Bionano Prep SP Amnio and CVS Culture DNA Isolation Protocol

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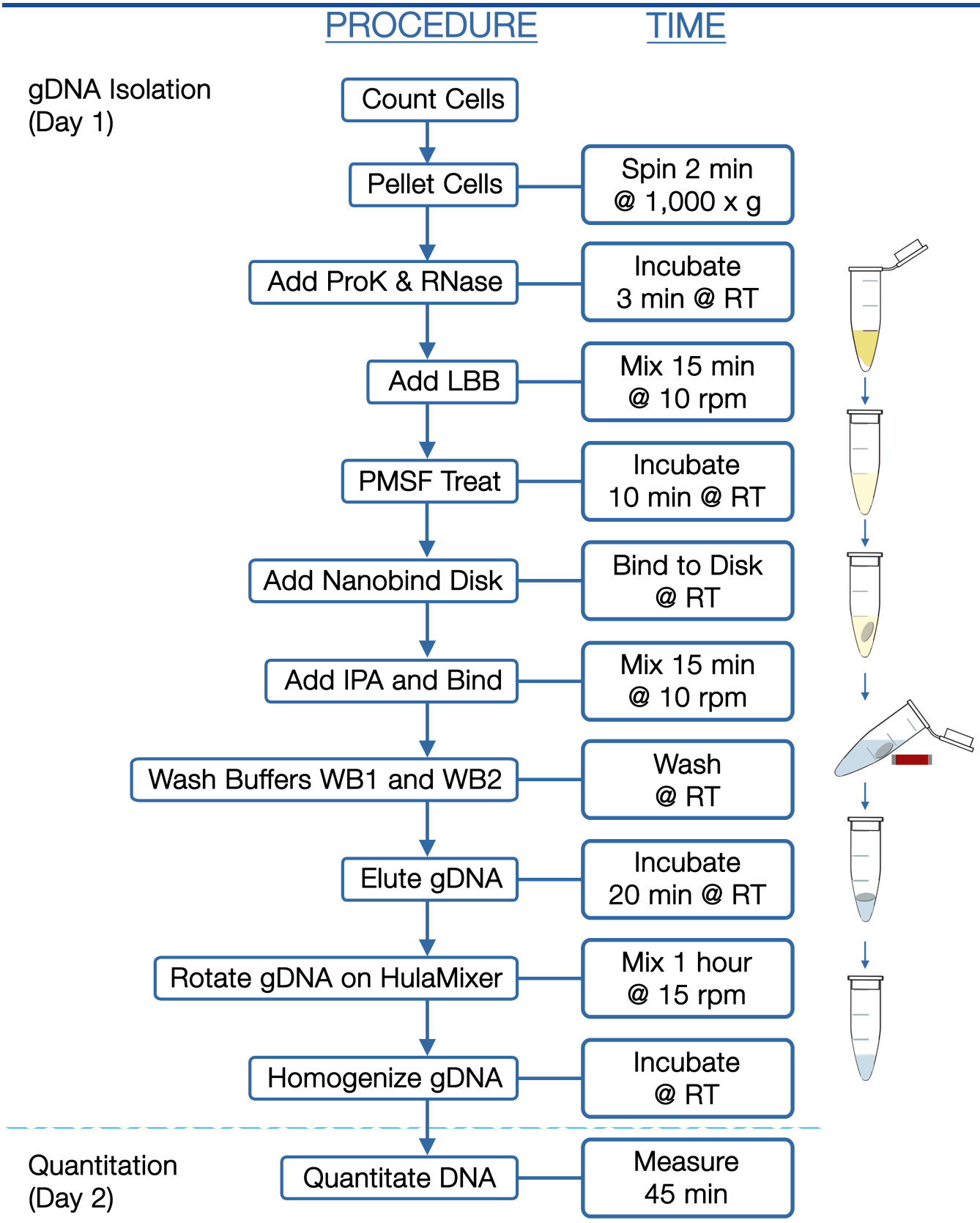
Revision History

Revision	Notes
1	Beta Release
2	Revised Appendix to change when cryopreserved cells are placed on ice. Specified transfer to room temperature after addition of PK and RNAse
3	Added disclaimer for limited commercial release. Updated Trypsin-EDTA material information. Added instructions for handling sample input between 0.5 million – 1 million cells. Removed spin step and volume removal after transfer to 0.5 ml Protein LoBind tube.
A	Added T25 flasks to user-supplied materials. Specified light protection for PMSF aliquots. Specified that 15 ml conical tubes and 1.5 ml microcentrifuge tubes are only needed if processing fresh culture.

