DNA Isolation with Qiagen Genomic-Tips

This protocol applies to: Neuroblastoma (NBL; prior to 2013), and Wilms Tumor (WT)

The protocol herein describes the procedures used by Nationwide Children's Hospital to process disease tissues for RNA and/or DNA subsequently used for characterization in the NCI's TARGET initiative. All nucleic acid samples used in TARGET projects were quality tested for consistency using picogreen quantification and SSTR genotyping methods, regardless of where the nucleic acid was originally extracted.

I. PRINCIPLE

QIAGEN Blood & Cell Culture DNA Kits and QIAGEN Genomic-tips with the Genomic DNA Buffer Set, provide an easy, safe and reliable method for the isolation of pure high molecular weight genomic DNA, direct from whole blood, lymphocytes and tissues. The procedure is based on optimized buffer system for lysis of cells and/or nuclei, followed by binding of genomic DNA to QIAGEN Anion Exchange Resin under appropriate low salt and pH conditions. RNA, proteins, dyes and low-molecular-weight impurities are removed by a medium-salt wash. Genomic DNA is eluted in a high-salt buffer and concentrated and desalted by isopropanol precipitation.

II. SPECIMEN

A. Type

Tumor or normal tissue – snap frozen

B. Handling Conditions

Standard precautions must be followed when handling all neuroblastoma tissue. Samples must be stored at -80°C prior to DNA isolation.

C. Sample Preparation

Tissue is obtained from the BPC in a 2 mL safe-lock tube. The weight of tissue requested is 50-60 mg.

D. Indications for Study

DNA isolated from tissue is to be used for Children's Oncology Group (COG) related research; this protocol is most commonly used for LOH analysis.

III. REQUIRED EQUIPMENT, SUPPLIES AND REAGENTS

A. Equipment

Centrifuge, Capsule
Centrifuge, Table Top with 15 mL and 50 mL tube holders
Pipet (electric)
Pipetters - Adjustable, 1-10 μL, 5-20 μL, 20-200 μL, 100-1000 μL
Vortex Mixer
Water Bath at 37°C
Water Bath at 50°C
TissueLyser (120V, 50/60 Hz) (Qiagen, Catalog# 85210)
TissueLyser Adapter sets (2 X 24)
TEC label printer

B. Supplies

QIArack
Ice Bucket
Floating Tube Rack
Forceps, disposable (Fisher, Catalog# F-1205-2)
Aerosol Barrier Pipet Tips
Markers
15 and 50 mL Conical Bottom Polypropylene Centrifuge Tubes
PPE (Gloves, Lab Coat, Safety Glasses)
Labels
2 mL Safe-Lock Tubes (Eppendorf, Catalog# 2236335-2)
1.5 mL Microcentrifuge Tubes
2 mL Microcentrifuge Tubes
Microcentrifuge Tube Rack
Racks
Sterile Plastic Pipets for Large Volumes (2, 5, 10 and 25 mL)
Dry Ice
Stainless Steel Beads, 5mm (Qiagen, Catalog# 69989)
1.5 mL Micrewtubes, round bottom (Milian, Catalog# T338-5S)
0.5 mL Micrewtubes, self-standing (Milian, Catalog# T338-2S)
Blue inserts (Milian, TIL-28)
Red inserts (Milian, TIL-14)
Pipet tips (20 µL, 200 µL, 1000 µL)
Reach pipet tips (1000 µL)
TEC printer labels

C. Supplies for tissue smashing (alternate method)

Safety shield
Dull rubber hammer
Super-Polyfoil, 4X4, 100/box (Fisher, Catalog# 22029118)
Dewars (2)
Liquid Nitrogen
Hearing Protection
14” Tweezers

