Bionano Prep Animal Tissue DNA Isolation Fibrous Tissue Protocol

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Revision History

Revision	Notes
D	Standardized quantitation section for consistency with other protocols, minor edits for clarity.

Bionano Prep Animal Tissue DNA Isolation Kit & User-Supplied Materials

Table 1: Bionano Prep Animal Tissue DNA Isolation Kit Contents (Part # 80002)

Item	Volume	Part Number
Bionano Prep Animal Tissue Homogenization Buffer	68 ml	20278
Bionano Prep Lysis Buffer	30 ml (2x)	20255
Bionano Prep 5X Wash Buffer	90 ml (2x)	20256

Table 2: User-Supplied Equipment and Consumables

Item	Description	Catalog #
Tissue handling and homogenization – Day 1; Plug lysis – Day 1 and 2		
Rotor-Stator homogenizer (with disposable probes)	Qiagen	9001271 (and # 990890)
Formaldehyde 36.5%-38% in H_2O	Sigma-Aldrich or equivalent	F8775
15 ml conical tube(s)	VWR or equivalent	21008-918
Ice bucket and ice	General lab supplier	
Dissecting forceps	VWR or equivalent	89259-946
Razor blade (optional)	General lab supplier	
Sterile surface, chilled (optional)	General lab supplier	
Liquid Nitrogen (optional)	General lab supplier	
Conical bottom cryogenic vial (optional)	General lab supplier	
Heat blocks or water baths set to 43°C and 70°C	General lab supplier	
Thermomixer	Eppendorf or equivalent	5382000023
50 ml Thermoblock	Eppendorf or equivalent	5365000010
Orbital Platform Shaker	Cole-Parmer or equivalent	EW-51820-30
Proteinase K enzyme, 5 ml	Qiagen	158920
RNase A solution, 5 ml	Qiagen	158924
Green screened caps for 50 ml conicals	Bio-Rad	1703711
Agarose plug molds and plug mold plunger	Bio-Rad	1703713
Agarose, CleanCut 2% solution	Bio-Rad, Catalog #	1703594
Microcentrifuge tubes	VWR or equivalent	87003-294
50 ml conical tube(s)	VWR or equivalent	21008-951
TE Buffer, pH 8.0	Thermo Fisher	AM9849
DNA Recovery - Day 2		
Metal spatula	VWR	82027-530
Agarase (0.5 unit/µl)	Thermo Fisher	EO0461
0.1 µm dialysis membrane	Millipore or equivalent	VCWP04700
Petri dish, 6 cm	VWR	28384-092
Pipette Tips, 200 µl, nonfiltered and beveled	USA Scientific	1111-1810
Pipette Tips, 200 µl, wide-bore	VWR	46620-642
DNA Quantitation - Day 3		
Bath Sonicator (recommended)	General lab supplier	
Vortexer	General lab supplier	
Qubit 3.0 Fluorometer	Thermo Fisher or equivalent	Q33216
Qubit BR Assay Kit	Thermo Fisher or equivalent	Q32850
Qubit Assay Tubes	Thermo Fisher or equivalent	Q32856

Introduction and Important Notes

Introduction:

The Bionano Prep Animal Tissue DNA Isolation Kit enables the isolation of megabase containing genomic DNA from a broad range of animal tissue types for next-generation mapping using the Bionano® System. The kit provides critical reagents for high molecular weight DNA isolation. However, separate protocols are available for processing two categories of starting animal tissue types. The following protocol is for DNA isolation from “fibrous” or elastic tissues such as lung, muscle, and colon. An additional protocol is available for DNA isolation from “soft” tissues such as liver, kidney, prostate, and brain (Bionano Prep Animal Tissue DNA Isolation, Soft Tissue Protocol - Document Number 30077). Both protocols use the same Bionano Prep reagents.

Overview

The Bionano Prep Fibrous Tissue Protocol is a four step process that involves: 1) brief formaldehyde fixation to strengthen nuclei and protect DNA against mechanical shearing, 2) homogenization of the tissue with a rotor stator, 3) embedding of the crude homogenate into agarose plugs for subsequent DNA purification, and 4) recovery of high molecular weight (HMW) DNA.