Workflow Overview



Bionano Prep SP DNA Isolation Kit v2 and User-Supplied Materials

Bionano Prep SP Blood and Cell DNA Isolation Kit v2 Contents (Part # 80042, 10 preps)

Item	Amount	Part Number	Storage
4 mm Nanobind Disks	10 disks	20402	Room Temp (18-25°C)
Protein LoBind Microcentrifuge Tubes, 1.5 ml	10 tubes	20422	Room Temp (18-25°C)
Protein LoBind Microcentrifuge Tubes, 0.5 ml	10 tubes	20421	Room Temp (18-25°C)
RNase A Enzyme	200 µl	20373	Refrigerate (4°C)
DNA Stabilizer**	20 µl	20397	Room Temp (18-25°C)
Microcentrifuge Tubes, 2.0 ml	10 tubes	20396	Room Temp (18-25°C)
Cell Buffer	50 ml	20374	Room Temp (18-25°C)
Proteinase K Enzyme	0.5 ml	20372	Room Temp (18-25°C)
Lysis and Binding Buffer (LBB)*	2.5 ml	20375	Room Temp (18-25°C)
Wash Buffer 1 Concentrate (2.5X) (WB1)*	3.25 ml	20376	Room Temp (18-25°C)
Wash Buffer 2 Concentrate (2.5X) (WB2)	5 ml	20377	Room Temp (18-25°C)
Elution Buffer (EB)	1.1 ml	20378	Room Temp (18-25°C)
Magnetic Disk Retriever Plastic Sheath	10	20381	Room Temp (18-25°C)

* See Important Notes Section for hazardous waste information
**Used to wash fresh or cryopreserved cells and resuspending cell pellets for SP DNA isolation preps

User-Supplied Materials

Item	Supplier	Catalog #
Day 1 – Counting, Pelleting, gDNA Isolation and Homogenization		
Bionano Prep SP Magnetic Retriever (2 pack)	Bionano Genomics	80031
Phase Contrast Microscope	General Lab Supplier	
Hemocytometer and Coverslip and 2 Key Differential Counter or Automated Cell Counter	General Lab Supplier	
Trypan Blue Solution (0.4%) for Counting Viable Cells	Thermo Fisher or Equivalent	15250061
DynaMag-2 Magnetic Tube Rack	Thermo Fisher	12321D
HulaMixer Sample Mixer	Thermo Fisher	15920D
Microcentrifuge Tubes, 1.5 ml, Nuclease Free	VWR	87003-294
Cryogenic Vials with Screw Cap	General Lab Supplier	
Phenylmethylsulphonyl Fluoride Solution (PMSF),100 mM	Sigma-Aldrich	93482
Ethanol, 200 Proof, Molecular Biology Grade	Sigma-Aldrich	E7023
Isopropanol (IPA), ≥ 99.5%, Molecular Biology Grade	Fisher Scientific	A461-212
Disinfectant Concentrate, TexQ TX651	Texwipe	TX651
1x Trypsin-EDTA (0.25%)	Thermo Fisher or Equivalent	25200056
Dimethyl Sulfoxide (DMSO)	Sigma-Aldrich or Equivalent	D2650
T25 Cell Culture Flasks	Thermo Fisher or Equivalent	156367
Cryogenic Tubes/Storage Vials (1 ml)		
Cell Freezing Container	Corning or Equivalent	432001
Phosphate Buffered Saline (PBS), pH 7.2	Thermo Fisher or Equivalent	20012027
AmnioMAX II, Complete Medium	Thermo Fisher or Equivalent	11269016
Bleach for Cell Media Disposal	General Lab Supplier	
Conical Centrifuge Tubes, 50 ml, PP	Thermo Fisher or Equivalent	14-432-22
Conical Centrifuge Tubes, 15 ml, PP	Fisher Scientific	05-539-12
Centrifuge with 1.5 ml Tube Rotor	General Lab Supplier	
Centrifuge with a Swinging Bucket Rotor for 15 ml Conical Tubes to Concentrate Cells from Medium	General Lab Supplier	
Refrigerated Benchtop Microfuge for 1.5 ml Tubes	Eppendorf or Equivalent	5425R
Ice Bucket and Ice	General Lab Supplier	
Sterile 5 and 10 ml Disposable Pipettes (TD+)	General Lab Supplier	
Mini Benchtop Microcentrifuge	Labnet	C1301B
Pointed Forceps	Electron Microscopy Sciences, or Equivalent	78141-01
Wide-Bore Pipette Tips, Filtered, Aerosol, 200 µl	VWR or Rainin Equivalent	46620-642

Extra Long 1000 µl Tips, Sterile	VWR or Rainin Equivalent	16466-008
Gel Loading Tips, 200 µl	General Lab Supplier	
Pipettes (10, 20, 200, and 1,000 µl) and Nuclease Free, Filtered Pipette Tips	General Lab Supplier	
Day 2 - Quantitation		
Benchtop Vortexer	General Lab Supplier	
Bath Sonicator (recommended)	Branson or Equivalent	CPX 952-119R
15 ml Conical Tube	Fisher Scientific	05-539-12
Fluorometer, Qubit	Thermo Fisher or Equivalent	Q33216
Qubit® BR (Broad Range) dsDNA Assay Kit	Thermo Fisher or Equivalent	Q32853
Qubit Assay Tubes	Thermo Fisher	Q32856
Positive-Displacement Pipette MR-10 (optional)	Rainin or Equivalent	17008575
Pipette Tips, 10 ul, C-10 for Pos. Displ. Pipette (optional)	Rainin or Equivalent	17008604

Introduction and Important Notes

Introduction:

This Bionano Prep® SP Amnio and CVS Culture DNA Isolation Protocol can provide ultra-high molecular weight (UHMW) gDNA in less than 4 hours targeting 1.0 million cells from cultured Amniocytes or Chorionic Villus Samples (CVS). The process utilizes a lyse, bind, wash, and elute procedure that is common for silica-based gDNA extraction technologies in combination with a novel paramagnetic disk. Unlike magnetic beads and silica spin columns, which shear large gDNA, the Nanobind Disk binds and releases gDNA with significantly less fragmentation, resulting in UHMW gDNA. High gDNA binding capacity is the result of a novel nano structured silica on the outside of the thermoplastic paramagnetic disk. This protocol was tested using cultured cells from amniotic fluid or CVS. gDNA prepared using this protocol has been tested with DLS labeling. See Training Video for technically critical steps and troubleshooting; the steps mentioned in the video correspond to the Frozen Blood Protocol, but overall the steps and processes are the same for this protocol.

