**TNA, Tissue Manual Extraction Procedure**

1. **PRINCIPLE:**
   1. Extraction of DNA from tissue and body fluid samples is critical for the success of most assays in the Molecular Genomic Pathology (MGP) Laboratory. The quality of DNA produced is dependent on the starting material, as well as the method of preparation. High quality DNA can be prepared from fresh, alcohol-fixed, or frozen tissue. DNA of quality satisfactory for PCR can usually be obtained from standard formalin-fixed, paraffin-embedded (FFPE) tissue block samples. To aid in purification of DNA from tissue, samples are typically digested with the active protease, Proteinase K, in the presence of ionic detergent and the chelating agent EDTA. This protocol rapidly and irreversibly inhibits DNases and results in DNA of sufficient quality for all assays performed in the laboratory. This digestion step also results in the rapid inactivation of conventional infectious agents. This method adapts well to the isolation of DNA from FFPE blocks, following removal of paraffin by treatment with xylene and ethanol.
   2. Of note, this procedure directly results in the extraction of Total Nucleic Acid (TNA), including DNA and RNA. However, the TNA product can be used for most assays in the MGP lab that require DNA. In some cases, residual RNA is removed from tissue samples by treatment with Ribonuclease A.
   3. Automated extraction of DNA from FFPE is performed with the Promega Maxwell Instrument and magnetic bead purification. Automated extraction of DNA from blood and bone marrow aspirate specimens is carried out on the Qiagen QIAcube Instrument (See DNA Isolation QIAcube Procedure).
2. **SAMPLE:**
   1. Sample types: Formalin-fixed, paraffin embedded (FFPE) tissue, Fresh Frozen, or OCT-Embedded Tissue
3. **REAGENTS:**
   1. 1XPBS: Dulbeccos Phosphate Buffered Saline from Life Technologies. Cat #14190144. Store at room temperature.
   2. Cell Lysis Solution: Qiagen Cat #1045696. Store at room temperature.
   3. Proteinase K (PK): Sigma Catalog #3115828001. 15mg/ml. Store at 4°C.
   4. TE: Life Technologies Cat# AM9849
   5. Phenol/Chloroform: Add Tris bottle (comes attached), shake well, allow layers to separate. Take the reagent from the lower layer. Store at 4°C for one year.
   6. Chloroform: Store at room temperature in flammable cabinet for up to 5 years.
   7. Xylene: Store at room temperature in flammable cabinet for up to 5 years.
   8. Ethanol, 100%: Store at room temperature in flammable cabinet for up to 2 years.
   9. Ethanol, 70%: Store at room temperature in flammable cabinet for up to 2 years.
   10. Isopropanol, 100%: Store at room temperature in flammable cabinet for 2 years.
   11. 5M Ammonium acetate: Store at room temperature for up to 2 years. To prepare, dissolve 385grams of ammonium acetate in 800ml of sterile double distilled water. Adjust the volume to 1000ml.
   12. AE: Qiagen Catalog # 19077. Store at room temperature.
   13. Distilled Water (DH20): Reagent grade. Store at room temperature.
4. **TNA, Tissue Manual Extraction: Formalin-fixed, paraffin embedded (FFPE) tissue**
   1. Carry out Organic portions of procedure in the fume hood.
   2. **Cut Block/Scrape Slides Action (Day 1)**
      1. Log into Soft Molecular.
      2. Open Extractions by using the Extractions tile on the dashboard.
      3. Highlight the Cut Block or Scrape Slides branch in the action tree.
      4. Highlight the Barcode# field. Scan the Soft Molecular specimen label and press **Enter** on the keyboard. Repeat this step for all applicable specimens.
      5. For each sample, change the protocol from **MAXEXTR** to **ORGEXTR** using the dropdown menu in the Protocol column on the child level.
