Bionano Prep Blood DNA Isolation Protocol

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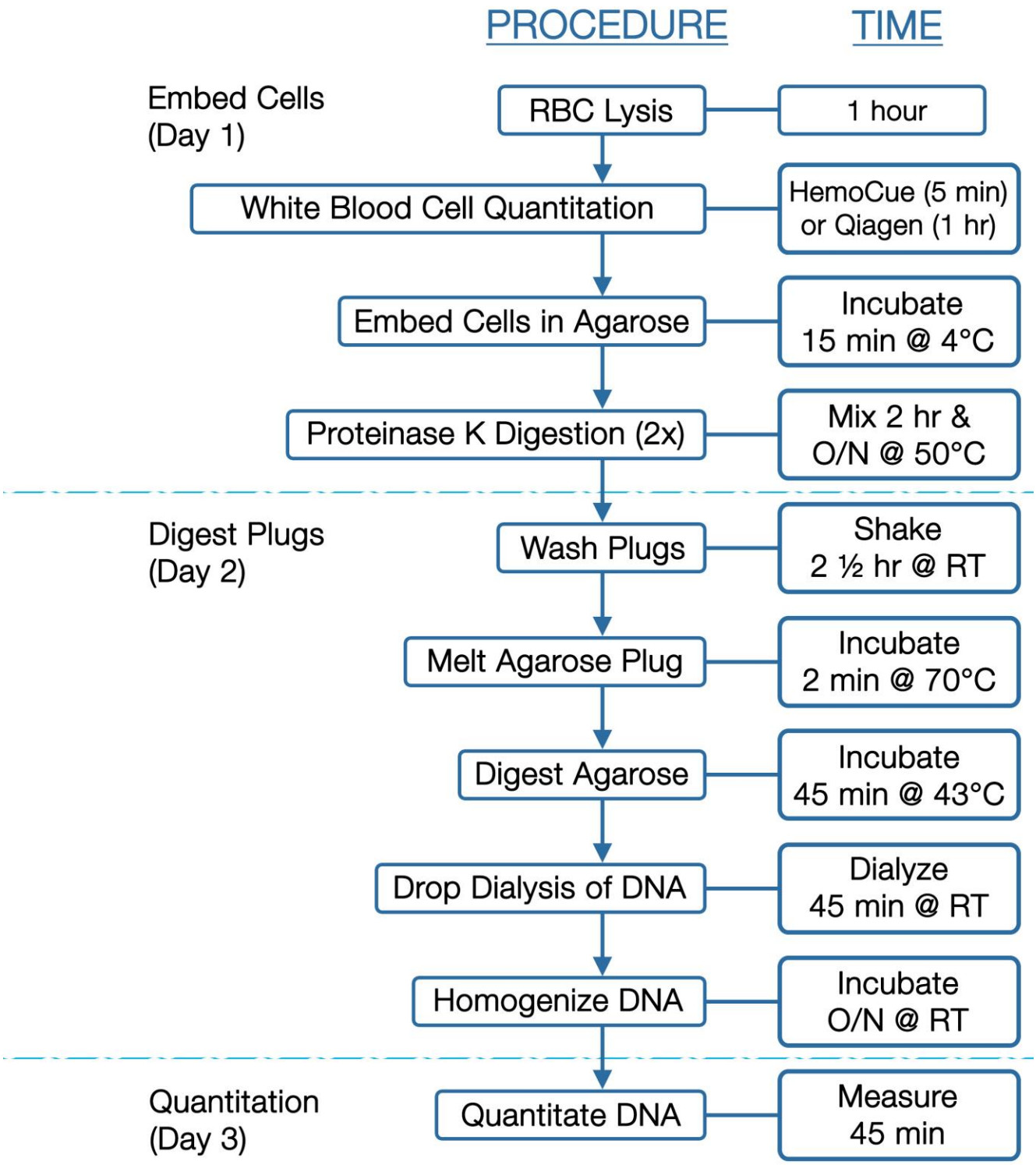
Bionano Prep Blood DNA Isolation Protocol

Workflow Overview



Experienced User Card: Blood

RBC lysis (1 hour)

1. Mix blood by gently rocking 10 min at RT.
2. Transfer **3 ml blood** per 15 ml tube containing **9 ml RBC lysis solution**.
3. Mix by gently rocking 10 times. Incubate 5 min at RT, and gently rock 10 times during and 10 times at end of incubation.
4. Spin 2 min at 2000 x g at 4°C & decant sup.
5. **Quickly** suspend pellet in **3 ml Cell Buffer** (0.3 ml resuspension, then + 2.7 ml).
6. Add **9 ml RBC lysis solution**.
7. Repeat steps 3-5.
8. **Remove insoluble particulates with pipette tip**.
9. Spin and pipet out liquid removing last drop.
10. **Quickly** resuspend pellet in **563 µl Cell Buffer** (200 µl resuspend, then + 363 µl) and **place on ice.**

WBC Quantitation (5 min or 1 hour)

Option 1: WBC count with HemoCue (~5 min)

11. Pipet mix WBC (Step 10). Transfer 7.5 µl to new tube and mix with 32.5 µl Cell Buffer.
12. Determine total WBC count per 65 µl.
13. **If count = 1.2×10^6 – 1.9×10^6**, go to Step 17. If different adjust to 1.2×10^6/65 µl Cell Buffer and proceed.