Overview:

Cell lysis and Proteinase K digestion occurs in a chaotropic buffer and the released gDNA binds to the Nanobind Disk upon the addition of isopropanol. After three wash steps, the disk is transferred to a fresh tube and the gDNA is eluted from the disk. The recovered UHMW gDNA is subjected to limited shearing to make the UHMW gDNA more homogeneous. The gDNA is then mixed and equilibrated overnight at room temperature to facilitate DNA homogeneity and the concentration is determined. Typical gDNA size range is from 50 Kbp to ≥ 1 Mbp.

Important Notes:

DNA Homogeneity

- Recovered gDNA is subjected to pipette mixing with a 200 µl standard pipet tip to increase homogeneity, ensuring consistent DNA sampling for labeling.

gDNA Quantitation

gDNA quantitation is used to measure concentration and serves as a gauge of UHMW gDNA homogeneity. Qubit quantitation is preferred over other quantitation methods since it can also be used for measuring gDNA concentration of the labeling reaction. The Qubit Broad Range (BR) dsDNA Assay measures gDNA concentration after isolation, while the High Sensitivity (HS) dsDNA Assay measures gDNA concentration after labeling.

To gauge gDNA homogeneity, it is essential to measure the concentration of gDNA at multiple positions in the solution. Since viscous gDNA is difficult to pipet, follow guidelines in the Important Notes and gDNA Quantitation sections below for accurate pipetting. Standard assays for quantitation of gDNA concentration will not provide accurate measurements of long gDNA due to its viscous nature.

- Effective fragmentation of sampled gDNA via sonication or extensive vortexing is necessary for accurate quantitation.
- The coefficient of variation (CV) from three unique samplings should be less than 0.30.
- Typical gDNA concentration is 50-120 ng/µl.

Pipetting Viscous Genomic DNA (gDNA)

To draw viscous gDNA, hold the stock tube for close-up visualization, depress the pipette plunger until the first stop, submerge the pipette tip and carefully and slowly release the plunger to start drawing the viscous gDNA into the tip while carefully monitoring uptake. Keep the tip submerged even after the viscous solution stops moving upward and levels off. Be patient. Viscous gDNA can take a few seconds to fill 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 tip has leveled off and while the tip is still submerged in the gDNA solution, scrape the tip against the bottom of the tube 3-5 times using a circular motion. Remove the tip from the gDNA 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 gDNA strands from the tip, can produce a bubble at the tip of the pipette tip indicating under-sampling (start over if this happens).

gDNA Handling

- Mixing of recovered gDNA is always carried out with a wide bore pipette tip to prevent shearing.
- Recovered gDNA should never be frozen or vortexed.
- Pipetting of recovered gDNA for accurate sampling is always carried out with a standard tip or positive displacement pipette.

Characteristics of High Quality gDNA for Bionano Mapping

- A clear gDNA solution is ideal, but an unclear solution does not always correlate with poor sample quality.
- Recovered gDNA in solution is viscous.
- Presence of mega base size gDNA is measured by pulsed field gel electrophoresis (PFGE).
- Recovered gDNA is homogenous as measured with Qubit gDNA quantitation assay with CV < 0.30.

Using the Bionano Prep SP Magnetic Retriever

a. Hold a plastic sheath on the sides near the top and insert the Bionano Prep SP Magnetic Retriever into the sheath, positioning it such that it is sitting at the bottom of the sheath.
b. Insert the sheathed retriever into the Protein LoBind microcentrifuge tube to attract the Nanobind Disk to the sheathed retriever.
c. Carefully lift the sheathed retriever with the bound disk out of tube and insert the sheathed retriever into a new Protein LoBind microcentrifuge tube.
d. Holding the sheath on the side near the top, with one hand pull the retriever up until the Nanobind Disk disassociates from the sheath and drops into the new tube.
e. Change sheath for each new sample.

Workflow and Batch Size

- To obtain at least 1.0 million cells, we recommend growing Amniocyte or CVS cultures in T25 flasks targeting 90% confluence.
- We recommend processing no more than 6 samples per batch in a single day if performing cell harvesting, cell counting, and SP DNA isolation from the fresh cell pellets.
- If starting with cryopreserved cells, two batches of 6 samples can be processed within an 8-hour workday.
- It is most convenient to have 1.0 million cells of each of the culture samples to be processed in 15 ml polypropylene conicals or 1.5 ml microcentrifuge tubes and centrifuged as a group to concentrate the cells.

