Enzyme) into a 200 µl thin-wall PCR tube.
4. Using a **Wide-Bore Tip**, pre-mix the starting DNA by gently pipetting up and down 5 times, avoiding the introduction of air bubbles, and centrifuge briefly for 2 seconds.
5. Using a **Standard Tip** or **Positive Displacement Pipette**, transfer **300 ng/600 ng/900 ng** of DNA to each ***Nicking Master Mix*** reaction tube following the methods for accurate pipetting of viscous DNA described in the DNA Handling section of the Important Notes.
6. Mix using the Xplorer Plus Electronic Pipettor set to **8 µl/16 µl/24 µl** at the lowest speed (**1**) by pipetting up and down 4 times and centrifuge briefly for 2 seconds if any liquid is visible on the wall of the tube.

   Note: Pipette mixing ensures a homogeneous solution for efficient nicking. Moving the tip of the electronic pipettor in a circular manner during the first draw can help pick up the viscous sample for subsequent mixing. To avoid substantial bubble formation, lift the tip of the Xplorer Plus out of the solution before the "blowout phase" of the expulsion.

   Note: If an Xplorer Plus Electronic Pipettor is not available make sure to minimize DNA shearing by employing extremely slow up and down stokes with a manual pipettor.

   Note: Very viscous DNA may require additional mixing.
7. Incubate in a thermal cycler for **2 hours at 37°C** using a heated lid set at 10°C above the block temperature (or ON if no temperature choice is available), holding the reaction at 4°C. Before proceeding immediately to the labeling step, centrifuge briefly for 2 seconds if any condensation is visible on the tube wall.

Continue to Labeling…

Labeling (15 µl/30 µl/45 µl reaction, 1 hour 15 minutes)

Reminder: Minimize exposure to light and use very slow, but thorough, pipetting as DNA is fragile.

Prepare Labeling Master Mix

8. On ice, prepare the ***Labeling Master Mix*** for the required number of reaction volumes making sure the minimum number is satisfied and following proper guidelines for buffer and enzyme addition in the important notes section, mix by pipetting up and down 5 times and centrifuge briefly for 2 seconds.

Labeling Reaction				*Master Mix (add extra ½ reaction)*
Reaction Size	300 ng	600 ng	900 ng	
10X Labeling Buffer	1.5 µl	3.0 µl	4.5 µl	µl
10X Labeling Mix	1.5 µl	3.0 µl	4.5 µl	µl
Taq DNA Polymerase [5U/µl]	1.0 µl	2.0 µl	3.0 µl	µl
Ultrapure Water	1.0 µl	2.0 µl	3.0 µl	µl
Final volume	5 µl	10 µl	15 µl	µl

9. Add **5 µl/10 µl/15 µl** of the ***Labeling Master Mix*** to each nicked reaction tube.

10. Mix the labeling reaction using the Xplorer Plus Electronic Pipettor set to **13 µl/25 µl/40 µl** at the lowest speed (**1**) by pipetting up and down 4 times, to ensure a homogenous solution for efficient labeling, and pulse centrifuge briefly for 2 seconds if any liquid is visible on the wall of the tube.

    Note: Controlled stirring can be substituted to maximize DNA size. Go to https://bionanogenomics.com/support-page/dna-labeling-kit-nlrs/ and view Videos describing tips for Bionano Prep Labeling.

11. Incubate in a thermal cycler for **60 minutes at 72°C** using a heated lid set at 10°C above the block temperature (or ON if no temperature choice is available), holding the reaction at 4°C. Before proceeding immediately to the repair step, centrifuge briefly for 2 seconds if any condensation is visible on the tube wall.

Continue to Repair…

Repair (20 µl/40 µl/60 µl reaction, 45 minutes)

Reminder: Minimize exposure to light and use very slow, but thorough, pipetting as DNA is fragile.