Or, Option 2: DNA Recovery (~1hr)

14. Pipet mix WBC from Step 10. Transfer 65 µl to tube, add 135 µl Cell Buffer, & isolate DNA (QIAamp).
15. Determine total DNA yield per 65 µl WBC.
16. **If DNA yield = 4.3-6.9 µg**, go to Step 17. If different, adjust WBC to 6 µg per 65 µl Cell Buffer and proceed.

WBC plugs (~1hr)

17. Pipet mix WBC (from Step 10) and transfer 65 µl per plug to new tube.
18. Equilibrate WBC at 43°C for **only** 10 min.
19. Add 39 µl 2% agarose per 65 µl WBC & proceed quickly.
20. Pipet mix WBC-agarose mixture 10 times, and cast plugs.
21. Incubate 15 min at 4°C to solidify agarose.

Proteinase K digestion

22. For each sample, make PK solution: 167 µl Proteinase K + 2.5 ml Lysis Buffer.
23. Dislodge plugs into PK Solution, cap and incubate for 2 hours at 50°C with intermittent mixing (or overnight first if desired).
24. After 2 hours, replace PK Solution with freshly made PK Solution, incubate overnight at 50°C with intermittent mixing (or overnight incubation followed by 2 hours).

Wash plugs to stabilize DNA

25. Prepare 70 ml 1x Wash Buffer per 1-5 plugs.
26. Drain PK Solution and rinse 3 times with 10 ml 1x Wash Buffer.
27. Wash 4 times with 10 ml 1x Wash Buffer by shaking 15 min at 180 rpm. Do not discard last wash. Plugs are stable in 1x Wash Buffer at 4°C.

DNA recovery

28. Wash plugs 5 times in 10 ml TE by shaking 15 min at 180 rpm.
29. Discard last TE wash. Scoop plug with metal spatula, drain excess liquid and transfer to 1.5 ml tube.
30. Melt plug at 70°C for 2 min.
31. Equilibrate at 43°C for 5 min.
32. Add 2 µl of Agarase and stir mixture gently with pipet tip for 10 sec.
33. Incubate at 43°C for 45 min.

Drop dialysis and DNA homogenization

34. Dialyze DNA 45 min at RT on floating dialysis membrane in 15 ml TE and transfer to 1.5 ml tube.
35. If DNA is viscous, pipet mix up to 9 times until entire sample is taken up in a continuous flow and incubate overnight at RT.

Quantitation

36. Pipet mix DNA 5 times with wide bore tip, and transfer 2 µl from top, middle and bottom into Qubit tubes containing 18 µl BR Buffer.
37. Sonicate 10 min in water bath, briefly spin, vortex 5 sec at max speed and briefly spin.
38. Add 180 µl Qubit BR Dye Reagent + Buffer mix. Vortex tubes continuously at max speed for 5 sec and briefly spin.
39. Incubate 5 min at RT and read on Qubit reader.
40. Proceed to DNA labeling.

Bionano Prep Blood and Cell Culture DNA Isolation Kit (Part # 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
Blood processing – Day 1; Plug lysis – Day 1 and 2	
Vari-Mix™ Test Tube Rocker	Thermo Fisher Scientific, Catalog # M48725Q or equivalent
Ice bucket and ice	General lab supplier
15 ml conical tube(s)	VWR, Catalog # 21008-918 or equivalent
RBC Lysis Solution, 450 ml	Qiagen, Catalog # 158902
One of the following is required for quantitation:	
HemoCue WBC Analyzer (recommended) + microcuvettes	Fisher Scientific, Catalog # 22-601-017 Fisher Scientific, Catalog # 22-601-018
	-or-
QIAamp DNA Blood Mini Kit or equivalent + ethanol (non-denatured; 96-100%)	Qiagen, Catalog # 51104 General lab supplier
Microcentrifuge tubes, 1.5ml	VWR, Catalog # 87003-294 or Equivalent
50 ml conical tube(s)	VWR, Catalog # 21008-951 or Equivalent
Centrifuge, Swing-bucket Refrigerated with 15 ml Tube Adapters	Eppendorf 5804R or Equivalent
Heat blocks or water baths set to 43°C and 70°C	General lab supplier
Agarose, CleanCut 2% solution	Bio-Rad, Catalog # 1703594
Agarose plug molds and plug mold plunger	Bio-Rad, Catalog # 1703713
Green screened caps for 50 ml conicals	Bio-Rad, Catalog # 1703711
Proteinase K enzyme, 5 ml	Qiagen, Catalog # 158920
Thermomixer	Eppendorf, Catalog # 5382000023 or Equivalent
50 ml Thermoblock	Eppendorf, Catalog # 5365000010 or Equivalent
Orbital Platform Shaker	Cole-Parmer, Catalog # EW-51820-30 or Equivalent
Microwave (for agarose preparation)	General lab supplier
UltraPure™ DNase/RNase-Free Distilled Water	Thermo Fisher Scientific, Catalog # 10977-015
TE Buffer, pH 8.0	Thermo Fisher Scientific, Catalog # AM9849
DNA Recovery - Day 2	
Metal spatula	VWR, Catalog # 82027-530
Agarase (0.5 unit/µl)	Thermo Fisher Scientific, Catalog # EO0461
0.1µm Dialysis membrane	Millipore, Catalog # VCWP04700 or Equivalent
Petri dish, 6 cm	VWR, Catalog # 28384-092
Pipette Tips, 200 µl, Nonfiltered and beveled	USA Scientific, Catalog # 1111-1810
Pipette Tips, 200 µl, Wide-Bore	VWR, Catalog # 46620-642 or Rainin Equivalent
DNA Quantitation - Day 3	
Bath sonicator	General lab supplier
Microcentrifuge	General lab supplier
Vortexer	General lab supplier
Qubit 3.0 Fluorometer	Thermo Fisher, Catalog # Q33216 or Equivalent
Qubit BR 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
Positive-Displacement Tips, 10 µl, C-10 (optional)	Rainin, Catalog # 17008604 or Equivalent