D. Reagents

QIAGEN Blood & Cell Culture DNA Kit Midi (Qiagen, Catalog# 13343)
70% Ethanol - Stored at -20°C (Sigma-Aldrich, Catalog# E7023)
Isopropanol (Sigma, Catalog# I0398)
Water Molecular Biology Reagent (Sigma, Catalog# W4502)
Tris-EDTA Buffer 100X Concentrate (Sigma, Catalog# T9285)
QIAGEN Proteinase K (Qiagen, Catalog# 19133)
RNase A (100 mg/mL) (Qiagen, Catalog# 19101)

IV. WARNINGS / PRECAUTIONS

A. Use Standard Precautions when handling all body fluids, tissues and cell cultures. Refer to the Specimen Handling and Collection procedure, GEN-1, for guidelines specific for the Molecular Genetics Laboratory and samples.

V. REAGENT PREPARATION (INCLUDING STORAGE CONDITIONS)

A. The QIAGEN Blood & Cell Culture DNA Kits and QIAGEN Genomic-tips and reagents are used within one year of opening.

B. The water and C1 buffer are stored at 4°C. Water and C1 buffer are used within one year of opening.

C. Isopropanol is stored at room temperature in a flammables cabinet and is used within 6 months of opening.

D. 70% ethanol is made by mixing 350 mL ethanol and 150 mL Sigma water. Record preparation information into the Quality Control notebook. 70% ethanol is stored at room temperature in the flammable cabinet and maintained on ice prior to use.

VI. QUALITY CONTROL

A. Specimen identification is assured though each phase of handling by assignment of a specific color to each sample. That color is placed on each tube and column used throughout the procedure.

   1. NBL specimens are also labeled with the NBL #, patient initials, COG # and BPC #.

   2. Wilms tumor specimens are also labeled with the ACC #, COG # and BPC #.

B. Only one tissue sample is opened at a time during the initial placement of the tissue into a labeled 2 mL safe-lock tube for tissue disruption (or tube of digestion buffer, if using the cell-smashing method).

C. At each step in the isolation, the supernatant or pellet that does not contain the DNA is retained until after isolation and quantitation is completed. The 15 mL tubes are kept in the following order in the rack until the samples have been quantitated:

3	6
2	5
1	4

D. The QIAGEN Blood & Cell Culture DNA Kits and QIAGEN Genomic-tips are manufactured in compliance with current FDA Good Manufacturing Practices and undergo quality assurance testing.

E. All new lots of reagents will be tested in parallel with the one in current use before a new lot is put into use. The lot numbers and date of QC are recorded in the QC logbook.

VII. PROCEDURE – STEPWISE

A. NBL DNA Isolation Worksheet (Follow DNA Isolation worksheet)

1. Fill out the NBL DNA isolation worksheet (*internal document*) with information from the Freezerworks database and the isolation board. Include NBL#, BPC#, COG#, patient initials, sample weight, aliquot ID, tumor percent and percent alive. To do this:

   a. Get the COG# for the samples to be isolated from the isolation board. Do not isolate those that have not been signed out yet.

   b. Log into Freezerworks.

c. To search for a patient, go to Search, Search samples, put in the search field as the COG Reg # and the value is the COG#.

d. Write down the demographic information on the isolation worksheet.

e. Check that a tissue sample was received for this patient by looking in Aliquots | Shipping. The first sample sent to MGL is for DNA, if the two samples came from the same source. If the DNA and RNA pieces came from different sources, the RNA cannot be from the fresh piece. Write down the weight and aliquot ID.

f. Go to the Review 3 screen, to find the tumor percent and the percent alive.

2. Type all the information from the worksheet except the weight into the NBL Excel spreadsheet. Check with the signing Director if samples with 0% tumor should be isolated.

B. Wilms Tumor DNA Isolation Worksheet

1. Use the information from the shipping log or electronic case log to complete the Wilms Tumor Tissue DNA Isolation sheet (*internal documents*) enter the ACC #, BPC #, COG #, aliquot ID and tissue weight.