      6. **If a sample is being re-extracted, expand the child level and change the Volume in the child level tube to 4800. This is necessary to ensure adequate volume in the system for performing any testing.**
      7. Select **Save**.
         1. Two aliquot labels per sample will automatically print upon saving.
            1. If only one 1.5ml tube is being utilized, discard the second aliquot label in the appropriate bin.
      8. Select **Back** in the Extractions window.
      9. Exit Soft Molecular application.
      10. Label a 1.5ml tube with an aliquot label for each specimen.
   3. **Cut Block/Scrape Slides**
      1. Collect between 10 and 20 sections cut 5 microns thick from the tissue block and place them in a labeled 1.5ml screw-cap microfuge tube.
   4. **Begin the TNA, Tissue Manual Extraction**
      1. Add 1mL xylene to the tube and vortex several times over 15 minutes.
      2. Centrifuge for 3 min. at 25°C at 14,000 rpm in a microfuge.
      3. Remove supernatant by aspiration with a plastic transfer pipette tip.
         1. **If at this step, or any of the following steps prior to adding the cell lysis solution, you do not see any tissue, save the aspirated supernatant in a 1.5ml tube. You may use this later to spin down and collect any tissue that may have been aspirated off. Label tubes appropriately.**
         2. If necessary, centrifuge for 3 minutes at 14, 000 rpm to recover any aspirated tissue.
      4. Add 1mL xylene, vortex briefly, and incubate for 5 min. at room temperature.
      5. Centrifuge for 3 min. at 25°C at 14,000 rpm in a microfuge.
      6. Remove supernatant by aspiration with a plastic fine tip transfer pipette.
      7. Add 1ml 100% ethanol and vortex briefly.
      8. Centrifuge for 3 min. at 25°C at 14,000 rpm in a microfuge.
      9. Remove supernatant by aspiration with a plastic fine tip transfer pipette.
      10. Add 1ml of 100% ethanol and vortex briefly.
      11. Centrifuge for 3 min. at 25°C at 14,000 rpm in a microfuge.
      12. Remove supernatant by aspiration with a plastic fine tip transfer pipette.
      13. Air dry for 30 min. or place in heat block for 5 min.
      14. Add 100ul proteinase K per 0.5ml Cell lysis solution and mix gently, making sure the tissue is dislodged from the bottom of the tube.
      15. Close cap and incubate at 55°C overnight or up to 72 hours.
   5. **Record your extraction reagents: Organic TNA Extraction Reagents (Day 1)**
      1. Log into Soft Molecular.
      2. Open Extractions by using the Extraction tile on the dashboard.
      3. Highlight the Organic TNA Extraction Reagents branch on the action tree.
      4. Highlight the Barcode# field. Scan the aliquot label and press **Enter** on the keyboard. Repeat this step for all applicable specimens.
      5. Select the Spec/Tube Reagents field. In the dropdown, scan the appropriate TQC reagent labels to add the reagent lot numbers to each specimen.
      6. Select **OK** in the Spec/Tube Reagent window.
      7. Mark the **Completed** checkbox and select **Save**.
      8. Select **Back** in the Extractions window.
      9. Exit Soft Molecular application.
   6. **Organic TNA Extraction Action (Day 1)**
      1. Log into Soft Molecular.
      2. Open Extractions by using the Extraction tile on the dashboard.
      3. Highlight the Organic TNA Extraction branch on the action tree.
      4. Highlight the Barcode# field. Scan the aliquot label and press **Enter** on the keyboard. Repeat this step for all applicable specimens.
      5. Enter the final elution volume in the Prod Vol column. The final volume should reflect the anticipated TNA retrieval. For small pieces of tissue, enter 50ul. Larger pieces of tissue may be resuspended in a larger volume. See step H15 below.
      6. Click on the empty space in the Extraction window.
      7. Select **Save**.
         1. Do not complete the Organic TNA Extraction action.
      8. Select **Back** in the Extractions window.
      9. Exit Soft Molecular application.
   7. **Organic TNA Extraction Action (Day 2)**
      1. Log into Soft Molecular.
      2. Open Extractions by using the Extraction tile on the dashboard.
      3. Highlight the Organic TNA Extraction branch on the action tree.
      4. Highlight the Barcode# field. Scan the aliquot label and press **Enter** on the keyboard. Repeat this step for all applicable specimens.