Introduction

The Bionano Prep Blood DNA Isolation Protocol targets recovery of white blood cells (WBC) using differential red blood cell (RBC) lysis. The resulting WBCs are quantitated, immobilized in agarose plugs, and subjected to plug lysis treatment for high molecular weight DNA recovery. WBC quantitation enables targeting the proper number of cells per plug to stay within the optimal range for plug lysis.

Procedure

Fresh blood is subjected to two rounds of differential RBC lysis to get clean WBC preparations. Cell quantitation for proper targeting of WBC per plug is achieved by means of a bench top automated counting instrument, HemoCue, which counts WBCs within a few minutes. Alternatively, a DNA-recovery strategy can be used with Qiagen's DNA Blood Mini Kit (or equivalent), a process that takes about 1 hour to complete. High molecular weight DNA 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 mixing step to ensure sample homogeneity prior to quantitation.

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 reaction. The Qubit Broad Range (BR) dsDNA Assay measures DNA concentration following plug lysis, while the High Sensitivity (HS) dsDNA Assay measures DNA concentration after labeling. The Picogreen-based Quan-iT™ kit can be used for quantitating DNA following plug lysis; however, it is not appropriate for DNA quantitation after labeling.

To gauge DNA homogeneity, it is essential to measure DNA concentration at multiple positions (top, middle, and bottom) in the DNA solution. Since viscous DNA is difficult to pipet, follow guidelines in the Important Notes and DNA Quantitation sections below for accurate pipetting. Standard assays for quantification of DNA concentration will not provide accurate measurements of long DNA due to its viscous nature. A sonication step to fragment the gDNA has additionally been added to facilitate accuracy and consistency.

Important Notes

Blood Considerations

- Blood should be non-frozen, EDTA stabilized, and **processed within 5 days of draw (including transport time)**.
- Blood should be stored at 4°C promptly after collection and shipped on ice. It is best to process blood samples immediately after receiving. However, a full day's work is required to isolate WBCs and embed in plugs for overnight proteinase K digestion (see Workflow Overview). If time constraints occur on the day blood is received, store blood at 4°C for processing the following day.
- Do not use blood showing signs of hemolysis or clotting.
- Blood should be adequately mixed and warmed to room temperature for a uniform cell distribution before sampling for WBC isolation.
- A uniform suspension of WBC, representing 4-6 µg worth of DNA (3 µg of DNA if limited material), evenly dispersed in agarose matrix is **required** for recovering quality megabase containing DNA.
- It is preferable to process WBCs into plugs rather than freezing, which can lead to cell clumping and render the thawed aliquot difficult for future DNA isolation.
- WBCs embedded in agarose are not stable; they must be carried through overnight Proteinase K digestion and DNA stabilization washes. Plugs in 1x Wash Buffer can be subsequently processed for DNA recovery, stored at 4°C for later use, or shipped at room temperature or 4°C for processing at distant facilities.
- RNAse treatment of plugs is not necessary for the Bionano Prep Blood DNA Isolation Protocol.

Safety Precautions For Handling Blood

- Direct blood manipulations should be done in a biosafety cabinet.
- Centrifugations involving blood are best carried out in buckets with bio-containment lids.
- All solutions that come in contact with plugs containing blood cells up to (and including) the Proteinase K digestion step should be treated as biohazardous liquid and discarded per your site's guidance for biohazardous waste stream disposal (such as collecting liquid in a waste container filled with bleach before discarding, autoclaving, etc).
- Contaminated solid waste should also be discarded as biohazard waste.
- A lab coat, disposable gloves, and protective goggles should be worn.