Workflow Flexibility: Preparing Cryopreserved Cells

- SP DNA isolation does not necessarily need to be performed on the day the cells are harvested.
- After harvesting and counting cells, 1.0 million cells can be stored at -80°C or liquid N_2 as a frozen resuspension in cryopreservation medium until it is convenient to proceed to SP DNA isolation (see Appendix).

Hazardous Waste Disposal

Buffers LBB and WB1 contain guanidine hydrochloride (GuHCl). GuHCl is harmful if swallowed or inhaled and causes skin and eye irritation. DO NOT mix with bleach or acidic reagents. Liquid waste containing GuHCl should be safely decontaminated with a quaternary ammonium disinfectant before disposal in a hazardous waste stream. We recommend bleach for decontamination of pellet supernatant and TexQ for decontamination of all solutions mixed with GuHCl. This conforms to disposal requirements in the state of California, US, but may be different for your location. Please consult local requirement for decontamination and disposal.

Bionano Prep SP Amnio and CVS Culture DNA Isolation Protocol

Preparation for gDNA Isolation

Before First Use

- Verify access to tabletop centrifuge with swinging bucket rotor that can accommodate 15 ml polypropylene conicals to concentrate cells from medium.
- Verify refrigerated benchtop microfuge spin speed is 1000 x g (fresh cultures) or 2200 x g (cryopreserved cells).
- PMSF decomposes rapidly in aqueous solutions. Create aliquots of 80 µl in 1.5 ml screw cap tubes and store stock and aliquots at 4°C. Protect from light. Each aliquot will be sufficient for ten gDNA isolations.
- Add 100% Ethanol to Wash Buffers (WB1 and WB2) and mix thoroughly:
    - Add 5 ml of 100% Ethanol to Wash Buffer 1 (WB1) for a final volume of 8.25 ml.
    - Add 7.5 ml of 100% Ethanol to Wash Buffer 2 (WB2) for a final volume of 12.5 ml.

Set Up

- Gather materials (see “User Supplied Material” section above).
- For waste disposal, prepare:
    - One 50 ml conical with 5 ml bleach + 20 ml water; invert several times to mix.
        - Supernatants from cell cultures and pellets should go into dilute bleach for decontamination before disposal in a sink.
    - One 50 ml conical with 100 µl TexQ decontaminant per sample (to be disposed as hazardous waste).
        - Supernatants from SP protocol binding reaction and first wash need to be decontaminated by the quaternary ammonium disinfectant and treated as hazardous waste.
- For each sample, label one 0.5 ml Protein LoBind Tube (Bionano), one 1.5 ml Protein LoBind Tube (Bionano), and one 2.0 ml Microcentrifuge Tube (Bionano).
    - If processing fresh cell culture, for each sample label one 15 ml polypropylene conical and one 1.5 ml microcentrifuge tube.
- Invert tubes of PMSF, RNase A (Bionano) and Proteinase K (Bionano) three times to mix, pulse spin briefly. Place PMSF and RNase A on ice.

Fresh Cultured Cell Processing for gDNA Isolation

Cell Harvesting, Counting and Pelleting (0.5 - 1.5 hours)

- For each sample, prepare 350 ul Stabilizing Buffer by mixing Cell Buffer (Bionano) with DNA Stabilizer (Bionano):
    - *350 µl Stabilizing Buffer = 343 µl Cell Buffer + 7 µl DNA Stabilizer, vortex to mix and pulse spin. Store on ice*.
- To obtain at least 1.0 million cells, we recommend growing Amniocyte or CVS cultures in T25 flasks targeting 90% confluence.
- **Harvesting Cells from Fresh Culture(s)**

  Note: if processing multiple cultures, harvest up to two at a time

    - Thaw/prewarm growth medium (6 ml/flask) and thaw/prewarm 1x Trypsin-EDTA (2ml/flask).
    - Remove medium and rinse culture(s) with 5 ml 1x Phosphate Buffered Saline (PBS).
    - Remove the PBS, add 1.5 ml 1x Trypsin-EDTA and gently swirl around the flask(s) to disperse.
    - Place in the 37°C CO_2 incubator.
    - After 3 minutes, remove the flask(s) from the incubator and swirl the 1x Trypsin-EDTA across the flask(s) to help dislodge the cells.
    - Check under the microscope to make sure most of the cells have detached.
    - Add 4.5 ml of growth medium and pipet medium against the growth surface of the flask(s) several times to fully remove cells from the plastic substratum.
    - Transfer the ~ 6 ml to 15 ml polypropylene conical(s).
    - After the sample(s) are in labeled conicals, pellet the cells by centrifugation using a swinging bucket rotor at 500 x g for 2 minutes at room temperature.
    - Remove all supernatant and resuspend the pellet(s) until fully resuspended by repeated pipetting with one ml of growth medium using a P1000 pipet. Transfer to labeled 1.5 ml microcentrifuge tube(s).

- **Counting Cells**

  Note: if processing multiple cultures, count one at a time

    - For each sample, pipet isolated cells in medium repeatedly until fully resuspended.
    - Remove a 5 ul aliquot from the middle of the cell suspension and mix well with 45 ul of Trypan Blue solution in a 1.5 ml microcentrifuge tube.
    - Count viable cells with a cell counting device (i.e., Hemocytometer or an automated cell counting device).