      5. Click **Completed** checkbox.
      6. Navigate to the **Print** tab and click the **Print Product Labels** dropdown menu. Select the ‘**…**’ button that appears.
      7. Select **OK** when asked to save before print.
      8. In the Print Product Labels window, verify the correct printer and product label template is selected (PROD LBL V1). Change the # of labels to 2. Click **Print**.
      9. Select **Back** in the Extractions window.
      10. Exit Soft Molecular application.
      11. Label one 1.5ml tube and one 2ml tube with a product label for each specimen.
   8. **Continue TNA, Tissue Manual Extraction**
      1. Add 500ul of 50% phenol:50% chloroform and vortex vigorously for 30 seconds.
      2. Centrifuge for 3 min. at 25°C at 14,000 rpm in a microfuge.
      3. Immediately transfer aqueous phase (top layer) to a new labeled 1.5ml microfuge tube, add 500ul of chloroform, and vortex 30 seconds.
      4. If a large interface precludes transferring at least 300ul of sample, increase the volume transferred, as follows:
         1. Add 200 ul TE.
         2. Vortex briefly.
         3. Re-centrifuge for 5 min. at 25°C at 14,000 rpm in a microfuge.
      5. Centrifuge for 3 min. at 25°C at 14,000 rpm in a microfuge.
      6. Immediately transfer aqueous phase to a new 2ml microfuge tube labelled with a product label.
      7. If the starting volume from step D.14. was 0.5ml of cell lysis solution, add 50ul of 5M ammonium acetate to the tube and 1.5ml of 100% ethanol. Vortex briefly.
         1. Adjust according to the volume of lysis solution. For example, if 1ml of cell lysis solution was used in D.14., use 100ul of NH₄ acetate to 3ml of 100% ethanol.
         2. If the total volume will exceed 1.5ml, divide the aqueous phase into 2 tubes before adding the ammonium acetate and ethanol, keeping the proportion of ammonium acetate to ethanol.
      8. Incubate at -20°C for at least 30 min.
      9. Centrifuge for a minimum of 5 min. at 14,000 rpm in a microfuge. For samples with a very low concentration of DNA, centrifuge for 15 minutes.
      10. Remove supernatant with a fine-tip micropipette.
      11. Slowly add 1ml of 70% ethanol (pre-chilled at -20°C). Vortex.
      12. Centrifuge for 5 min. at 14,000 rpm in a microfuge.
      13. Remove supernatant with a fine-tip micropipette.
      14. Dry in heat block for 5 min. with cap open.
      15. Resuspend in 50-200 ulTE or AE (from Qiagen) and vortex (see Director/Pathologist for any questions about the volume). Place in heat block for 5 min. and vortex again. Longer incubation at 55°C may be necessary if the entire DNA is not dissolved.
   9. **Refer to the NanoDrop 2000 Instrument Procedure to measure the nucleic acid concentration of each sample.**
      1. Soft Molecular will automatically calculate dilutions to 50ng/ul.
      2. When the original reading is greater than 500ng/ul, dilute to 100ng/ul and utilize an internal note in Soft Molecular to state original nanodrop reading and how much buffer was added.
      3. If the sample has a DNA concentration of greater than 100ng/ul but less that 500ng/ul, dilute the sample to 50ng/ul. Then, proceed with the appropriate clinical assay.
      4. If a given sample has a DNA concentration of less than 100 ng/ul, proceed with the appropriate clinical assay**.**
   10. Proceed to the **Quantitation Action** below.
5. **TNA, Tissue Manual Extraction: Fresh Frozen or OCT Embedded Tissue**
   1. **NOTE**: Carry out Organic portions of procedure in the fume hood.
   2. **Cut Block/Scrape Slides Action (Day 1)**
      1. Log into Soft Molecular.
      2. Open Extractions by using the Extractions tile on the dashboard.
      3. Highlight the Cut Block or Scrape Slides branch in the action tree.
      4. Highlight the Barcode# field. Scan the Soft Molecular specimen label and press **Enter** on the keyboard. Repeat this step for all applicable specimens.
      5. Change the protocol from **MAXEXTR** to **ORGEXTR** using the dropdown menu in the Protocol column on the child level for each sample.
