Bionano Prep Cell Culture DNA Isolation Protocol

Document Number: 30026

Document Revision: F

Contents

	Page
Experienced User Card	5
User Supplied Materials	6
Introduction	7
Important notes	8-9
Protocol	
Determining Starting Number of Cells to Embed	10
Embedding Cells	11-12
Proteinase K Digestion	13
RNase Digestion and Washing	14
DNA Recovery	15-16
Measuring DNA Concentration	17
Troubleshooting	18-20

Legal Notices

For Research Use Only. Not for use in diagnostic procedures.

Patents

Products of Bionano Genomics may be covered by one or more U.S. or foreign patents.

Trademarks

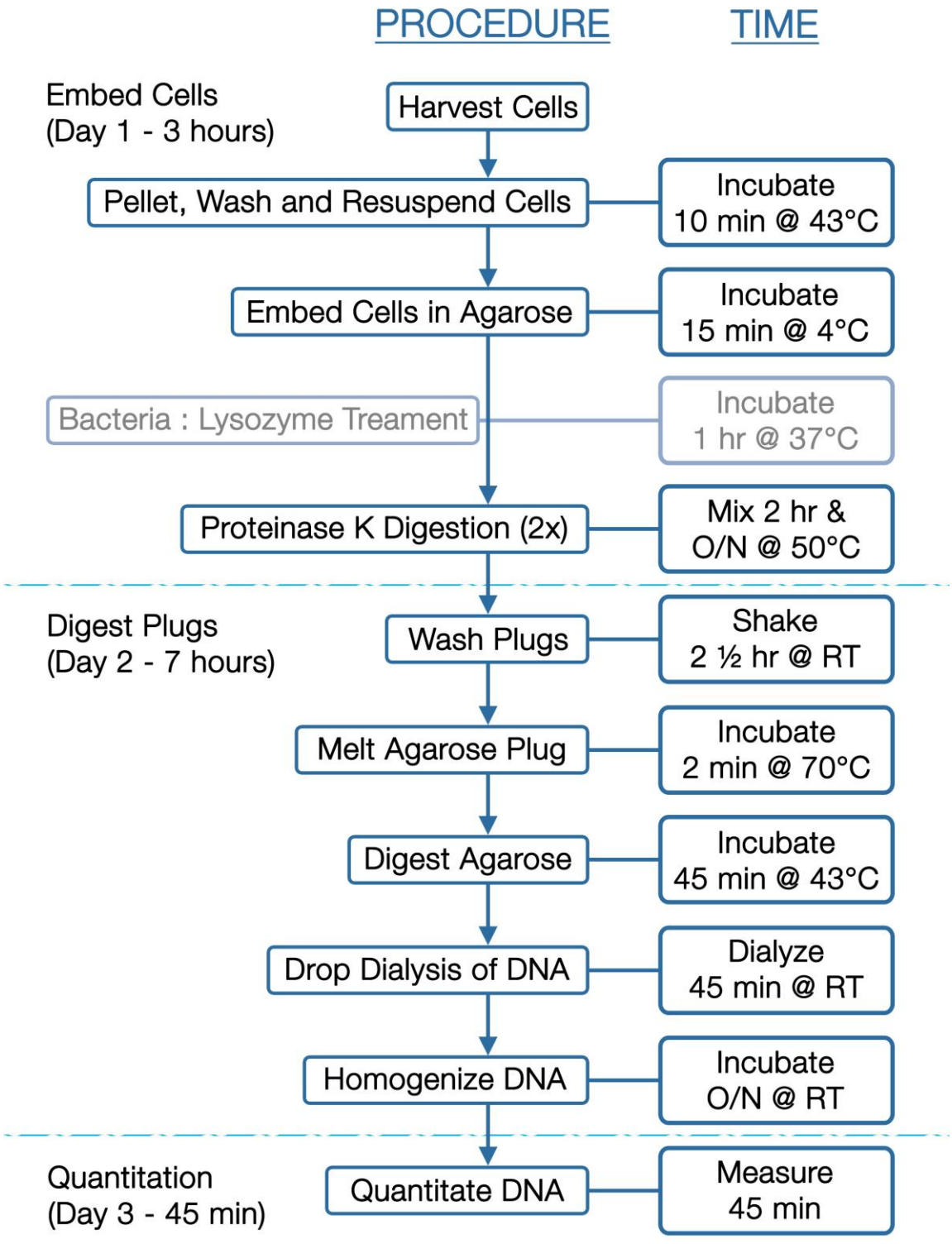
The Bionano Genomics logo and names of Bionano Genomics products or services are registered trademarks or trademarks owned by Bionano Genomics in the United States and certain other countries.