2. Type the information from the worksheet into the electronic case log (see above for location). Order patients by DNA # (ACC # - T for tumor or N for normal). The tumor information goes in the tab labeled Tumor DNA, and the normal tissue information goes in the tab labeled Normal DNA.

C. TissueLyser Tissue Extraction (preferred method)

1. Completely cover samples until processing starts. Label appropriate tubes (a 2 mL snap lock tube, a 50 mL conical vial and six 15 mL conical vials) and worksheet for processing specimens:

    - for NBL, include color, NBL #, BPC #, COG # and patient initials;
    - for Wilms Tumor, include color, BPC #, COG # and ACC #.

2. Prepare the digestion solution. For each sample, prepare a labeled 50 mL tube containing

    - 9 mL of Buffer G2
    - 19 µL RNase A and
    - 500 µL QIAGEN Proteinase K solution

3. Add 0.5 mL of Buffer G2 into the labeled 2 mL safe-lock tube.

4. Remove samples from the -80°C freezer and place in an ice bucket containing dry ice.

5. Working with one sample at a time, carefully unwrap the sample confirming that the information on the sample corresponds to the worksheet while unwrapping. Make sure the sample is resting on the dry ice.

6. Using new disposable forceps for each sample, carefully pick up the tumor tissue and place it in the 2 mL Eppendorf safe-lock tube containing 0.5 mL Buffer G2. If you accidently touch a sample, change your gloves before you move on to the next sample. Repeat until all the samples have been placed in the appropriately labeled tubes.

7. Opening only one at a time, un-cap each sample tube and place one stainless steel bead with the sample. Place the sample tubes into the TissueLyser adapter sets.

8. Fix the adapter sets into the clamps (arms) of the TissueLyser. Disrupt the samples for 30 seconds at 20 Hz. Short disruption times are recommended for DNA purification, in order to prevent shearing. For optimal operation, the TissueLyser should always be balanced.

9. Spin the lysed samples in a capsule centrifuge, and – using a Reach pipet and tips – transfer samples one at a time into the labeled 50 mL polypropylene tubes containing G2, RNase A and Qiagen Proteinase K (from step C1).

10. Vigorously vortex the samples for 10 seconds and place into a 50°C waterbath for at least 2 hours until the solution becomes clear (See Qiagen Genomic DNA Handbook). Longer incubations including overnight incubation are acceptable and preferred.

11. Continue to section E.

D. Smashing Tissue Extraction (use rarely – if TissueLyser is out of service)

1. Label tubes (a 50mL; six 15mL conical vials) and worksheet for processing specimens:

    - for NBL, include color, NBL #, BPC #, COG # and patient initials;
    - for Wilms Tumor, include color, BPC #, COG # and ACC #.

2. Prepare the digestion solution. For each sample, prepare a labeled 50 mL tube containing 9.5 mL of Buffer G2, 19 µL RNase A and 500 µL QIAGEN Proteinase K solution. Add 0.5 mL of Buffer G2 into a 2 mL safe-lock tube.

3. Remove samples from the -80°C freezer and place in an ice bucket containing dry ice.

4. Safety glasses MUST be worn during all steps that involve liquid nitrogen. Fill 2 liquid nitrogen dewars approximately halfway with LN_2 and place the wrapped tissue samples in it; allow to incubate for 10 minutes.

5. Wrap a chop block in aluminum foil and place behind a plexiglass shield. Place an additional, loose sheet of aluminum foil on the chop block (this piece will be replaced for each sample). Put on/in hearing protection.

6. Working with one sample at a time, remove the samples from the liquid nitrogen using 14" tweezers and place on the chop block.

7. While wearing hearing protection, pound the sample 3 – 4 times with an aluminium-foil wrapped dead blow hammer, using only one side of the hammer (use the clean side for the next sample and replace foil every other sample).