Prepare Repair Master Mix

12. On ice, prepare the ***Repair Master Mix*** for the required number of reaction volumes, making sure the minimum number is satisfied and following proper guidelines for buffer and enzyme addition in the important notes section, mix by pipetting up and down 5 times and pulse centrifuge briefly for 2 seconds.

Repair Reaction				*Master Mix (add extra ½ reaction)*
Reaction Size	300 ng	600 ng	900 ng	
10X ThermoPol Rxn Buffer	0.5 µl	1.0 µl	1.5 µl	µl
50X Repair Mix	0.4 µl	0.8 µl	1.2 µl	µl
NAD^+ [50 mM]	0.4 µl	0.8 µl	1.2 µl	µl
Taq DNA Ligase [40U/µl]	1.0 µl	2.0 µl	3.0 µl	µl
Ultrapure Water	2.7 µl	5.4 µl	8.1 µl	µl
Final volume	5 µl	10 µl	15 µl	µl

13. Add **5 µl/10 µl/15 µl** of the ***Repair Master Mix*** to each nick-labeled reaction tube.

14. Mix the repair reaction using the Xplorer Plus Electronic Pipettor set to **18 µl/35 µl/55 µl** at the lowest speed (**1**) by pipetting up and down 4 times, to ensure a homogenous solution for efficient repair, and pulse centrifuge briefly for 2 seconds if any liquid is visible on the wall of the tube.

    Note: Controlled stirring can be substituted to maximize DNA size. Go to https://bionanogenomics.com/support-page/dna-labeling-kit-nlrs/ and view Videos describing tips for Bionano Prep Labeling.

15. Incubate in a thermal cycler for **30 minutes at 37°C** using a heated lid set at 10°C above the block temperature (or ON if no temperature choice is available), holding the reaction at 4°C. Before proceeding immediately to the next step, centrifuge briefly for 2 seconds if condensation is visible.

Continue to Stain…

Staining NLR (60 µl/120 µl/180 µl reaction, Overnight)

Reminder: Minimize exposure to light **Prepare Staining Master Mix.**

Note: The Staining Master Mix is prepared at room temperature. Always stain the entire 300 ng/600 ng/900 ng NLR reaction.

16. Label a **0.6 ml Amber Tube** for each NLR reaction.

17. Before proceeding, allow ***Staining Master Mix*** components to equilibrate to room temp.

18. In a separate **Amber Tube**, prepare the ***Staining Master Mix*** for the required number of reactions, mix by vortexing and centrifuge briefly.

*** Note:** The Saphyr System requires higher stain amounts than the Irys System; if preparing samples for Saphyr System, use the Saphyr stain volumes as outlined in the table below.

Stain Reaction				*Master Mix (add extra ½ reaction)*
Reaction Size	300 ng	600 ng	900 ng	
Stop Solution	1 µl	2 µl	3 µl	µl
4X Flow Buffer	15 µl	30 µl	45 µl	µl
5X DTT	12 µl	24 µl	36 µl	µl
DNA Stain Irys **or** (*Saphyr)	3 (4.8) µl	6 (9.6) µl	9 (14.4) µl	µl
Ultrapure Water	9 µl	18 µl	27 µl	µl
Final volume Irys (*Saphyr)	40 (41.8) µl	80 (83.6) µl	120 (125.4) µl	µl

19. Transfer **the entire volume** of the ***Staining Master Mix*** into a **0.6 ml amber tube** per reaction.

20. Using a **Standard Pipette Tip**, transfer all **20 µl/40 µl/60 µl** of each Nicked-Labeled-Repaired reaction into the amber tube containing the ***Staining Master Mix***. Centrifuge briefly for 2 seconds.

21. Using a **200 µl Wide-Bore Tip** set to **50 µl/100 µl/150 µl**, gently mix the NLRS DNA by pipetting up and down 5 times, being careful not to generate bubbles. Centrifuge briefly for 2 seconds.

22. Place staining reactions at **4°C Overnight** to ensure uniform DNA staining.

23. Perform **Quantitation** before using the NLRS sample on the Irys System. **Quantitation** is an optional QC step for the Saphyr System workflow.