Bionano Genomics®, Irys®, IrysView®, IrysChip®, IrysPrep®, IrysSolve®, Saphyr™, Saphyr Chip™, and Bionano Access™ are trademarks of Bionano Genomics, Inc. All other trademarks are the sole property of their respective owners.

No license to use any trademarks of Bionano Genomics is given or implied. Users are not permitted to use these trademarks without the prior written consent of Bionano Genomics. The use of these trademarks or any other materials, except as permitted herein, is expressly prohibited and may be in violation of federal or other applicable laws.

Protocol for High Molecule Weight (HMW) DNA Isolation from Cells

Workflow Overview – 3 Days



Experienced User Card: Cell Culture

Embed Cells in Agarose (1 hour)

1. Calculate number of cells with 6 µg of DNA (or 1 x 10^6 cells), aliquot into new tube/conical.
   a. Novel users target 9, 6 and 3 µg of DNA.
   b. If cells limited, extract for 3 µg of DNA.
2. Spin cells 10 minutes at 300 x g.
3. Remove supernatant, resuspend in 500 µl cold Cell Buffer, spin 2,000 x g for 3 min, and remove supernatant. Repeat 1 additional time.
4. Remove all supernatant with pipette and resuspend each pellet to a total volume of 66 µl with Cell Buffer.
5. For each 66 µl cell suspension, add 40 µl 2% Agarose and mix 10 times with pipette.
6. Pipet 100 µl of mixture immediately into cold plug mold until liquid is flush. Repeat for each sample.
7. Place plug mold at 4°C for 15 minutes.

Proteinase K Digestion (2 hours, O/N)

8. For each sample, combine 167 µl Qiagen Proteinase K with 2.5 ml Lysis Buffer in a 50 ml conical.
9. Dislodge plugs into Proteinase K/Lysis Solution, cap and incubate for 2 hours at 50°C in thermomixer with intermittent mixing.
10. After 2 hours, replace Proteinase K/Lysis solution with freshly made solution, incubate overnight at 50°C, intermittent mixing.

RNase A Digestion (1 hour)

11. Remove conical from thermomixer and let stand at room temp for 5 minutes.
12. Add 50 µl Qiagen RNase A to conical. Cap and incubate in thermomixer for 1 hour at 37°C with intermittent mixing.

Wash Plugs, Stabilize DNA (1 hr 30 min)

13. Prepare 70 ml 1x Wash Buffer per 1-5 plugs.
14. Rinse 3 times with 10 ml 1x Wash Buffer.
15. Wash 4 times with 10 ml 1x Wash Buffer by shaking 15 min at 180 RPM on orbital shaker. Do not discard last wash. Plugs are stable in 1x wash at 4°C.

DNA recovery (1 hour 30 minutes)

16. Wash plugs 5 times in 10 mL TE by shaking 15 min at 180 RPM.
17. After last wash, scoop each plug out of conical with spatula, remove excess liquid and transfer to 1.5 ml tube.
18. Pulse spin to bring plug to bottom of tube.
19. Melt plug at 70°C for 2 minutes.
20. Transfer plug to 43°C for 5 minutes.
21. To each tube, add 2 µl Agarase into melted plug and stir with tip for 10 seconds.
22. Incubate plug for 45 minutes at exactly 43°C.