Proteinase K Digestion

- Embedding cells in agarose does not stabilize the DNA. The optimum stopping point for fully stabilized DNA within agarose plugs is after the 1x Wash Buffer steps following Proteinase K digestion.
- Use of the Lysis Buffer developed by Bionano Genomics is important for maximal lysis and Proteinase K digestion.
- Up to 5 plugs can be simultaneously treated with Proteinase K within the same conical tube.

DNA Recovery

- Recovered DNA is subjected to pipette mixing with a carefully selected standard pipet tip to increase homogeneity, ensuring consistent DNA sampling for labeling.
- It is important to remove as much residual buffer from an agarose plug before the plug melting step.

Plug Handling

- Plugs must be fully submerged during lysis and wash steps, with intermittent mixing employed during enzymatic steps and continuous mixing during wash steps. Horizontal, orbital style mixers are employed during lysis and wash steps. Rotating, rotisserie style mixers such as a hybridization oven, are not recommended.
- Plugs are very fragile and should be handled carefully to prevent fragmentation. The corners of plugs may be clipped during the protocol without significant loss of plug volume. In rare instances, a plug may be sheared into two or more large pieces during normal processing. Large pieces can be collected at the end of processing and combined into a single microfuge tube for plug melting.
- Always use a flat, blunt tool, such as a metal spatula, to retrieve, transport, or deposit an agarose plug. Avoid pointed objects, such as pipette tips, which will easily fragment the agarose plug.
- When attempting to pick up an agarose plug with a metal spatula, always decant buffer first. The plug will adhere to the wall of the conical and the metal spatula is then slid under the plug between the plug and wall of the conical and can be transported safely.
- Agarose plugs are extremely prone to sticking to the sides of 50 ml conical tubes and to the bottom of the green screened cap (side facing inside of conical) during washing.

  Note: *It is critical to always check* ***during and after every wash*** *or enzymatic digestion step, to make sure* ***all*** *agarose plugs are collected in bottom of tube and are fully submerged in solution. If possible, always try to count the number of submerged plugs after each step. Failure to do so, can lead to poor quality plugs due to lack of enzymatic digestion or washing, or due to excessive drying.*

Drop Dialysis

- Make sure to pipet DNA volume onto center of drop dialysis membrane. Pipetting DNA onto edge of membrane risks membrane submersion and sample loss.
- Avoid pushing down on membrane with excessive force from pipette tip, which can lead to membrane submersion and sample loss. Surface tension between the pipette tip and DNA solution is generally sufficient to allow collection of DNA from membrane without directly touching the membrane with pipette tip.

Pipetting Viscous Genomic DNA

- To draw viscous gDNA, hold the stock DNA tube for close-up visualization, depress the pipette plunger until the first stop, submerge the pipette tip toward the top, middle, or bottom of the viscous solution, and carefully release the plunger, as **slowly** as possible, to start drawing 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. Be patient. Viscous DNA can take more than 30 seconds to fill the tip to the 2µl mark. Releasing the plunger too fast can produce a bubble in the tip leading to 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 3-5 times using a circular motion. Remove the tip from the DNA solution and visually inspect to confirm that it is filled to the 2 µl mark. Removing the pipette tip from the gDNA solution too early, or ineffectively 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).

DNA Handling

- Mixing of recovered DNA is always carried out with a wide bore pipette tip to prevent DNA shearing.
- Pipetting of recovered DNA for accurate sampling is always carried out with a standard bore tip.

DNA Quantitation

- Sonication of sampled DNA is necessary for accurate quantitation.
- DNA having a concentration between 35 ng/µl and 200 ng/µl should be used for labeling.
- The coefficient of variation (CV) from three unique samplings (from the top, middle, and bottom of the DNA solution) should be less than 0.25. CV = standard deviation/mean.

Characteristics of High Quality DNA for Bionano Mapping

- A clear DNA solution is ideal, but an unclear solution does not always correlate with poor sample quality.
- DNA in solution is viscous in appearance.
- Contains mega base size DNA as measured by pulsed field gel electrophoresis (PFGE).
- Homogenous as measured with Picogreen or Qubit® DNA quantitation assay with CV < 0.25.

Blood DNA Isolation Protocol

Day 1: RBC Lysis, WBC Quantitation, WBC Plugs, and Proteinase K Digestion (5 hours)

Before First Use

1) 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 aliquots in microfuge tubes that can be stored at 4°C. Use one or more 500 µl aliquots for each new procedure.

Before Each Use

2) Gather materials (see 'User Supplied Material' section above).
3) Set up heat blocks/water baths:
    a) Set a heat block or water bath to 70°C.
    b) Set another heat block or water bath to 43°C.
4) Set centrifuge with swinging bucket rotor and 15 ml tube adapters to 4°C.
5) Pre-chill the Cell Buffer and plug mold(s)/plunger to 4°C.
6) Melt 2% Agarose aliquot(s) in a 70°C water bath or heat block for 10 minutes and equilibrate at 43°C for at least 15 minutes to prepare WBC plugs. If using heat blocks, ensure presence of water in wells equilibrated to desired temperature, for efficient heat transfer while in use.
7) Equilibrate a Thermomixer fitted with 50 ml adapter to 50°C for Proteinase K digestion.