Typical gDNA Yield

Tissue Type	Starting Tissue	Tissue / Plug	Estimated DNA Yield / Plug
Rat lung	10-40 mg	5-10 mg	up to 5 µg
Hummingbird muscle	10-20 mg	5-10 mg	up to 8 µg

Important Notes:

- Before starting, make sure reagents and equipment are available for disrupting tissue and embedding homogenate in plug(s) for subsequent DNA purification - see the ‘User-Supplied Materials’ section above.
- Work promptly through tissue homogenization, embedding of homogenate in agarose, and addition of Lysis Buffer-Proteinase K solution to stabilize DNA.
- It is best if freshly harvested tissue is flash-frozen in liquid nitrogen to minimize nuclease activity. Large tissue chunks can be broken into smaller pieces, within the desired weight and size range as specified in this protocol, by fragmentation under liquid nitrogen using a mortar and pestle. Excess pieces can then be stored at -80ºC for later use.
- Prior knowledge of starting tissue weight is important to target proper amount of tissue homogenate per plug. This protocol is designed to work with a small amount of tissue (10- 40 mg), however, it is possible to scale to larger tissue amounts.
- For efficient disruption of tissue samples using the rotor-stator, the size of the sample must not be greater than half the diameter of the rotor-stator probe. If using the QIAGEN TissueRuptor, the tissue sample size should not be greater than 3 mm in any one dimension. To achieve the desired sample size, we recommend trimming the tissue by holding the tissue piece with surgical tweezers on a sterilized surface on ice and cutting with a razor blade to reduce size.
- Choice of tissue amount to embed per plug depends on DNA content and extracellular bulk. Target 3-8 µg worth of DNA per plug. ***For tissues that have not been tested, process 5, 10 and 15 mg tissue equivalents per plug.***
- Following tissue dissociation, the homogenate is split into 10 mg (or more) aliquots for subsequent processing in microcentrifuge tubes. Since 5 to 10 mg of most animal tissues contain DNA in the optimal range for plug lysis, each aliquot can make either one plug at 10 mg or two plugs at 5 mg each.
- DNA recovered by the Bionano Prep Animal Tissue DNA Isolation kit is ready for labeling.

Bionano Fibrous Tissue gDNA Isolation Protocol

Day 1: Tissue Fixation and Homogenization / Embedding in Agarose / Proteinase K Digestion

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.
   b) Set another heat block or water bath to 43°C.
3. Melt an aliquot of 2% agarose at 70°C for 15 minutes and then equilibrate to 43ºC for at least 15 minutes.
4. Pre-chill plug molds/plunger, forceps, 1.5 ml tubes, and a microcentrifuge to 4°C.
5. Pre-chill Bionano Prep Animal Tissue Homogenization Buffer.
6. Immobilize rotor-stator to a vertical stand.
7. Equilibrate a Thermomixer fitted with 50 ml adapter to 50ºC for proteinase K digestion.

Tissue Fixation

Tissue fixation is carried out in a fume hood. Up to four tissue samples can be fixed sequentially by weighing/chopping one sample at a time and submerging in fixing solution. Keep rest of tissue samples in freezer, or on dry ice, until ready to process.

8. For each tissue sample, prepare 1 ml of **Fixing Solution** in a 15 ml conical tube, by adding **Formaldehyde** to **Bionano Prep Animal Tissue Homogenization Buffer** to a final concentration of 2% (e.g., 52.7 µl of 38% formaldehyde per 1 ml of buffer). Place conical tube(s) on ice.
9. Retrieve frozen tissue from the -80°C freezer/dry ice and immediately weigh on a precision scale, if weight is not known (weight will be used to determine the proper tissue homogenate input per plug).
10. Chop tissue into pieces 3 mm or smaller in any one dimension for effective fixing (if necessary). To chop tissue, place on sterile surface (e.g. Petri dish) on ice, hold with forceps, and cut with a razor blade.
11. Immediately transfer tissue to 15 ml conical tube containing Fixing Solution, cap tube and incubate in a hood, on ice, for 30 minutes without mixing. Repeat steps 8-10 for the remaining samples.