Drop Dialysis, Homogenize DNA (1 hour)

23. Float 0.1 µm dialysis membrane on top of 15ml TE in petri dish; wet membrane for 10 minutes.
24. Pipet mix entire volume of DNA 2 times with a wide-bore pipette tip and dispense as a single drop to the top of the membrane.
25. Cover petri dish and let dialyze for 45 minutes.
26. Transfer DNA to 1.5 ml tube with wide-bore tip.
27. If DNA is viscous, pipette mix up to 9 times with standard pipette tip until entire sample is taken up in a continuous flow. Do not exceed 9 times.
28. Incubate DNA overnight at room temp (25°C).

Quantitation (45 mins)

Protect from light

29. Equilibrate DNA and Qubit standards to room temperature for 30 minutes.
30. Gently mix DNA 5 times with wide-bore tip set to 50 µl.
31. Remove 2 µl aliquots from the Top, Middle and Bottom to separate Qubit Assay tubes. Add 18 µl of Qubit BR Buffer.
32. Sonicate 10 minutes in a bath sonicator.
33. Add 10 µl of each Qubit standard to separate Qubit Assay tubes. Add 10 µl of qubit buffer to each.
34. Add 180 µl Qubit fluorescent working solution (Qubit dye + Qubit Buffer).
    Vortex 5 seconds. Pulse spin 2 seconds.
35. Incubate in dark at least 2 minutes at room temperature. Quantitate with Qubit.
36. DNA concentration to target:
    35-200 ng/µl. CV < 0.25
37. Proceed to DNA labeling.

Bionano Prep Blood and Cell Culture DNA Isolation Kit Contents (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
Embed Cells/Proteinse K Digestion – Day 1	
Hemocytometer	General Lab Supplier
2% CleanCut Agarose (12 ml)	Bio-Rad, Catalog # 1703594
Plug Molds, Disposable (5 x 50 plugs)	Bio-Rad, Catalog # 1703713
Bacterial DNA Module Kit (only if processing bacterial cells)	Bio-Rad, Catalog # 1703597
1.5 ml Microcentrifuge Tubes	VWR, Catalog # 87003-294
Green Screened Caps for 50 ml Conicals (individual ordering)	Bio-Rad, Catalog # 1703711 (recommend ordering 2-4)
Conical Centrifuge Tubes, 50 ml, PP	Thermo Fisher, Catalog # 14-432-22 or Equivalent
Centrifuge, Refrigerated with 1.5 ml Tube and Conical Rotor	General Lab Supplier
2 Heat Blocks and Heaters for 1.5 ml Tubes, or 2 Water Baths	General Lab Supplier
Thermomixer with Intermittent Mixing	Eppendorf, Catalog # 5382000023 or Equivalent
Thermomixer 50 ml Conical Adapter	Eppendorf, Catalog # 5382000028 or Equivalent
UltraPure, Nuclease Free Water	Thermo Fisher, Catalog # 10977-015 or Equivalent
Proteinase K Enzyme, 5 ml	Qiagen, Catalog # 158920
Ice Bucket and Ice	General Lab Supplier
Vortexer	General Lab Supplier
Microcentrifuge	General Lab Supplier
Pipettes and Sterile, Filtered Tips	General Lab Supplier
Microwave for Boiling Water	General Supplier
RNase Digestion/Plug Washes/Plug Lysis/Drop Dialysis – Day 2	
Rnase A Solution, 5 ml	Qiagen, Catalog # 158924
TE Buffer, 10 mM Tris, 1 mM EDTA, pH8.0	Thermo Fisher, Catalog # AM9849
Orbital Platform Shaker, 180 RPM	Cole-Parmer, Catalog # EW-51820-30 or Equivalent
Agarase Enzyme, 100 Units, 0.5 U/µl	Thermo Fisher, Catalog # EO0461
Dialysis Membrane Filter, MF, 0.1 µm	Millipore, Catalog # VCWP04700
Blunt-End Forceps	General Lab Supplier
Spatula, Metal	Thermo Fisher, Catalog # 82027-530
Petri Dishes, Sterile, 60 x 15 mm	Thermo Fisher or (VWR), Catalog # 1431160N or (25384-092)
Wide-Bore Pipette Tips, Filtered, Aerosol, 200 µl	VWR, Catalog # 46620-642 or Rainin Equivalent
Quantitation – Day 3	
Bath Sonicator	Branson, Catalog # CPX 952-119R or Equivalent
Fluorometer, Qubit 3.0	Thermo Fisher, Catalog # Q33216 or Equivalent
Qubit® BR (Broad Range) dsDNA Assay Kit	Thermo Fisher, Catalog # Q32853 or Equivalent
Qubit Assay Tubes	Thermo Fisher or (Axygen), Catalog # Q32856 or (10011-830)
Pipette Tip, 200 uL, Nonfiltered and beveled tip	USA Scientific, Catalog # 1111-1810 or Equivalent for your pipettes
Positive-Displacement Pipette MR-10 (optional)	Rainin, Catalog # 17008575 or Equivalent
Pipette Tips, 10 uL, C-10 for Positive Displacement (optional)	Rainin, Catalog # 17008604 or Equivalent