RBC Lysis (1 hour)

Two rounds of differential Red Blood Cell (RBC) lysis

8) Place blood sample on a test tube rocker (set to: speed 20 rpm, max rocking angle) and mix by rocking for 10 minutes at room temperature to ensure a homogenous WBC distribution prior to sampling for differential RBC lysis.
9) Dispense **9 ml RBC Lysis Solution (Qiagen)** into a 15 ml conical tube for each sample. Add 3 ml mixed blood and cap each tube, then wrap with parafilm.

    Note: If processing <3 ml blood make up volume difference with Cell Buffer. Protocol is optimized for 3 ml processing.

10) Mix by gently rocking 10 times using test tube rocker. Incubate 5 minutes at room temperature. Approximately halfway through incubation, mix by gently rocking an additional 10 times.
11) After incubation, mix by gently rocking a final 10 times.
12) Spin 2 minutes at 2,000 x g at 4°C, then carefully decant the supernatant into an appropriate blood waste container (see Safety Precautions for Handling Blood) without disturbing the White Blood Cell (WBC) pellet.
13) *Immediately* resuspend WBC pellet in 300 µl **Cell Buffer (Bionano)** by gently pipette mixing 5 times. Then add an additional 2.7 ml Cell Buffer without any additional mixing.

    Note: An insoluble particulate may become apparent in solution after the initial Cell Buffer addition (300 µl) or after the second Cell Buffer addition (2.7 ml); continue to Step 14. The insoluble particulate will carry through to the Cell Buffer wash where it will be removed at Step 16. The insoluble particulate may vary in size from sample to sample.

14) Add **9 ml RBC Lysis Solution** and cap each tube.

15) Repeat steps 10-13 for a second round of differential RBC lysis. The end result is WBC recovered from 3 ml of blood resuspended in 3 ml of Cell Buffer.

Wash and Resuspend White Blood Cells (5 min)

16) If a particulate is present, remove by dragging on tube side with pipette tip until free of solution and discard.

17) Spin 2 minutes at 2,000 x g at 4°C, and carefully remove the supernatant with a 1,000 µl pipette tip without disturbing the WBC pellet. Tilt tube to pipet out the last drop of liquid, and proceed *quickly* to next step.

18) Resuspend in 200 µl Cell Buffer by gently pipet mixing 5 times. Transfer the entire volume to a new microfuge tube. Add 363 µl Cell Buffer and gently pipet mix the entire volume another 5 times. Keep cells on ice until ready to embed in agarose.

**Note: Step 18 is calculated for WBC recovered from 3 ml of blood. If procedure began with <3 ml blood, decrease the amount of Cell Buffer from 563 µl to a proportionally lower amount. Divide the amount of blood used by 3 ml to determine that proportion.*

WBC Quantitation (Option 1: ~5 min; Option 2: ~1 hour)

*Two options: Option 1 is based on **WBC counting** (HemoCue), Option 2 is based on **DNA recovery** (QIAmp DNA Blood Mini)*

Option 1: WBC count (5 min)

Option 1 requires a HemoCue (see User Supplied Materials table). Use of a hemocytometer to count WBC's is highly variable and discouraged.

19) Pipet mix entire volume of WBC from Step 18 gently 5 times with a 1,000µl pipette to ensure a uniform suspension. ***Immediately*** transfer 7.5 µl to a new microfuge tube containing 32.5 µl **Cell Buffer** for HemoCue counting. Keep rest of WBC on ice.

20) Use the **HemoCue WBC count (___×10^9 WBC/L)** to determine **total # WBC per 65 µl** WBC Suspension:

(_____×10^9 WBC/L) × (0.000001 L/µl) × (5.33) × (65 µl) → (_____ $WBC_{65µl}$)

21) If **#WBC per 65 µl Cell Suspension = 1.2×10^6 – 1.9×10^6, go to Step 25**. If different, adjust to 1.2×10^6 WBC per 65 µl as shown next, then proceed to Step 25:

i. Given the **total #WBC per 65 µl** and 1.2×10^6 WBC target, calculate the **WBC volume /plug (µl)**:

65 µl ÷ (_____ $WBC_{65µl}$) × (1.2×10^6) → (_____ µl $WBC_{1.2×10^6\ WBC/plug}$)

ii. Using **WBC volume/plug (µl)** and desired **# of plugs**, calculate **total WBC volume (µl)**:

(_____ µl $WBC_{1.2×10^6\ WBC/plug}$) × (_____ plugs) → (_____ µl WBC_{total})

iii. Pipet mix WBC from Step 18 gently 5 times with a 1,000 µl pipette and transfer **total WBC volume (µl)** to a new tube. Adjust its volume to achieve **final volume (µl)**:

65 µl × (_____ plugs) → (_____ µl final volume)

- To concentrate WBC, spin cells at 400 × g for 10 minutes at room temperature, remove the supernatant, and resuspend pellet in new Cell Buffer.
- To dilute WBC, add Cell Buffer to final volume.

