**TNA Cytology 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).
   4. Cytology smears and liquid-based cytology slides may be used as a source of cells for nucleic acid extraction. The material on these slides is usually dried and fixed using a variety of techniques and reagents (such as alcohols). In addition, coverslips are added to the slide using adhesives. To process the slide for nucleic acid extraction, this coverslip must be removed with xylene and the tissue/cells must be isolated.
2. **SAMPLES**:
   1. Sample type: Cytology Preparation Slides
3. **REAGENTS**:
   1. Cell Lysis Solution: Qiagen Cat #1045696. Store at room temperature.
   2. Proteinase K (PK): Sigma Catalog #3115828001. 15mg/ml. Store at 4°C.
   3. TE: Life Technologies Cat# AM9849
   4. 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.
   5. Chloroform: Store at room temperature in flammable cabinet for up to 5 years.
   6. Xylene: Store at room temperature in flammable cabinet for up to 5 years.
   7. Ethanol, 100%: Store at room temperature in flammable cabinet for up to 2 years.
   8. Ethanol, 70%: Store at room temperature in flammable cabinet for up to 2 years.
   9. 5M Ammonium acetate: Store at room temperature for up to 2 years.
      1. To prepare, dissolve 385grams of ammonium acetate in 800ml of sterile double distilled water.
      2. Adjust the volume to 1000ml.
   10. AE: Qiagen Catalog # 19077. Store at room temperature.
   11. Distilled Water (DH20): Reagent grade. Store at room temperature.
4. **TNA Cytology Manual Extraction**
   1. **Soak in Xylene Action (Day 1)**
      1. Log into Soft Molecular.
      2. Open Extractions by using the Extractions tile on the dashboard.
      3. Highlight the Soak in Xylene 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. Remove the label from the slide box and affix the label to the Coplin jar.
      6. In the Comment field, enter the date and time the slide was added to the xylene followed by the initials of the technologist performing the action.
      7. Select **Save.**
      8. Select **Back** in the Extractions window.
      9. Exit Soft Molecular application.
   2. **Place Cytology Prep slide in xylene**
      1. Using a solvent-resistant marker, turn the slide to the back and circle the area marked by the pathologist to be scraped. On the back of the frosted area of the slide, write the surgical number using this same marker in case the label with the patient information bleeds or falls off.
      2. Soak the slide in clean xylene in a Coplin jar under the fume hood. Most cover slips should be easily removed by sliding it off with a pair of forceps within 48 hours. Do not force the cover slip off. If the cover slip is not easily removed, continue to soak in 24-hour increments until it can be removed.
   3. **Soak in Xylene Action (Day 2-3)** 
      1. Log into Soft Molecular.
      2. Open Extractions by using the Extractions tile on the dashboard.
      3. Highlight the Soak in Xylene branch in the action tree.
      4. Highlight the Barcode# field. Scan the Soft Molecular specimen label, found on the Coplin jar, and press **Enter** on the keyboard. Repeat this step for all applicable specimens.
      5. Mark the **Completed** checkbox and select **Save**.
      6. Select **Back** in the Extractions window.
      7. Exit Soft Molecular application.
   4. **Cytology Slide Scrape Action (Day 2-3)**
      1. Log into Soft Molecular.
      2. Open Extractions by using the Extractions tile on the dashboard.
      3. Highlight the Cyto Slide Scrape branch in the action tree.
      4. Highlight the Barcode# field. Scan the Soft Molecular specimen label, found on the Coplin jar, and press **Enter** on the keyboard. Repeat this step for all applicable specimens.
      5. **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.**
      6. Select **Save**.
         1. **Note:** Two aliquot labels per sample will automatically print upon saving.
      7. Select **Back** in the Extractions window.
      8. Exit Soft Molecular application.
   5. **Begin TNA, Cytology Manual Extraction (Day 2-3)**
      1. Soak the de-coverslipped slide for an additional 5 minutes in xylene to remove any residual mounting medium. Then, air dry on a paper towel under the fume hood.
      2. Label a 1.5ml tube with the printed aliquot label and add 1ml of 100% ethanol to the tube.
      3. Move the slide to the extraction area. Using a transfer pipette, add a few drops of 100% ethanol to the slide to moisten it and use a scalpel to scrape the circled area.
      4. Dip the scalpel into the labeled tube with the pre-aliquoted ethanol to facilitate the removal of tissue from the scalpel.
      5. Centrifuge the specimen for 3 min at 14,000 RPM.
      6. Remove supernatant by aspiration with a plastic fine-tip transfer pipette.
         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.
      7. Air dry for 30 min. or place in heat block for 5 min.
      8. 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.
      9. Close cap and incubate at 55°C overnight or up to 72 hours.
   6. **Record your extraction reagents: Organic TNA Extraction Reagents (Day 2-3)**
      1. Log into Soft Molecular.
      2. Open Extractions by using the Extractions tile on the dashboard.
      3. Highlight the Organic TNA Extraction Reagents branch in 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.
   7. **Cytology Prep Organic Extraction Action (Day 4-5)**
      1. Log into Soft Molecular.
      2. Open Extractions by using the Extractions tile on the dashboard.
      3. Highlight the Cytology Prep Organic Extraction branch in 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. Mark the **Completed** checkbox.
      7. Navigate to the **Print** tab and click the **Print Product Labels** dropdown menu. Select the ‘**…**’ button that appears.
      8. Select **OK** when asked to save before print.
      9. 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**.
      10. Select **Back** in the Extractions window.
      11. Exit Soft Molecular application.
      12. Label one 1.5ml tube and one 2ml tube with the printed product labels.
   8. **Continue TNA, Cytology Manual Extraction (Day 4-5)**
      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 1.5ml microfuge tube labelled with a product label, 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 200ul 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 E.8. was 0.5ml of cell lysis solution, add 50ul of 5M ammonium acetate and then 1.5ml of 100% ethanol to the tube. Vortex briefly.
         1. Adjust according to the volume of lysis solution. For example, if 1ml of cell lysis solution was used in step E.8., 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 the 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 20ul TE or AE (from Qiagen) and vortex. If necessary, discuss with Director for alternate volumes, based on the cellularity of the original slide. 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. **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.
   11. **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.
   12. **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.
5. **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.
6. **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.
7. **REVISIONS:**
   1. 4/14/2020: Added instructions to change the volume on re-extracted specimens.
   2. 12/14/2020: Updated reagents and clarified steps that are used with 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.