- Calculate the volume of original cell stock required for 1.0 million cells.

Note: If there are < 1.0 million cells but > 0.5 million cells continue with the pelleting and subsequent DNA isolation. With < 1.0 million cells there may be an increased risk of low DNA yield or gDNA fall offs (however, the gDNA can be rebound to the Nanobind - see Troubleshooting).

- Re-pipet cells in medium repeatedly until fully resuspended, and transfer the volume for 1.0 million (or transfer total volume if > 0.5 million) cells to a labeled 1.5 ml Protein LoBind tube.

- **Pelleting Cells**
  - Pellet cells by centrifugation at 1,000 x g for 2 minutes at 4°C.
  - Remove as much of the supernatant as possible without disturbing the cell pellet.
  - Using a P1000 pipet, resuspend the pellet in 250 ul ice-cold Stabilizing Buffer by pipetting repeatedly until fully resuspended.

Note: Keep all samples on ice until beginning gDNA Isolation (Lyse and Digest Cells)

- Pellet cells by centrifugation at 1,000 x g for 2 minutes at 4°C
- Remove as much of the supernatant as possible with a without disturbing the cell pellet. Use a gel loading tip to remove any residual volume.
- Proceed to Step 1 below for gDNA Isolation **(Cell Pellet Resuspension).**

Cryopreserved Cell Processing for gDNA Isolation

Note: Preparation of Cryopreserved Cells from fresh culture is described in the Appendix

- **Cryopreserved Cells (1.0 million cells)**
  - For each sample prepare 1.6 ml of Stabilizing Buffer by mixing Cell Buffer (Bionano) with DNA Stabilizer (Bionano):
    - *1.6 ml Stabilizing Buffer = 1568 ul Cell Buffer + 32 ul DNA Stabilizer. Vortex to mix and pulse spin. Store on ice.*
  - For each sample, prechill a 1.5 ml Protein LoBind tube on ice.
  - Remove cryotube(s) from -80°C (or liquid N_2) and thaw by swirling in 37°C water bath until the ice has melted (30 seconds - 2 minutes) and then place on ice.

Note: Keep all samples on ice until beginning gDNA Isolation (Lyse and Digest Cells)

- Using a P1000 pipet, pipet mix the entire sample volume 3 times and transfer the entire volume into a prechilled 1.5 ml Protein LoBind tube.

- Centrifuge at 2,200 x g for 2 minutes at 4°C, balanced, with lid hinges facing the outside of the rotor.
- Carefully remove as much of the bulk supernatant as possible with a pipet without disturbing the cell pellet, drawing the solution away from the pellet from the side opposite of the tube hinge. Then, use a gel loading tip to remove the residual liquid from the pellet, drawing the residual into the gel loading tip from the side opposite of the tube hinge. Place the tubes on ice.
- Add 750 ul ice-cold Stabilizing Buffer onto the top of each pellet.
- Disrupt the pellet with a 1000 µl tip and resuspend the pellet by pipetting up and down 3 times with this tip.
- Add another 750 ul of ice-cold Stabilizing Buffer, cap the tube and mix by inversion 3 times.
- Centrifuge at 2,200 x g for 2 minutes at 4°C balanced, with lid hinges facing the outside of the rotor.
- Carefully remove as much of the bulk supernatant as possible with a pipet without disturbing the cell pellet, drawing the solution away from the pellet from the side opposite of the tube hinge. Then, use a gel loading tip to remove the residual liquid from the pellet, drawing the residual into the gel loading tip from the side opposite of the tube hinge.
- Proceed to Step 1 below for gDNA Isolation **(Cell Pellet Resuspension)**

gDNA Isolation from Fresh Cells and Cryopreserved Cells (3 hours)

Cell Pellet Resuspension

1. Add 40 µl of ice-cold Stabilizing Buffer on top of each pellet.
2. Disrupt the pellet with a 200 µl standard bore tip, then continue to resuspend the pellet by pipetting up and down 10 times.

Lyse and Digest Cells

3. Add 35 µl of Proteinase K and 13 µl of RNase A to each of the Protein LoBind tubes containing resuspended cells. Transfer to room temperature. **DO NOT PIPET MIX.**
4. Incubate at room temperature for 3 minutes.
5. Add 150 µl Buffer LBB to each sample with a standard 200 µl tip. Cap and invert tube 15 times to mix.

   Note: Buffer LBB is a viscous and foamy solution which will adhere to pipette tip. Dispense slowly and change tips between dispensing to ensure accuracy of dispense volume.
6. Rotate sample on HulaMixer for 15 minutes at room temperature at 10 rpm. No shaking/vibration.

7. Pulse spin tube for 2 seconds to collect liquid at the bottom of the tube.
8. Add 6.7 µl of 100 mM PMSF into the liquid portion of tube. Cap and invert tube 5 times to mix, pulse spin tube for 2 seconds to collect liquid at the bottom of the tube.
9. Incubate at room temperature for 10 minutes.

gDNA Bind, Wash and Elute

10. Using forceps, carefully transfer a single Nanobind Disk to the lysate.

    Note: Disks can sometimes stick together.