      6. Select **Save**.
         1. Two aliquot labels per sample will automatically print upon saving.
            1. Discard the second aliquot label in the appropriate bin.
      7. Select **Back** in the Extractions window.
      8. Exit Soft Molecular application.
      9. Label a 15ml polypropylene tube with an aliquot label for each specimen.
   3. **Process the Fresh Frozen or OCT Embedded Tissue**
      1. For Fresh or frozen tissue that has been thawed, proceed to step 5.
      2. Remove frozen tissue from -80°C freezer.
      3. If using entire piece of tissue, open foil and let OCT melt away from tissue.
      4. To aid in melting of OCT, you can put tissue into PBS. Use a sterile disposable petri dish for PBS.
         1. For small pieces of tissue, place the specimen in a 1.5ml tube and add the PBS to the tube.
            1. Centrifuge the sample for 3 minutes at 14000rpm.
            2. Using a fine tip pipette, aspirate off the supernatant
            3. **If at this step, or any of the following steps prior to adding the cell lysis solution, you do not see any tissue, save the aspirated supernatant in a 1.5ml tube. You may use this later to spin down and collect any tissue that may have been aspirated off. Label tubes appropriately.**
            4. If necessary, centrifuge for 3 minutes at 14, 000 rpm to recover any aspirated tissue.
      5. Once tissue has been liberated from OCT fixative, blot on an absorbent paper and weigh it.
      6. Using a tared weigh boat, mince tissue finely with a scalpel and place in a labeled polypropylene 15ml tube.
      7. For 100 to 200 mg of tissue, add 1ml of DNA lysis buffer and 100ul proteinase K. For each additional 100mg of tissue, add another 1ml lysis buffer and 100ul of proteinase K to a separate tube.
      8. Perform the lysis at 55°C overnight in Hybridizer oven in the Post-PCR area.
   4. **Record your extraction reagents: Organic TNA Extraction Reagents (Day 1)**
      1. Log into Soft Molecular.
      2. Open Extractions by using the Extraction tile on the dashboard.
      3. Highlight the Organic TNA Extraction Reagents branch on the action tree.
      4. Highlight the Barcode# field. Scan the aliquot label and press **Enter** on the keyboard. Repeat this step for all applicable specimens.
      5. Select the Spec/Tube Reagents field. In the dropdown, scan the appropriate TQC reagent labels to add the reagent lot numbers to each specimen.
      6. Select **OK** in the Spec/Tube Reagent window.
      7. Mark the **Completed** checkbox and select **Save**.
   5. **Organic TNA Extraction Action (Day 1)**
      1. Log into Soft Molecular.
      2. Open Extractions by using the Extraction tile on the dashboard.
      3. Highlight the Organic TNA Extraction branch on the action tree.
      4. Highlight the Barcode# field. Scan the aliquot label and press **Enter** on the keyboard. Repeat this step for all applicable specimens.
      5. Enter the final elution volume in the Prod Vol column.
      6. Click on the empty space in the Extraction window.
      7. Select **Save**.
         1. Do not complete the Organic TNA Extraction action.
      8. Select **Back** in the Extractions window.
      9. Exit Soft Molecular application.
   6. **Organic TNA Extraction Action (Day 2)**
      1. Log into Soft Molecular.
      2. Open Extractions by using the Extraction tile on the dashboard.
      3. Highlight the Organic TNA Extraction branch on the action tree.
      4. Highlight the Barcode# field. Scan the aliquot label and press **Enter** on the keyboard. Repeat this step for all applicable specimens.
      5. Click **Completed** checkbox.
      6. Navigate to the **Print** tab and click the **Print Product Labels** dropdown menu. Select the ‘**…**’ button that appears.
      7. Select **OK** when asked to save before print.
      8. In the Print Product Labels window, verify the correct printer and product label template is selected (PROD LBL V1). Change the # of labels to 5. Click **Print**.