    Note: Make sure tissue is submerged during fixation process.

12. After 30 minutes, carefully pipet out the Fixing Solution and discard, leaving each sample in its tube. Take care to avoid pipetting up tissue pieces.
13. Add 1 ml chilled **Bionano Prep Animal Tissue Homogenization Buffer** to each 15 ml conical tube, swirl gently, and pipet out the buffer. Repeat this wash 2 more times.
14. Add 2 ml of chilled **Bionano Prep Animal Tissue Homogenization Buffer** to each 15 ml conical tube and place on ice.

Tissue Disruption with a Rotor-Stator

15. Attach probe to rotor-stator, and make sure probe is fully locked.
16. Submerge probe tip, as per manufacturer's instructions, in buffer while ensuring that the tissue pieces are located beneath the probe tip for effective blending. Blend for 10 seconds at maximum speed and set on ice.

    Note: If tissue pieces are not all underneath probe tip, submerge tissue using probe tip before blending.
17. Examine both the homogenate and the probe tip for intact tissue pieces. If any remain, blend for another 10 seconds as before.

    Note: Do not blend more than 2 times. Tissue particulates that persist after the second blending can be successfully processed downstream.
18. Repeat steps 15-17 for any additional samples, using a new probe for each sample.
19. Gently pipette mix tissue homogenate and aliquot 10 mg tissue equivalent per chilled 1.5 ml microcentrifuge tube.

    Note: If desired, more than 10 mg tissue equivalent may be aliquoted per tube. For tissues that have not been tested, process 5, 10, and 15 mg tissue equivalents per plug.
20. Spin at 1,500 x g for 5 minutes at 4°C. Pipet off and discard the supernatant.
21. To each pellet, add 1 ml of **Bionano Prep Animal Tissue Homogenization Buffer**. Resuspend gently with a regular pipette tip. Spin again at 1,500 x g for 5 minutes at 4°C. Pipet off and discard the supernatant.

Embedding in Agarose

22. To each homogenate pellet, add the appropriate volume of **Bionano Prep Animal Tissue Homogenization Buffer** as shown in the table below, to form 1 plug at 10 mg (or 10 mg+) tissue equivalent, or 2 plugs at 5 mg tissue equivalent each. Resuspend gently with a regular pipette tip. Do not add agarose yet!

Tissue Amount per Plug	Homogenization Buffer	2% Agarose (43°C)
5 mg (2 plugs)	125 µl	75 µl
10 mg or 10 mg+ (1 plug)	66 µl	40 µl

23. Equilibrate each tissue homogenate suspension to room temperature for 5 minutes.
24. Mix tissue homogenate suspension with agarose, one tube at a time:
    a) Pipette mix the tissue homogenate suspension 3 times.
    b) Add the proper volume of 43°C equilibrated **2% Agarose** (0.75% final concentration) per table above.
    c) Carefully pipette mix the agarose-tissue homogenate suspension 10 times, avoid forming bubbles, and immediately add mixture to 4°C pre-chilled plug molds. For casting of two plugs, withdraw entire 200 µl and sequentially fill two plug molds.
25. Place plug molds at 4°C or on ice and incubate for 15 minutes to solidify agarose.

Digest with Proteinase K (2 hours)

Up to five plugs can be processed simultaneously per 50 ml conical tube if each plug is from the same sample with the same homogenate 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.

26. Prepare fresh **Proteinase K Solution** by mixing 200 µl of **Qiagen Proteinase K Enzyme** with 2.5 ml of **Bionano Prep Lysis Buffer** per 1-5 plugs to be processed in each 50 ml conical tube.
27. Transfer plugs to conical tube containing 2.7 ml of **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.
28. 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).
29. Near the end of the incubation, prepare fresh **Proteinase K Solution** by mixing 200 µl of **Proteinase K Enzyme** with 2.5 ml of **Bionano Prep Lysis Buffer** per 1-5 plugs to be processed per tube.
30. 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.
31. Remove the screened cap, and add 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: RNase Digestion, DNA Stabilization Washes, DNA Recovery, Drop Dialysis

Set Up

32. Gather materials (see 'User Supplied Materials' section above).
33. 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.