Option 2: DNA Recovery (1 hour)

22) Pipet mix entire volume of WBC from Step 18 gently 5 times with a 1,000 µl pipette to ensure a uniform suspension. ***Immediately*** transfer 65 µl to a microfuge tube containing 135 µl Cell Buffer for DNA isolation as per Qiagen QIAamp DNA Blood Mini Kit, 'Blood or Body Fluid Spin Protocol'. Keep the rest of the WBC on ice.

23) Use **QIAamp conc. (ng/µl)** and **elution vol. (µl)** to determine **total (µg) DNA per 65 µl** WBC Suspension:

(_____ ng/µl) × (_____ µl) × (0.001 µg/ng) → (_____ µg $DNA_{65µl}$)

24) **If total DNA yield per 65 µl is 4.3 – 6.9 µg, go to Step 25**. If different, adjust to 6 µg per 65 µl as shown next, then proceed to Step 25:

i. Given the **total (µg) DNA per 65 µl** WBC and 6 µg DNA target, calculate **WBC volume/plug (µl)**:

65 µl ÷ (_____ µg $DNA_{65µl}$) × (6 µg) ⟶ (_____ µl $WBC_{6µg/plug}$)

ii. Using **WBC volume/plug (µl)** and desired **# of plugs**, calculate **total WBC volume (µl)**:

(_____ µl $WBC_{6µg/plug}$) × (_____ plugs) ⟶ (_____ µl WBC_{total})

iii. Pipet mix WBC from Step 18 gently 5 times with a 1,000 µl pipette and transfer **total WBC volume (µl)** to a new tube. Adjust its volume to achieve **final volume (µl)**:

65 µl × (_____ plugs) ⟶ (_____ µl final volume)

- To concentrate WBC, spin cells at 400 × g for 10 minutes at room temperature, remove the supernatant, and resuspend pellet in new Cell Buffer.
- To dilute WBC, add Cell Buffer to final volume.

WBC Plugs (~ 1hr)

Note: *Perform next steps rapidly to avoid solidification of the WBC-agarose mixture before pipetting into plug mold. Limit total incubation time of resuspended WBC-agarose mixture at 43°C to 10 minutes max. WBC resuspended in Cell Buffer are stable on ice if multiple rounds of gel plug casting are necessary in order to avoid exposure of cells to 43°C for more than 10 minutes.*

25) Use table (from Step 26 below) to determine number of plugs to be made based on WBC amount. If only a portion of the WBC is needed, gently pipet mix WBC (from Step 18) 5 times with a 1,000 µl pipette and *immediately* transfer the proper amount to a new tube.

26) Equilibrate cells in 43°C water bath/heat block for **only 10 minutes** before use. During equilibration, place the appropriate number of **Plug Molds (Bio-Rad)** and pipettes (1000 µl & 200 µl) near 43°C water bath or heat block. Find appropriate volumes per table below, and preset pipettes to efficiently process steps 27-28.

# plugs	1	2	3	4	5	6	7	8
WBC in Cell Buffer	65 µl	130 µl	195 µl	260 µl	325 µl	390 µl	455 µl	520 µl
2% agarose	39 µl	78 µl	117 µl	156 µl	195 µl	234 µl	273 µl	312 µl

27) Quickly add **2% Agarose** to cell suspension, and gently mix 10 times with a 1,000 µl pipette, avoiding bubble formation.

28) *Immediately* fill first well of the plug mold by pipetting ~95 µl of cell-agarose mixture with 200 µl pipette until liquid is flush with top of the well. Make sure to place the WBC-agarose mixture back at 43°C when not in use.

29) For each subsequent plug mold, mix entire volume of the WBC-agarose mixture an additional 3 times with a 200 µl pipette, and immediately pipet ~95 µl of the cell-agarose mixture with a second 200 µl pipette, into next well of plug mold.

30) Place plug cast at 4°C for at least 15 minutes to solidify. Alternatively, place plug cast on ice or on an inverted metal microfuge block on ice for 15 minutes, to avoid potential contact of agarose with ice.

Digest with Proteinase K (2 hours + Overnight)

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

31) Prepare fresh **Proteinase K Solution** by mixing 167 µl of **Puregene Proteinase K Enzyme (Qiagen)** with 2.5 ml of **Bionano Prep Lysis Buffer** per 1-5 plugs to be processed in each 50 ml conical tube.
32) Transfer plugs to conical tube containing **Proteinase K Solution**, by first removing tape from bottom of plug mold followed by dislodging plugs into conical tube with **Plug Mold Plunger** (Bio-Rad). Make sure all plugs are fully submerged. Use spatula or blunt edged instrument to submerge plugs if they become stuck to conical tube walls.
33) Cap each tube and incubate in Thermomixer (fitted with adaptor for 50 ml conical tubes) 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).
34) Near the end of the incubation, prepare fresh **Proteinase K Solution** by mixing 167 µl of **Puregene Proteinase K Enzyme** with 2.5 ml of **Bionano Prep Lysis Buffer** per 1-5 plugs to be processed per tube.
35) 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 digestion 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.
36) Remove the screened cap, and add ~2.6ml of freshly mixed **Proteinase K Solution**. Account for all plugs and ensure that they are completely submerged in the Proteinase K Digestion 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 digestion workflow was chosen.