Introduction

The Cell Culture DNA Isolation Protocol involves immobilizing a low complexity biological material in an agarose matrix for subsequent Proteinase K digestion followed by RNAse treatment and washes. The plug is then melted and the agarose matrix is digested in order to recover megabase containing genomic DNA, which is further cleaned by drop dialysis.

Overview

During day 1, isolated cells are embedded into agarose and digested with Proteinase K overnight to stabilize the DNA in the plugs. Removal of RNA and recovery of DNA occur on the second day. Quantitation of DNA is performed on the third day following overnight equilibration at room temperature to achieve DNA homogeneity.

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

Embedding Cells/Nuclei in Agarose Plugs

- A uniform suspension of cultured cells, 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.
- For first-time users, those unfamiliar with the protocol, or for novel cell types, titrations of 9 µg, 6 µg and 3 µg are recommended in order to give the highest probability of success.
- Cultured animal cells, nuclei and bacteria (i.e. *E. coli*) can be embedded directly into agarose plugs.

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 steps following Proteinase K and RNase 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 and RNase digestion 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 during enzymatic steps and continuous mixing during wash steps. Horizontal, orbital style mixers are 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.
- Additional information and how-to videos can be found in the support section at the bionanogenomics.com website.
- 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 up to 2 µl. 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 2 µl. 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 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.
- Presence of mega base size DNA is measured by pulsed field gel electrophoresis (PFGE).
- Homogenous as measured with Picogreen or Qubit® DNA quantitation assay with CV < 0.25.

Cell Culture DNA Isolation Protocol

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

Before First Use

1) Dilute 5x Wash Buffer (Bionano) to 1x.
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 aliquots 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) Pre-chill the Cell Buffer (Bionano), plug mold/plunger (Bio-Rad), and a microcentrifuge to 4°C.
6) Equilibrate a Thermomixer fitted with 50 ml adapter to 50°C for Proteinase K digestion.
   Note: If processing bacterial cells first equilibrate Thermomixer to 37°C for Lysozyme digestion; then set to 50°C for Proteinase K digestion.

Determining Number of Cells to Embed in Cell Buffer

7) Target 6 µg worth of DNA per 66 µl **Cell Buffer** per plug for standard workflow.
   For new users, target one plug each at 9 µg, 6 µg and 3 µg of DNA per 66 µl Cell Buffer per plug to reduce failures from uncertain genomic content. Cell viability can affect DNA yield.
   If material is limited, target only 3 µg worth of DNA per 66 µl Cell Buffer per plug.
    a. **For diploid human cells:**
       To target 6 µg worth of DNA resuspend 1 x 10^6 cells per 66 µl Cell Buffer.
       If cells are polyploid, adjust cell count accordingly (i.e. 5 x 10^5 cells if tetraploid; 2 x 10^6 cells if haploid). If partial ploidy titrate on diploid number.

    b. **For other diploid cells and/or nuclei with known genomic content**:
       Input picograms of DNA per cell into formula below to determine cells per 66 µl Cell Buffer. DNA content determination assumes 1 Gbp = 1 pg for a diploid genome, if ploidy is different adjust result accordingly.