8. Wrap the sample with a new piece of aluminum foil and place into the second liquid nitrogen dewar.

9. Repeat steps 5 – 8 until all samples have been smashed and let the samples incubate for another 10 minutes.

10. Working with only one sample at a time, remove from the liquid nitrogen using 14" tweezers and place on the aluminum foil-wrapped chop block behind a plexiglass shield with a new sheet of aluminum foil on top. Wearing hearing protection, re-smash the samples by pounding 3 – 4 times with a foil-wrapped dead blow hammer.

11. Unwrap the sample, taking notice of the sample identification information on the inner Super-Polyfoil wrap.

12. Pipet 750 µL of digestion solution (from step 2) onto the smashed tissue (in the polyfoil), and carefully collect the sample into the labeled 50 mL conical vial. Repeat until the entire sample has been transferred.

13. For each sample, a labeled 15 mL conical vial should be prepared with 9.5 mL of Buffer G2, 19 µL RNase A and 500 µL Qiagen Proteinase K.

E. DNA Isolation

1. After incubation, remove samples and vortex for 10-30 seconds. Centrifuge samples in a fixed-angle rotor at 8000 x g for 10 min at 4°C.

2. Get out one QIArack for every 4 samples. Clean the rack with gauze and isopropanol. Prepare G100 Genomic-tips by placing them in the QIArack. Label the genomic tip with:

    - for NBL, include color, NBL #, BPC #, COG # and patient initials;
    - for Wilms Tumor, include color, BPC #, COG # and ACC #.

3. Equilibrate each G100 QIAGEN Genomic-tip with 4 mL of Buffer QBT and allow the QIAGEN Genomic-tip to empty by gravity flow. Discard the flow-through in the sink and flush with water.

4. Place 5 mL of Buffer QF for each sample plus one extra in a labeled 50 mL centrifuge tube and place in a 50°C waterbath; the pre-warmed buffer will be used in Step 18.

5. After centrifugation, pour the sample onto the equilibrated Genomic-tip. Once the QIAGEN Genomic-tip is loaded, flow will begin unassisted. Allow gravity to determine the flow rate.

    - The flow rate will depend on the sample source and the number of cells from which the DNA sample was prepared. Particularly concentrated genomic DNA lysates may exhibit diminished flow rates due to increased viscosity.
    - Flow can be assisted by the application of gentle positive pressure using a disposable syringe and the appropriate adapter. When using positive pressure, do not allow the flow rate to exceed 4-10 drops/min.
    - It might also be helpful to dilute the sample with an equal volume of Buffer QBT prior to loading.
    - Collect and keep the flow-through in a labeled 15 mL centrifuge tube.

6. Wash the QIAGEN Genomic-tip with 7.5 mL of Buffer QC. Collect and keep the flow-through in a new labeled 15 mL centrifuge tube.

7. Repeat the wash step while collecting the flow-through into another new labeled 15 mL centrifuge tube.

8. Elute the genomic DNA with 5 mL of Buffer QF, which has been heated to 50°C. Collect the flow-through which contains the genomic DNA in a labeled 15 mL centrifuge tube.

F. DNA Precipitation

1. Precipitate the DNA by adding 3.5 mL (0.7 X volume) room temperature isopropanol to the eluted DNA. Mix by inversion several times and vortex for a few seconds.

2. Recover the precipitated DNA by centrifugation in a fixed angle rotor at 8000 x g for 15 minutes at 4°C. Isopropanol pellets have a glassy appearance and may be more difficult to see than the fluffy, salt-containing pellets that result from ethanol precipitation. Marking the outside of the tube before centrifugation allows the pellet to be more easily located. Isopropanol pellets are also more loosely attached to the side of the tube and care should be taken when removing the supernatant.

3. Carefully decant the supernatant into a labeled 15 mL centrifuge tube and save.

4. Wash the DNA pellet with 2 mL of cold 70% ethanol. Vortex for 10 seconds and centrifuge at 8000 x g for 10 minutes at 4°C.