Quantitation (45 minutes)

Note: Before loading the sample on the Bionano Chip, determine the final concentration of the NLRS DNA if using the Irys System, optional QC step for Saphyr System use. This is important for two reasons:

I. *Best nick, label, repair and stain results are dependent upon an accurate estimate of the amount of DNA going into the assay. If the final DNA concentration is outside the range of 3-10 ng/µl, there is a higher chance that the sample will either be over-nicked and over-stained, or under-nicked and under-stained.*

II. *On the Irys® System, the concentration time used to bring the DNA into the NanoChannels on the IrysChip is determined by the DNA concentration. Accurate measurement of the NLRS DNA concentration will help ensure optimal data collection.*

***Qubit dsDNA HS (High Sensitivity) Assay Kit & Qubit 3.0 Fluorometer*:**

Note: The standard Qubit dsDNA HS Assay protocol will not provide accurate measurements of concentration due to the extremely long lengths of the NLRS DNA. We have modified the protocol to include a sonication step to fragment the NLRS DNA to ensure accurate concentration measurements.

1. Let sample NLRS and Qubit HS standards come to room temperature (30 minutes).
2. Refer to the Qubit dsDNA HS Assay Kit user manual for full details. Briefly, prepare the **Working Solution** by diluting the **Dye Assay Reagent** into the **HS Dilution Buffer** (1:200); prepare 200 µl **Working Solution** for each of the two standards (400 µl total), and 200 µl for each sample aliquot (600 µl for each sample). For the Qubit DNA standards, add 10 µl of standard 1 and 2 to separate tubes and add 10 µl of **Qubit HS Buffer** to each.
3. Using a 200 µl pipette with a **Wide-Bore Tip** set to **50 µl/150 µl**, gently mix the NLRS DNA by pipetting up and down 3 times, being careful not to generate bubbles. Pulse centrifuge briefly for 2 seconds.
4. Using a **Narrow Bore Tip** or **Positive Displacement Pipette**, remove 2 µl aliquots from the Top, Middle and Bottom of the NLRS sample tube and place each in a separate **0.5 ml Qubit Assay Tube** used for the Qubit 3.0 Fluorometer containing 18 µL of **Qubit HS Buffer**. Place the NLRS DNA sample tubes in a floating rack and sonicate in most bath sonicators for 10 minutes. Spin down tubes briefly for 2 seconds, vortex tubes for 5 seconds, then spin down tubes again for 2 seconds.
    a. If a bath sonicator is not available, vortex for at least 30 seconds at maximum speed, then spin down briefly for 2 seconds.
5. Add 180 µl of **Working Solution** to each tube of sonicated NLRS DNA and Qubit DNA Standard plus HS Buffer, vortex for 5 seconds and centrifuge briefly to collect solution at the bottom of tubes.
6. Incubate samples in the dark for at least 5 minutes before quantitation on the Qubit Fluorimeter.

Note: The NLRS DNA concentration should fall between 3-10 ng/µl, targeting 5 ng/µl. Variation in the final concentration is due to the difficulties in accurately sampling the viscous starting gDNA. If the final NLRS DNA concentration is outside the acceptable range, re-qualify the concentration of the starting material and repeat the NLRS protocol. The CV between the Top, Middle and Bottom sampling should be < 0.25. If the CV is > 0.25, repeat the determination. If the CV remains > 0.25, proceed to loading or repeat the NLRS. CV = standard deviation/mean.