11. Add 227 µl 100% isopropanol to all tubes. Cap and invert tubes 5 times to mix.
12. Rotate sample on HulaMixer for 15 minutes at room temperature at 10 rpm. No shaking/vibration.

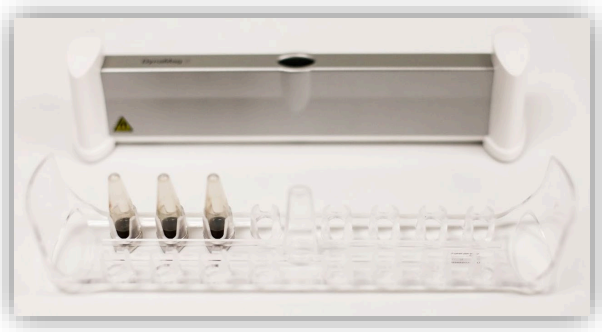
    Note: Ensure that the Nanobind Disk does not remain in the lid of the tube during initial rotations. If it does, turn off rotator and invert microcentrifuge tube until the Nanobind Disk goes back into the solution. Replace the tube on the HulaMixer and resume mixing.

13. Examine gDNA association with Nanobind Disk and invert to increase binding (See Training Video, 0:25):
    a. Place sample tubes into clear Dynamag tube rack and visually inspect all tubes in rack to ensure that gDNA is tethered to the Nanobind Disk.

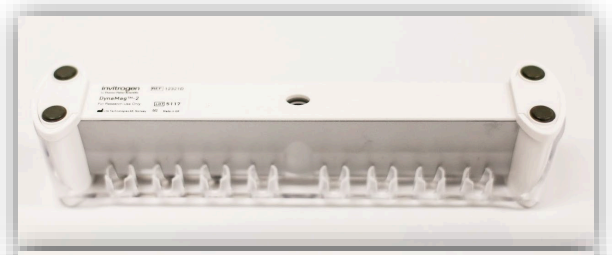
       Note: If gDNA is not tethered to the Nanobind Disk, aspirate liquid from the tube such that a minimal volume (~50 µl) remains above unbound gDNA and discard supernatant leaving the DNA in a minimal volume at bottom of the tube.
    b. If gDNA strands are visibly hanging low, quickly invert 180° to bring the gDNA into closer association with the Nanobind Disk.
    c. 180° inversions can be done many times until the gDNA association with the Nanobind Disk appears unchanged.
14. Combine clear rack with the magnetic base as outlined below, making sure Nanobind Disk is secured by the magnet near the top of the liquid level. If not, re-rack (See Training Video, 0:50).

    Note: The color of liquid in the pictures below was modified for illustrative purposes.

    a. Invert clear Dynamag tube rack and place upside down with sample lids touching the work surface. The tubes will be on the same row of the rack, and in the row furthest from you.

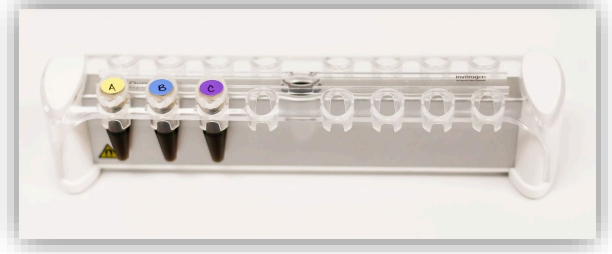


b. Invert Dynamag magnetic base and lower onto clear rack.

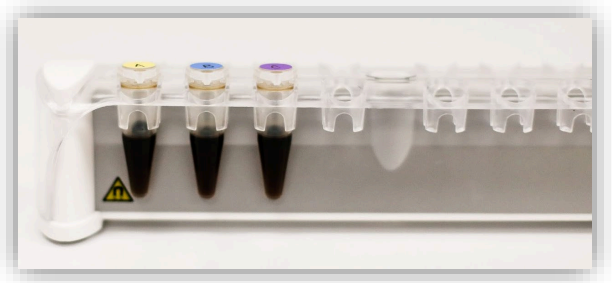


c. Tilt combined apparatus slowly 90° towards you while it continues to rest on surface. The tubes will now be horizontal and visible to you.

d. Tilt combined apparatus slowly 90° towards you while it continues to rest on surface, so that it stands fully upright, and tubes are facing you.



e. Make sure Nanobind Disk is held to the magnet near the top of the liquid level.



15. Set 1,000 µl pipette to 700 µl.
16. Remove supernatant as outlined below, careful not to aspirate the gDNA (See Training Video, 1:15):
    a. Angle entire rack at a 45° angle by holding in one hand (grasping the entire apparatus from below with tubes visible to you and lids towards your other hand).
    b. Wait 2 seconds for gDNA to lay on the Nanobind Disk.
    c. Slowly remove all liquid with a 1,000 µl extra-long tip angled away from the Nanobind Disk and/or gDNA to avoid disruption.
    d. Dispense supernatant into conical containing TexQ.

⚠ Ensure that the gDNA is not removed by visually inspecting the tip containing buffer before discarding. If gDNA is accidentally aspirated or becomes unbound from the disk, refer to Troubleshooting section below.