Day 2: DNA Stabilization Washes, DNA Recovery, Drop Dialysis

Set Up

1) Gather materials (see 'User Supplied Materials' section above).
2) Set up heat blocks/water baths:
    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.
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.

Washing Plugs to Stabilize DNA (1 hour 30 minutes)

4) Following the second Proteinase K digestion, remove tube (s) from the Thermomixer. Incubate at room temperature for 5 minutes.
5) Replace original cap with screened cap, drain 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.
6) **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.
7) **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 a horizontal platform mixer 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)

8) Discard final 1x Wash Buffer through the screened cap and tap plugs to the bottom of the tube.
9) **TE Wash** plugs **5 times** by:
    a. Adding 10 ml of **TE Buffer, pH 8.0 (Thermo Fisher Scientific)** per wash through the screened cap and capping tube.
    b. Continuous shaking for 15 minutes on a horizontal platform mixer at 180 rpm.
    c. Discarding the wash through the screened cap.
    d. Tapping plugs to bottom of tube before adding the next wash.

10) Discard the last TE wash through the screened cap and tap plugs to bottom of tube.

11) 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 1.5 ml microcentrifuge tube.

12) Pulse spin each microcentrifuge tube briefly to collect the plug at the bottom of the tube.

13) Melt each agarose plug in a water bath or heat block set at 70°C for 2 minutes.

14) Immediately transfer each tube to a water bath or heat block set at 43°C, incubate for 5 minutes.

15) Process one tube at a time: add **2 µl** of **0.5 U/µl Agarase (Thermo Fisher Scientific)** enzyme to tube and stir mixture gently with a pipette tip for 10 seconds.

16) 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)

17) Place 15 ml of **TE Buffer** into 6 cm **Petri Dish (VWR)** per each DNA sample.

18) 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.

19) Pipette mix the entire volume of DNA solution 2 times with a **Wide Bore Tip (VWR)** 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.

20) Place cover on the Petri dish and let the sample dialyze for 45 minutes at room temperature.

21) Transfer DNA to a 1.5 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)

22) Test DNA viscosity by slowly withdrawing the entire DNA volume into a **non-filtered beveled 200 µl tip (USA Scientific)** while slowly releasing pipette plunger. If the DNA is not viscous, do not pipette mix. If the DNA is 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.

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.

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

Day 3: Quantitation

Qubit Quantitation - BR dsDNA Assay

1) Equilibrate Qubit BR Assay kit standards to room temperature from 4°C.
2) Create 200 µl **Qubit BR Working Solution** for each sample assay tube (3 per sample) and each Qubit BR Assay Kit Standard (2 total) by diluting **Qubit dsDNA BR Reagent** in **BR Buffer** (1 : 200 dilution).
3) Aliquot 18 µl of **Qubit BR Buffer** per Qubit assay tube, preparing three tubes per sample.
4) Carefully pipette mix the entire volume of DNA 5 times with a **Wide Bore Tip,** avoid bubble formation.
5) Using a **Standard Bore Tip** or **Positive Displacement Pipette**, aspirate 2 µl from the top, middle and bottom of each DNA sample and dispense each draw into separate Qubit assay tubes (from step 2 above) following the methods described in the 'Pipetting Viscous Genomic DNA' section, to ensure for accurate pipetting of viscous DNA for quantitation. Use a different tip for each draw.
6) Sonicate tubes in sonicating bath for 10 minutes to fragment DNA and then briefly spin to collect solution at bottom of tubes.
7) Vortex tubes continuously at maximum speed for 5 seconds and then briefly spin to collect solution at bottom of tubes.
8) Add 180 µl of diluted **Qubit BR Working Solution** (Dye Reagent + Buffer). Vortex tubes continuously at maximum speed for 5 seconds and then briefly spin tubes to collect solution at bottom of tubes.
9) Incubate samples, light-protected, for 5 minutes and proceed with quantitation on Qubit reader.
10) Coefficient of variation (CV) from the three separate readings should be < 0.25.

    ***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 beveled 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. See 'Kits and Consumables' section at https://bionanogenomics.com/support/ for applicable kits and protocols.