Experienced User Card: NLRS 300 ng

For multiple chips, use 600-900 ng of DNA, see page 6

Nicking (10 µl, 2 hours 30 minutes)

1. Equilibrate DNA 30 minutes at room temperature. Pipette mix 5 times with wide-bore tip.
2. Prepare **Nicking Master Mix**
   One **10 µl rxn:** 1 µl buffer + Nickase + 300 ng DNA in a 200 µl thin-wall PCR tube.
   - *Blood* — *Nt.Bss**S**I: 40U (NEB3.1)*; *Nb.Bsp**Q**I: 8U (BNG3)*
   - *Cell line* — *Nt.Bss**S**I: 40U (NEB3.1)*; *Nb.Bsp**Q**I: 8U (BNG3)*
   - *Other* — *Nt.Bss**S**I: 40U (NEB3.1)*; *Nb.Bsp**Q**I: 10U (BNG3)*; *Nb.Bbv**C**I: 2U (BNG2)*
3. Mix 4 times with **Xplorer Plus Pipettor** set to **8 µl** at lowest speed. Pulse spin 2 seconds. *Very viscous DNAs may require additional mixing.*
4. Incubate in a thermal cycler for **2 hours at 37°C** with heated lid. Pulse spin 2 seconds.

Labeling (15 µl, 1 hour 15 minutes)

Protect from light

5. Prepare the **Labeling Master Mix**.
   10X labeling buffer: 1.5 µl
   10X labeling mix: 1.5 µl
   Taq 5 U/µl: 1 µl
   H_2O: 1 µl
   For one reaction
6. Add **5 µl Labeling Master Mix.**
7. Mix 4 times with **Xplorer Plus Pipettor** set to **13 µl** at lowest speed. Pulse spin 2 seconds. *Controlled stirring can be substituted to maximize DNA size.*
8. Incubate in a thermal cycler for **60 minutes at 72°C** with heated lid. Pulse spin 2 seconds.

Repair (20 µl, 45 minutes)

Protect from light

9. Prepare the **Repair Master Mix.**
   10X Thermo Pol: 0.5 µl
   50X Repair Mix: 0.4 µl
   50 mM NAD+: 0.4 µl
   Taq DNA ligase: 1 µl
   H_2O: 2.7 µl
   For one reaction
10. Add **5 µl** of the **Repair Master Mix**.
11. Mix 4 times with **Xplorer Plus Pipettor** set to **18 µl** at lowest speed. Pulse spin 2 seconds. *Controlled stirring can be substituted to maximize DNA size.*
12. Incubate in a thermal cycler for **30 minutes at 37°C** with heated lid. Pulse spin 2 seconds.
13. Place NLR reaction on ice.

Staining NLR (60 µl, 16 hours/overnight)

Protect from light

14. Equilibrate **Staining Master Mix** components to room temperature.
15. Prepare the **Staining Master Mix.**
    Stop solution**:** *1 µl*
    4X flow buffer: 15 µl
    5X DTT: 12 µl
    DNA stain: 3 µl (Irys) or 4.8 µl (Saphyr)
    H_2O: 9 µl
    For one reaction
16. Aliquot **40 µl** of the **Staining Master Mix** into a 0.6 ml amber tube.
17. Transfer NLR to Staining Mix with standard tip. Pulse spin 2 seconds.
18. Gently mix NLRS DNA 5x with **wide-bore tip** set to **50 µl**. Pulse spin 2 seconds.
19. Place staining reactions at 4°C **overnight** to ensure uniform DNA staining.

Quantitation (*optional for Saphyr*, 45 min)

Protect from light

1. Equilibrate NLRS and Qubit standards to room temperature for 30 minutes.
2. Gently mix DNA **5 times** with **wide-bore tip** set to **50 µl**. Pulse spin 2 seconds.
3. Remove 2 µl aliquots from the **Top, Middle, and Bottom** to separate Qubit Assay tubes. Add 18 µl of Qubit HS Buffer.
4. Sonicate 10 minutes in a bath sonicator.
5. Add 10 µl of each Qubit standard to separate Qubit Assay tubes. Add 10µl of TE to each.
6. Add 180 µl Qubit reagent mix. Vortex 5 seconds. Pulse spin 2 seconds.
7. Incubate at least 2 minutes at room temperature. Quantitate with Qubit Fluorimeter.

   *NLRS DNA concentration: **3-10 ng/µl**. CV < 0.25*

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