17. Perform Wash WB1 (See Training Video, 2:21):

a. Dispense 700 µl of Buffer WB1 directly onto the disks in the tubes and cap tubes.
b. Lift clear tube rack to separate from magnetic base.
c. Invert clear rack with tubes 180° 4 times to wash.
d. Re-rack clear tube rack and tubes with magnetic base as described in Step 21.
e. Remove supernatant as described in Step 23.

⚠ Ensure that the gDNA is not removed by visually inspecting the tip containing buffer before discarding. If gDNA is accidentally aspirated or becomes unbound from the disk refer to Troubleshooting section below.

18. Set the pipette to 500 µl (previously at 700 µl).
19. Perform Wash WB2 (See Training Video, 4:10):

    a. Dispense 500 µl of Buffer WB2 directly onto the disks in the tubes and cap.
    b. Lift clear rack to separate from magnetic base.
    c. Invert clear rack 180° 10 times to wash.
    d. Re-rack clear tube rack and tubes with magnetic base as described in Step 21.
    e. Remove supernatant as described in Step 23.

    ⚠ Ensure that the gDNA is not removed by visually inspecting the tip containing buffer before discarding. If gDNA is accidentally aspirated or becomes unbound from the disk refer to Troubleshooting section below.

20. Repeat Wash WB2 Step 26 (See Training Video, 5:50).

    Note: Remove buffer from 2 or 3 tubes at a time and process through Buffer EB incubation step in small batches to prevent the disk/gDNA from drying out.

21. Open tube lid fully (parallel to lab bench) and lift each tube apart from base.
22. In close proximity to a 0.5 ml Protein LoBind tube, transfer Nanobind Disk to the 0.5 ml Protein LoBind tube using Bionano Prep SP Magnetic Retriever (see Important Notes section for proper usage). Cap tube to prevent disk drying (See Training Video, 7:30).
23. Add 50 µl of Buffer EB to the 0.5 ml Protein LoBind tube.
24. Spin the tube on benchtop microcentrifuge for 5 seconds.
25. Using a 10 µl standard tip, gently nudge Nanobind Disk towards the bottom of the tube, making sure that it is fully submerged in liquid. The disk should remain parallel to the bench surface (See Training Video, 8:20).
26. Incubate submerged Nanobind Disk in Buffer EB at room temperature for 20 minutes.
27. Collect extracted gDNA by transferring eluate to a previously labeled 2.0 ml microcentrifuge tube with a 200 µl standard tip.

28. Spin the tube with the Nanobind Disk on benchtop microcentrifuge for 5 seconds and transfer all the remaining eluate containing viscous gDNA to the same standard 2.0 ml microcentrifuge tube as in previous step with a standard 200 µl tip. You may remove the disk before aspirating remaining elution buffer.

    Note: Almost all the viscous gDNA comes off the Nanobind Disk during the second spin.

Homogenization of gDNA Solution (70 minutes)

Homogenization of gDNA Solution

29. Slowly pipette the entire gDNA volume into a standard 200 µl tip, then slowly dispense the gDNA. Avoid creating bubbles.

- Repeat this process 3 times for a total of 4 strokes: (1 stroke = 1 aspiration and 1 dispense).

    Note: If gDNA uptake stalls due to high viscosity, it may be necessary to stir gently while slowly releasing the plunger to withdraw the gDNA.

30. Place standard 2.0 ml microcentrifuge tube containing gDNA in rack of Hula Mixer Sample Mixer and rotate at room temperature for 1 hour at 15 rpm.

    Note: During initial rotations, ensure that the gDNA gets drawn from the bottom of the microcentrifuge tube to reside in the lid of the tube during rotations. If the DNA solution remains in the bottom of the tube during initial rotations, turn off Hula Mixer and position rack so the microcentrifuge tube is oriented upside down. Gently flick the bottom of the microcentrifuge tube until the gDNA is drawn to into the lid and resume mixing.

31. Remove the 2.0 ml microcentrifuge tube from rack of Hula Mixer and spin tube on benchtop microcentrifuge for 2 seconds to bring the gDNA to the bottom of the tube. Allow the gDNA to equilibrate overnight at room temperature (25°C) to homogenize.

    Note: Most samples will become homogenous by the third day (from the start of the protocol), but samples may be labeled as soon as they become homogenous.

gDNA Quantitation (45 minutes)

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 new standard pipette tip or positive displacement pipette tip for each draw:

   Remove 2 µl aliquots from the left side, middle, and right side 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.
10. Coefficient of Variation (CV = standard deviation/mean) from three readings should be < 0.30.

    Note: If CV > 0.30, gently pipette-mix the entire volume of gDNA with five strokes (1 stroke = 1 up stroke + 1 down stroke) using a wide bore tip. Let the gDNA rest at least overnight at room temperature before repeating quantitation.

    Note: Typical DNA concentrations range from 50-120 ng/ul.

Sample ID	Left (ng/µl)	Middle (ng/µl)	Right (ng/µl)	Mean (ng/µl)	CV (stdev/mean)

Labeling

DNA is ready for Direct Label and Stain (DLS) labeling. See "Kits and Consumables" section at https://bionanogenomics.com/support/ for applicable kits and protocols.