Troubleshooting

Problem	Possible Causes	Actions
Insoluble precipitate forms upon first round of differential RBC lysis.	High fat content in original whole blood.	Remove insoluble precipitate with pipette tip at the Cell Buffer wash step.
Gel plugs appear non-homogenous after plug casting.	2% agarose did not completely melt before addition to suspended cells.	Increase melting time of 2% agarose at 70°C before equilibration at 43°C.
	Molten 2% agarose allowed to re-solidify before addition to suspended cells.	Make sure to keep molten agarose at 43°C before adding to suspended cells to prevent agarose from re-solidifying.
	Waiting too long to pipet mixture of cells and agarose into plug molds.	Limit the total time from mixing molten agarose with suspended cells to pipetting mixture into plug molds, to less than 1 minute.
	Improper mixing of molten agarose and suspended cells before pipetting into plug molds.	Make sure mixture of molten agarose and suspended cells is mixed thoroughly with 1,000 µl pipette immediately before pipetting into plug molds with a 200 µl pipette.
Gel plug never fully solidifies.	Agarose concentration may be too low.	See below: *Agarose concentration of plug is too low.*
	Not enough time to solidify plug molds.	Increase incubation time of plug molds on ice to 60 minutes.
Agarose concentration of plug is too low.	Concentration of stock 2% agarose has changed since initial use.	Use fresh bottle of 2% agarose. It is recommended to melt fresh bottle of 2% agarose and then store as 500 µl aliquots in microfuge tubes at 4°C to prevent change in stock agarose concentration over time.
	Improper ratio of agarose to suspended cells.	Check calculations to make sure to add enough 2% agarose to resuspended cells in order to yield 0.75% agarose plugs.
Gel plug damaged during purification.	Agarose concentration of gel plug may be too low.	See above: *Agarose concentration of plug is too low.*
	Mixing steps during lysis and washes may be too vigorous.	Lower mixing speeds during washes and lysis. Can reduce mixing speed to 100-120 RPM on orbital shaker or can manually mix by hand once, halfway through each 15min wash step to prevent further damage.
	Improper handling of plugs with spatula.	Drain as much liquid as possible using green screened cap before transferring plug with spatula. Then, make sure to insert spatula completely beneath entire length of plug before transferring.

Gel plug lost during purification.	Gel plug adhered to green screened cap or sides of 50 ml conical tubes during lysis or wash steps.	Check to make sure all plugs are accounted for during and after all wash and lysis steps by checking 1) plug number and 2) attachment of plugs to sides of 50 ml conical tubes and green screened cap.
Gel plug never becomes fully clear after lysis [**Note:** While a cleared gel plug is considered optimum, a plug that never clears, despite following all of the recommendations to the right, does not always correlate with poor sample quality. This phenomenon has been observed with samples requiring nuclei extraction or complex sample types such as plant or animal tissue. Plugs embedded with WBC's are typically clear after Proteinase K step.**]**	Too many cells embedded in plug.	See below: *Improper amount of starting material embedded in plug.*
	Starting material embedded as clumps instead of single cell suspension.	After final Cell Buffer wash and removal of supernatant from spin, tap tube vigorously to break up cell pellet before adding and mixing in Cell Buffer.
	Incomplete Proteinase K digestion.	Replace Proteinase K enzyme if enzyme becomes inactive or is expired.
Final purified DNA size (N50) too low.	Blood source stability has been compromised.	Obtain new blood draw, see Blood Considerations.
	Improper handling of purified DNA.	Make sure to avoid vortexing, rapid pipetting, or excessive pipetting with standard bore tips and use commercial wide-bore tips when appropriate.
	Improper storage of plugs.	Make sure to dialyze sample in TE with at least 1mM EDTA according to protocol in order to minimize DNase activity.
Final purified DNA concentration too low.	Loss of starting material due to improper centrifugation during washes before embedding.	Make sure initial wash centrifugation steps yield a tight pellet with clear supernatant.
	Inaccurate DNA quantitation.	Make sure to only use Qubit BR dsDNA or Picogreen assay to quantitate DNA concentration.
	Too much starting material embedded in plug.	See above: *Improper amount of starting material embedded in plug.*
Protein contamination of purified DNA sample.	Incomplete Proteinase K digestion.	See above: *Incomplete Proteinase K digestion.*
	Too much starting material embedded in plug.	See above: *Improper amount of starting material embedded in plug.*

Final purified DNA is not homogeneous.	Not enough time for DNA to rest before DNA quantitation or before use for stored DNA.	Pipet mix an additional time with proper tip (USA Scientific, #1111-1810) and leave DNA at 25°C to 2-3 days.
		Spin down DNA stored at 4°C and then equilibrate to room temperature, followed by mixing 5x with wide bore genomic tip before use.
Poor DNA quality metrics (label density, mapping, etc.)	Presence of inhibitors in purified DNA.	See above: *Incomplete Proteinase K digestion*.
		Increase duration and number of wash cycles.
		Increase duration and number of TE washes after 1x Wash Buffer series in order to remove excess EDTA, which is known to be inhibitory to labeling reactions.
	Improper ratio of purified DNA to labeling reagents.	Make sure proper ratio of purified DNA to labeling reagents are used. See above: *Inaccurate DNA quantitation.*
	DNA is not homogeneous	Make sure purified DNA is homogeneous (see above: *Final purified DNA is not homogeneous*).

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