5. Carefully decant the supernatant into a labeled 15 mL centrifuge tube and save.

6. Centrifuge the tube containing DNA at 8000 x g for 2 minutes at 4°C. Carefully remove the remaining 70% ethanol using a Reach pipet tip and combine with the centrifuge tube containing 70% ethanol from step 5.

7. Invert the tubes containing DNA and place on a fresh paper towel. Allow the DNA pellet to air dry for 30 minutes or until no ethanol droplets are visible. Be careful not to over dry the pellet.

8. Resuspend the DNA in 200 µL of 1X TE Buffer. Incubate overnight in a 37°C waterbath. You can use less TE buffer for samples with lower total DNA amounts (smaller cell pellets).

9. Centrifuge the resuspended DNA at 8000 x g for 1 minute at 4°C. Using a Reach pipet tip transfer the DNA to a labeled 1.5 mL micrewfuge tube containing a blue cap insert (tumor) or red cap insert (normal) with the BPC # written on the side of the tube. Make sure that the tube is labeled with the BPC # prior to transfer.

10. Determine the concentration of the DNA by measuring absorption at 260nm. (See Quantitation of Nucleic Acids, GEN-2).

11. If quantity is adequate discard processing tubes from the various collection steps. If nothing is recovered from the isolation, keep the collection tubes and try redigesting any residual material in the digestion tube or re-eluting the DNA from the column.

12. For NBL, DNA is aliquoted immediately:

    - If the amount of total DNA is more than 40 µg, four 5 µg aliquots will be made from that particular stock DNA. If the amount of DNA is between 30 - 39.99 µg, three 5µg aliquots are made.
    - If the amount of DNA is 20 – 29.99 µg, two 5 µg aliquots are made.
    - If the amount of DNA is 10 – 19.99 µg, one 5 µg DNA aliquot is made.
    - A general rule of thumb is not to use more than ½ of the total stock of neuroblastoma tissue DNA when making the 5 µg aliquots. NBLs are labeled with the BPC # written on the side. The label should include the BPC #, NBL #, STOCK or 5 µg, parent aliquot ID, DNA concentration in ng/µL and the date of isolation. Wilms are labeled with the BPC # on the side. The label should include the ACC #, BPC #, COG #, parent aliquot ID, DNA concentration in Label tubes with BPC #, NBL #, COG # and date of isolation, DNA concentration in ng/µL and the date of isolation. DNA samples are stored at 4°C indefinitely or until shipped at COG request.

13. The quality of the DNA is confirmed upon LOH testing (See procedure REF-28, NBL Loss of Heterozygosity 1p36 and 11q23; or procedure REF-33, Wilms Tumor LOH 1p16q)

14. Quality control can be performed with a CX26 PCR using 1 µL of undiluted DNA. If DNA concentration is less than 200 µg/mL use 2 µL of undiluted DNA. See procedure CLN-17, Connexin 26 – only perform the PCR section of the protocol; analyze on an agarose gel.

VIII. INTERPRETATION / ANALYSIS / DOCUMENTATION

A. For NBL samples, enter the following information into the ANBL00B1DNA_RNA log (*internal document*):
BPC #, NBL #, COG #, patient initials, amount of resuspension volume, A260 reading, A280 reading, A260/280 ratio, DNA concentration (µg/mL), DNA yield

(µg), tumor percent, percent viable, technologist initials, date of isolation, parent ID and any necessary additional comments.

B. For Wilms Tumor samples, enter A260 reading, A280 reading, A260/280 ratio, DNA concentration (µg/mL), DNA yield (µg), technologist initials, date of isolation, parent ID and any necessary additional comments in the appropriate spreadsheet of the Wilms Tumor Case Log (*internal document*)

C. DNA yields from 50 – 60 mg of tissue are generally greater than 80 µg.

IX. REFERENCES

1. QIAGEN Genomic DNA Handbook