Troubleshooting

See Training Video starting at 8:40 for video explanations of troubleshooting.

The gDNA comes unbound from the Nanobind Disk (DNA fall offs).

Evidence: gDNA is aspirated or becomes detached from disk during binding or during washes.

Steps to follow if sample is aspirated:

1. Leaving the sample tube racked on the magnet, dispense gDNA-containing liquid back into tube containing disk.
2. Remove racked tube from magnet and invert rack multiple times by hand to re-establish binding.

Alternatively:

1. Leaving the sample tube racked on the magnet, dispense gDNA-containing liquid back into tube containing disk.
2. Aspirate liquid from tube such that a minimal volume (~50 µl) remains above unbound gDNA and discard supernatant leaving the DNA in a minimal volume at bottom of the tube.
3. Carefully aspirate unbound gDNA containing the minimal liquid into pipet tip and pipet directly onto racked disk on magnet to re-establish binding.

The gDNA is not homogeneous before labeling

Evidence: The gDNA quantitation CV of three measurements (top, middle and bottom) is > 0.30.

Steps to Follow:

1. Aspirate and dispense sample using a wide bore tip for a total of 5 times.
2. Incubate the gDNA at room temperature for 1 to 3 days.
3. After incubation, again aspirate and dispense the sample using a wide bore tip 5 times.
4. Quantitate with Qubit BR Assay.

The gDNA is not viscous

Evidence: Sample consistency is very thin and easily pipetted, but concentration is > 35 ng/µL.

The sample is likely not to have high molecular weight gDNA.

Check sample using pulse field gel electrophoresis before labeling to confirm presence of high molecular weight gDNA.

Evaluate sample prep method and input material quality/age and repeat DNA isolation from biological sample.

Appendix

Preparing Cryopreserved Cells from Fresh Culture

To Prepare Cryopreserved Cells

1. Recommended input: 1.0 million cells from Amnio or CVS cultures per tube.
2. For every sample to be cryopreserved, prepare 0.6 ml of Cell Freezing Medium (AmnioMax II Complete Medium with DMSO added to a final concentration of 5%). Place prepared Cell Freezing Medium on ice.
3. Thaw/prewarm growth medium (6 ml/flask) and thaw/prewarm 1x Trypsin-EDTA (2ml/flask).
4. Remove medium and rinse culture(s) with 5 ml 1x Phosphate Buffered Saline (PBS).
5. Remove the PBS, add 1.5 ml 1x Trypsin-EDTA and gently swirl around the flask(s) to disperse.
6. Place in the 37°C CO_2 incubator.
7. After 3 minutes, remove the flask(s) from the incubator and swirl the 1x Trypsin-EDTA across the flask(s) to help dislodge the cells.
8. Check under the microscope to make sure most of the cells have detached.
9. Add 4.5 ml of growth medium and pipet medium against the growth surface of the flask(s) several times to fully remove cells from the plastic substratum.
10. Transfer the ~ 6 ml to 15 ml polypropylene conical(s).
11. After the sample(s) are in labeled conicals, pellet the cells by centrifugation using a swinging bucket rotor at 500 x g for 2 minutes at room temperature.
12. Remove all supernatant and resuspend the pellet(s) until fully resuspended by repeated pipetting with one ml of growth medium using a P1000 pipet. Transfer to labeled 1.5 ml microcentrifuge tube(s).
13. For each sample, pipet isolated cells in medium repeatedly until fully resuspended.
14. Remove a 5 ul aliquot from the middle of the cell suspension and mix well with 45 ul of Trypan Blue solution in a 1.5 ml microcentrifuge tube.
15. Count viable cells with a cell counting device (i.e., Hemocytometer or an automated cell counting device).
16. Calculate the volume of original cell stock required for 1.0 million cells.

    Note: If there are < 1.0 million cells but > 0.5 million cells continue with the pelleting and subsequent DNA isolation. With < 1.0 million cells there may be an increased risk of low DNA yield or gDNA fall offs (however, the gDNA can be rebound to the Nanobind - see Troubleshooting).
17. Re-pipet cells in medium repeatedly until fully resuspended, and transfer the volume for 1.0 million (or transfer total volume if > 0.5 million) cells to a labeled 1.5 ml Protein LoBind tube.

18. Centrifuge at 1000 x g for 2 minutes at 4°C.
19. Carefully remove as much of the supernatant as possible with a pipet without disturbing the cell pellet, drawing the solution away from the pellet from the side opposite of the tube hinge. Then, use a gel loading tip to remove the residual liquid from the pellet, drawing the residual into the gel loading tip from the side opposite of the tube hinge.
    Note: Once supernatants from all samples are removed, you may fill the bleach-containing conical to 50ml with water, cap conical, invert conical to mix, and dispose of the contents down the sink.
20. Resuspend the pellet with 500 ul of pre-chilled Cell Freezing Medium by pipetting 3x times with a P1000 pipet.
21. Transfer the entire volume of suspension to a labeled 1.0 ml cryotube with gasket and/or internal thread cap.

    Note: Keep all samples on ice until storing in cryocooler device at -80ºC
22. Place in a cryocooler device and store overnight at -80°C.
23. For long-term storage, continue storing samples in cryotubes at -80°C or transfer to liquid N_2.

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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