       For 6 µg DNA: # cells/nuclei per 66 µl buffer = $\frac{(3.0 \times 10^6 \text{ pg}) \times 1 \text{ cell}}{(\quad\quad \text{ pg DNA per cell})}$

    c. **If processing animal cells with unknown ploidy or DNA content**:
       Establish DNA content using standard Qiagen kit, targeting 6 µg worth of DNA per 66 µl Cell Buffer. Alternatively, titrate cell amount to hit desired yield (3-6 µg).

   Note: If processing plant or animal tissue, or other DNA-containing material, please use corresponding protocol or contact your Field Application Scientist for guidance.

Protocol Start:

- **Cultured Cells:** Start protocol at **Step 1** below.
    - For new users, or unfamiliar cell types, recommend additional plugs at 9 µg and 3 µg.
- **Nuclei:** Start protocol at **Step 6** below.
- **Bacteria:** Start protocol at **Step 1** below and continue to **Step 12**. Then proceed to **Box A, Step a**. After all the steps in Box A are completed, resume standard protocol at **Step 13**.

Melt Agarose

1. Melt 500 µl aliquot of **2% Agarose** (Bio-Rad) in 70°C water bath or heat block for 10 minutes until completely melted.
2. Equilibrate melted agarose in 43°C water bath for at least 10 minutes before use.

Harvesting Cells and Bacteria (20 minutes)

3. Count cells using hemocytometer and spin to pellet, targeting plugs at 6 µg (and additionally at 9 µg and 3 µg for new users or unfamiliar sample types). For human and other animal cells, spin for 10 minutes at 300 x g at 4°C. If working with bacteria, spin for 5 minutes at 2,300 x g at 4°C.
4. Remove supernatant and resuspend cell pellet in 0.5 ml cold **Cell Buffer** (Bionano) by gently pipetting up and down (no vortexing), and transfer to microfuge tube. Spin down in benchtop microcentrifuge for 3 minutes at 2,000 x g and then discard supernatant. If working with bacteria spin for 5 minutes at 2,300 x g.
5. Repeat **Cell Buffer** wash one additional time (for a total of 2 washes) to make sure that growth medium is completely removed. After final spin, remove the last drop of liquid with a pipette.
6. Resuspend cells, targeting 6 µg (and additionally 9 µg and 3 µg if needed) worth of DNA (as described above) with 66 µl cold **Cell Buffer** (Bionano) per plug.
7. To maintain exact agarose-cell suspension ratio, transfer exactly 66 µl of above cell mixture to a new tube and keep on ice. Proceed immediately to embed in agarose. Refer to table below if scaling up the reaction for multiple plugs.

Embed Cells in Agarose Plugs (1 hour)

Note: *Perform next steps rapidly to avoid solidification of the cell-agarose mixture before pipetting into plug mold. Limit total incubation time of cell-agarose mixture at 43°C for a maximum of 30 min. Cells 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 30 minutes.*

8. Place the appropriate number of chilled **Disposable Plug Molds** (10 plugs per strip) (Bio-Rad), and 1,000 µl & 200 µl pipettes near 43°C water bath or heat block.
9. Equilibrate cells in Cell Buffer in a 43°C water bath or heat block for 3 minutes if processing 1-2 plugs, for 3 or more plugs, incubate for 10 minutes.
10. **Immediately** add 40 µl of **2% Agarose** per 66 µl cells in Cell Buffer, according to table below, and quickly mix 10 times and pipette set to 90% solution volume, while avoiding bubble formation.