      9. Select **Back** in the Extractions window.
      10. Exit Soft Molecular application.
      11. Label four 15ml polypropylene tubes and one 1.5ml tube, with the printed product labels, per specimen.
   7. **Continue TNA, Tissue Manual Extraction**
      1. Solution should be viscous and there should be no pieces of tissue left when digestion is complete. If digestion is not complete, add another 100ul proteinase K and allow to digest another 3 hours at 55°C.
      2. Add an equal volume of 50% phenol:50% chloroform to aqueous DNA solution. Vortex gently for 1 minute.
      3. Centrifuge at 2000 rpm for 5 minutes.
      4. Using a disposable transfer pipette, remove the upper aqueous phase and place in a 15ml polypropylene tube labelled with Soft Molecular product label.
      5. Leave the interface from the first extraction.
      6. Discard phenol and chloroform into waste bottle.
      7. Repeat steps 2 through 4 with another phenol:chloroform extraction, followed by one chloroform extraction.
      8. After the final chloroform extraction, remove the upper aqueous DNA layer to a new labeled 15ml polypropylene tube and discard phenol and chloroform to a waste bottle.
      9. The final chloroform layer must be clear.
      10. Add 1/10 volume of 5M ammonium acetate.
      11. Add equal volume isopropanol, mix gently until DNA starts coming out of solution.
      12. Let tube stand at room temperature for approximately 5 minutes. DNA will float to the top.
      13. Incubate at -20°C for at least 30 minutes.
      14. Centrifuge for a minimum of 5 minutes at 14,000 rpm in a microfuge. For samples with a very low concentration of DNA, centrifuge for 15 minutes.
      15. Remove supernatant with a fine-tip micropipette.
      16. Slowly add 1ml of 70% ethanol (pre-chilled at -20°C).
      17. Centrifuge for 5 minutes at 14,000 rpm in a microfuge.
      18. Remove supernatant with a fine-tip micropipette.
      19. Let air dry overnight or in heat block for 5 to 10 minutes to remove residual alcohol.
      20. Resuspend in 100 – 200ul of TE or AE (Qiagen), depending on the amount of DNA or RNA, in a 1.5ml tube labelled with a Soft Molecular product label.
   8. **Refer to the NanoDrop 2000 Instrument Procedure to measure the nucleic acid concentration of each sample.**
   9. **Quantitation Action (Day 4-5)**
      1. Log into Soft Molecular.
      2. Open Extractions by using the Extraction tile on the dashboard.
      3. Highlight the correct Quantitation branch on the action tree.
      4. Select **Tools** tab followed by **Import** to import Nanodrop or Qubit results.
      5. Choose file location using the ‘**…**’ button next to the Directory field and confirm by clicking **OK**.
      6. Select the correct **INITIAL CONC** from the dropdown in the Template field.
      7. Choose file name using the ‘**…**’ button next to the File Name field and confirm by clicking **Open**.
      8. Select **Import**.
      9. In the Import Finished window select **OK**.
      10. Close the Import from Excel window using the ‘**X**’.
      11. If a dilution is not required, highlight the Barcode# field, scan the product label, and select Enter on the keyboard. Repeat this step for all applicable specimens. Select **Save**.
      12. Select **Back** in the Extractions window.
      13. Exit Soft Molecular application.
   10. **First Dilution**
       1. Mark the **Dilute(?)** checkbox for every patient sample that requires a dilution.
       2. Highlight the Barcode# field. Scan the product label and select **Enter** on the keyboard. Repeat this step for all samples pending Quantitation. Select **Save**.
       3. Highlight the correct Dilute and Repeat Quantitation branch on the action tree.
       4. Select **Tools** tab followed by **Import** to import Nanodrop or Qubit results.
       5. Choose file location using the ‘**…**’ button next to the Directory field and confirm by clicking **OK**.
       6. Select the correct **FINAL CONC** from the dropdown in the Template field.
       7. Choose file name using the ‘**…**’ button next to the File Name field and confirm by clicking **Open**.
       8. Select **Import**. If a second dilution is required, refer to L. Second Dilution.
       9. In the Import Finished window select **OK**.
       10. Close the Import from Excel window using the ‘**X**’.
       11. If a second dilution is not required, highlight the Barcode# field, scan the product label and select **Enter** on the keyboard. Repeat this step for all applicable specimens. Select **Save**.