RNase Digestion (1 hour)

34. 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.
35. 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 (10 seconds at 450 rpm followed by 10 minutes at 0 rpm).

Washing Plugs to Stabilize DNA (1 hour 30 minutes)

36. During RNase digestion, 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.

37. 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.
38. **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.
39. **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, 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)

40. Discard final 1x Wash Buffer through the screened cap and tap plugs to the bottom of the tube.
41. **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.
42. Discard the last TE wash through the screened cap and tap plugs to bottom of tube.
43. 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.
44. Pulse spin each microcentrifuge tube briefly to collect the plug at the bottom of the tube.
45. Melt each agarose plug in a water bath or heat block set at 70°C for 2 minutes.
46. Immediately transfer each tube to a water bath or heat block set at 43°C, incubate for 5 minutes.
47. 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.
48. 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)

49. Place 15 ml of **TE Buffer** into 6 cm **Petri Dish (Thermo Fisher/VWR)** per each DNA sample.
50. 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.

51. 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.

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

53. 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)

54. Test DNA viscosity by slowly withdrawing the entire DNA volume into a **non-filtered beveled 200 µl tip** 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.

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

Day 3: Quantitation

Qubit Quantitation - BR dsDNA Assay

Refer to the Qubit dsDNA BR Assay Kit user manual for kit details and follow the methods described in the "Pipetting Viscous Genomic DNA" section, to ensure accurate pipetting of viscous gDNA.

1. Equilibrate Qubit BR Assay Kit Standards to room temperature.

   Note: If the gDNA has been stored at 4ºC, equilibrate at room temperature before moving to the next step.

2. Add Qubit BR Buffer to 0.5 ml Qubit Assay Tubes:
   a. For each sample, add 18 µl of Qubit BR Buffer to three separate Qubit Assay Tubes.
   b. For the Qubit Standards, add 10 µl Qubit BR Buffer to two separate Qubit Assay Tubes.
3. Using a 200 µl pipette with a wide bore tip, gently mix the entire gDNA sample volume by pipetting up and down 5 times, being careful not to generate bubbles.
4. Using a fresh standard bore pipette tip or positive displacement pipette tip for each draw:

   Remove 2 µl aliquots from the top, middle, and bottom of each sample and dispense into BR Buffer of corresponding Qubit Assay Tube, rinsing tip when dispensing. Place Assay Tubes in a floating rack and sonicate for 10 minutes. Perform Steps 5 and 6 during sonication.

   Note: If a bath sonicator is not available, vortex for at least 30 seconds at maximum speed, then spin down briefly for 2 seconds.
5. Prepare Working Solution by diluting the Dye Assay Reagent into BR Dilution Buffer (1:200):
   a. 200 µl Working Solution for each of the two standards (400 µl total).
   b. 200 µl Working Solution for each sample aliquot (600 µl for each sample).
6. For the Qubit DNA standards, add 10 µl of Standards 1 and 2 to the Assay Tubes containing BR Buffer from Step 2b.
7. Once sonication is complete, retrieve assay tubes and pulse spin briefly. Vortex tubes for 5 seconds at maximum speed, then pulse spin again.
8. Add 180 µl of Working Solution to each sonicated DNA aliquot and Qubit DNA Standard aliquot. Vortex for 5 seconds, and pulse spin tubes.
9. Incubate samples for at least 2 minutes, then read on the Qubit Fluorometer. Record values in the table on the next page.
10. 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.

Sample ID	Top (ng/µl)	Middle (ng/µl)	Bottom (ng/µl)	CV (stdev/mean)

Labeling

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

Technical Assistance

For technical assistance, contact Bionano Genomics Technical Support.

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

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