Number of Plugs	1	2	3
Cell Pellet + Cell Buffer	66 µl	125 µl	190 µl
2% Agarose	40 µl	75 µl	114 µl

11. **Immediately** fill first well of the plug mold by pipetting 100 µl of the **Cell-Agarose Mixture** with 200 µl pipette until liquid is flush with top of the well. Make sure to place Cell-Agarose Mixture back in 43°C water bath or heat block when not in use.
12. For each subsequent plug mold well, mix Cell-Agarose Mixture an additional 3 times with a new tip and immediately dispense into next well of plug mold.
13. Place filled plug mold at 4°C or on ice and incubate for 15 minutes to solidify agarose.

Box A: For Bacteria Only - Cell Wall Removal (1 hour)

Equilibrate a Thermomixer fitted with 50 ml adapter to 37°C.

a. Prepare **Lysozyme Solution** by mixing 100 µl of **Lysozyme Enzyme** (Bio-Rad kit) with 2.5 ml of **Lysozyme Buffer** (Bio-Rad kit) per 1-5 plugs to be processed in a 50 ml conical tube. (Lysozyme solution is cloudy).

   ***Note**: If using gram-positive bacteria, please contact your Field Application Scientist regarding addition of achromopeptidase.*

b. Transfer plugs to conical tube containing **Lysozyme Solution** by first removing tape from bottom of plug cast followed by dislodging with **Plug Mold Plunger** (Bio-Rad). Make sure plugs are fully submerged.

c. Incubate in thermomixer for 1 hour at 37°C with intermittent mixing.
(Intermittent cycle: 10 seconds at 450 RPM followed by 10 minutes at 0 RPM).

d. Screw a **Green Screened Cap** (Bio-Rad) per 50 ml conical tube, and invert to drain lysozyme solution into liquid waste. Tap bottom of tube on bench surface several times with strong repetitive force to localize plugs to conical tube bottom.

e. With green screened cap attached, fill tube with **Nuclease-Free Water**, and then immediately invert to drain followed by tube tapping to localize plugs to tube bottom.

f. Fill tube again with molecular nuclease-free water, set aside and proceed immediately to the next step.

g. Resume standard protocol below from Proteinase K (step 13).

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 sample with the same homogenate input. Ensure all plugs are fully submerged with 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 digest 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.

14. 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.
15. Add **Proteinase K Lysis Solution** to plugs:
    a. **Cultured Cells:** 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). Make sure all plugs are fully submerged. Use spatula or blunt edged instrument to submerge plugs if they become stuck to conical tube walls.
    b. **Bacterial Cells:** Drain water from tube through green screen cap (Box A, step f). Tap bottom of tube on bench surface several times with strong repetitive force to localize plugs to tube bottom. Then add approximately 2.7 ml of **Proteinase K Lysis Solution** to tube containing plugs.
16. 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).
17. 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.
18. 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.
19. 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: RNase Digestion, 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.

RNase Digestion (1 hour)

3. Following the second Proteinase K digestion, remove each tube from the Thermomixer. Gently tap the tube to collect any condensation and incubate at room temperature for 5 minutes.
4. Remove cap, add 50 µl of **RNase A Solution (Qiagen)**, recap and incubate in the Thermomixer for 1 hour at 37°C with intermittent mixing as described above.

Washing Plugs to Stabilize DNA (1 hour 30 minutes)

5. During RNase digestion, prepare 70 ml of **1x Wash Buffer** for each 50 ml conical tube, using the **Bionano Prep 5x Wash Buffer** (or Bio-Rad 10x Wash Buffer) and nuclease-free water. Mix thoroughly and store at room temperature until use.
6. After RNase digestion, replace original cap with screened cap, drain RNase digestion 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.
7. **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.
8. **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)

9. Discard final 1x Wash Buffer through the screened cap and tap plugs to the bottom of the tube.
10. **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 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.
11. Discard the last TE wash through the screened cap and tap plugs to bottom of tube.
12. 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.
13. Pulse spin each microcentrifuge tube briefly to collect the plug at the bottom of the tube.
14. Melt each agarose plug in a water bath or heat block set at 70°C for 2 minutes.
15. Immediately transfer each tube to a water bath or heat block set at 43°C, incubate for 5 minutes.
16. 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.
17. 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)

18. Place 15 ml of **TE Buffer** into 6 cm **Petri Dish (Thermo Fisher/VWR)** per each DNA sample.
19. 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.
20. Pipet 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.
21. Place cover on the Petri dish and let the sample dialyze for 45 minutes at room temperature.
22. 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)

23. 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.