       12. Select **Back** in the Extractions window.
       13. Exit Soft Molecular application.
   11. **Second Dilution** 
       1. Highlight the correct sample and select the **Internal Note** icon in the Home menu.
       2. Select **Add** and enter ‘Initial DNA or TNA Concentration: XYZ ng/uL’ in the text window that appears.
       3. Click **OK** and **Save**.
       4. Mark the **+** sign button in the Initial DNA or RNA Conc field to open the multi-run for tube window. Select **New Run** button. Click **OK**.
       5. Enter the value from DNA or RNA Concentration field into the Initial DNA or RNA Conc field, so that Suggest Add Vol Diluent is calculated.
       6. Enter ‘**2**’ in the Dilution column with the red ‘X’ icon.
       7. Select **Tools** tab followed by **Import** to import Nanodrop or Qubit results.
       8. Choose file location using the ‘**…**’ button next to the Directory field and confirm by clicking **OK**.
       9. Select **FINAL CONC** from the dropdown in the Template field.
       10. Choose file name using the ‘**…**’ button next to the File Name field and confirm by clicking **Open**.
       11. Select **Import**. Repeat steps 1-10 for additional dilutions, as necessary.
       12. Highlight the Barcode# field. Scan the product label and select **Enter** on the keyboard. Repeat this step for each specimen. Select the **Completed** checkbox and **Save**.
       13. Select **Back** in the Extractions window.
       14. Exit Soft Molecular application.
   12. **Test Aliquot Action: BRAF and IGH-BCL2** 
       1. Log into Soft Molecular.
       2. Open Extractions by using the Extraction tile on the dashboard.
       3. Highlight the Test Aliquot branch on the action tree.
       4. Highlight the Barcode# field. Scan the product label and press **Enter** on the keyboard. Repeat this step for all applicable specimens.
       5. Click on the **+** button to expand the child level of each patient sample, so the aliquots are displayed.
       6. **If additional aliquots are required**:
          1. Highlight the correct sample.
          2. Select the **Plan Aliquot/Material** button.
          3. Enter the Aliquot value.
             1. **Note**: The Aliquot Value field is alphanumeric, so text (BSA, 1:4 Dil., etc.) can be added to the Aliquot Value column.
          4. Enter the numeric value (no units) in the Volume column.
          5. Attach the test (IGH-BCL2 or BRAF).
          6. A window will appear. select **Yes**.
          7. Select **ATST** in the Protocol field.
             1. **Note**: The Next Action field will automatically populate with Specimen Testing.
          8. Highlight the Barcode# field. Scan the product label and select **Enter** on the keyboard. Repeat this step for all applicable samples. Select **Save**.
             1. **Note**: When the Test Aliquot action has been completed and saved, the Sample ID will be assigned to the additional aliquots.
       7. Enter the volume with units for each aliquot in the Aliquot Value column.
       8. Enter the numeric value (no units) in the Volume column.
       9. Complete the Test Aliquot action by marking the **Completed** checkbox and select **Save**.
       10. Select **Back** in the Extraction window.
       11. Exit Soft Molecular application.
6. **TROUBLESHOOTING**
   1. For samples with inhibitors, such as melanin, refer to the PCR Interfering Substances BSA Method Procedure.
   2. For additional troubleshooting, refer to the Genomic DNA Clean and Concentrate Procedure.
7. **REFERENCES:**
   1. Forsthoefel, K.F., et al., Optimization of DNA Extraction from formalin fixed tissue and its clinical application in Duchenne muscular dystrophy, Amer. J. Clin. Pathol. 98: 98-104, 1995.
   2. Davis, L.G., Dibner, M.D., & Battey, J.F. "Basic Methods in Molecular Biology", Elsevier, New York, Amsterdam, London, 1986.
8. **REVISIONS:**
   1. 4/14/2020: Added instructions to change the volume on re-extracted specimens
   2. 12/14/2020: Added instructions to accommodate the Fusion assay.
   3. 7/6/2021: Added a step to retain supernatant in cases where no tissue is seen prior to adding cell lysis buffer.