24. 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. Create 200 µl **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).
4. Aliquot 18 µl of **Qubit BR Buffer** per Qubit assay tube, preparing three tubes per sample.
5. Carefully pipette mix the entire volume of DNA 5 times with a **Wide Bore Tip,** avoid bubble formation.
6. Using a **Standard Bore Tip** or **Positive Displacement Pipet**, 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.
7. Sonicate tubes in sonicating bath for 10 minutes to fragment DNA and then briefly spin to collect solution at bottom of tubes.
8. Vortex tubes continuously at maximum speed for 5 seconds and then briefly spin to collect solution at bottom of tubes.
9. 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.
10. Incubate samples, light-protected, for 5 minutes and proceed with quantitation on Qubit reader.
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. See 'Kits and Consumables' section at https://bionanogenomics.com/support/ for applicable kits and protocols.

Troubleshooting

Problem	Possible Causes	Actions
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.
	Suspended cells did not completely equilibrate to 43°C before adding to molten agarose.	Increase equilibration time of suspended cells at 43°C.
	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 just a few minutes.
	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 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 60min.
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 cultured cells and Gram-negative bacteria 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 removal of cell wall (Bacteria only).	Replace Lysozyme enzyme if enzyme becomes inactive or is expired.
		Increase Lysozyme incubation to 4hrs at 37°C.
	Incomplete Proteinase K digestion.	Replace Proteinase K enzyme if enzyme becomes inactive or is expired.
		Increase Proteinase K digestion to 24-72hrs by adding fresh Proteinase K Buffer and enzyme each day while maintaining incubation temperature at 50°C. (**Caution**: Long incubations at elevated temperature may lead to DNA fragmentation).
Final purified DNA size (N50) too low.	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.
	Poor quality of starting material.	Check quality of starting material for presence of apoptosis/necrosis with Trypan exclusion test, flow cytometry analysis, or other method before embedding in plugs.
	Over-handling of tissue samples.	Reduce liquid N2 grinding of tissue samples and make sure to freeze tissue immediately upon collection in order to minimize DNase activity.
	Improper storage of starting material.	Use fresh samples when feasible. For samples that must be stored, limit the number of freeze- thaw cycles of starting material. Storing samples at -80°C is more stable than freezing at -20°C.
	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.
		Once starting material is embedded into plugs, it is best to completely remove the cell wall (bacteria), followed by Proteinase K digestion and RNase digestion and extensive washing in 1x Wash Buffer before storage in 1x Wash Buffer at 4°C. The 1x Wash Buffer contains a high concentration of EDTA to prevent DNA degradation by nucleases. As a result, lysed plugs stored in 1x Wash Buffer can be stored for 1-2 weeks at 4°C without significant DNA degradation.

	Improper amount of starting material embedded in plug.	Check quantity or mass of starting material using hemocytometer, flow cytometry, counting of colonies, or other method, to make sure 4-6 µg worth of DNA embedded per plug. Both too little and too much starting material embedded in plug can be problematic for sufficient final labeled DNA concentration and sample contamination, respectively.
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.
	Incomplete cell lysis.	See above: *Gel plug never becomes fully clear after lysis.*
	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 1x 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 removal of cell wall (Bacteria only)*.
		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.
	Presence of active Proteinase K.	Add PMSF to 1 mM final concentration during second 1x Wash Buffer wash step after Proteinase K step to inactivate residual Proteinase K.
	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*).

Bionano Genomics, Inc.
9540 Towne Centre Drive, Suite 100
San Diego, CA 92121
Phone: (858) 888-7600
www.bionanogenomics.com