

Department of Forensic Science

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**FORENSIC BIOLOGY
PROCEDURES MANUAL
OF
POWERPLEX® FUSION AMPLIFICATION
AND LONG TERM STORAGE**

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1 MANUAL PCR SET UP AND AMPLIFICATION

A dedicated area, such as a biological hood or a separate room, should be used for preparing PCR amplification reactions. All equipment and supplies used to prepare amplification reactions should be kept in this dedicated "clean" area at all times. These items will not be used to handle amplified DNA or other potential sources of contaminating DNA. Trace amounts of amplified DNA, if carried over into other samples before amplification, can lead to results that can be misinterpreted. Amplified DNA or equipment and supplies used to handle amplified DNA will NOT be brought into the designated "clean" area. Clean disposable laboratory gloves will be worn while preparing samples for PCR amplification and will be changed frequently or whenever there is a chance they have been contaminated with DNA. Touching the inside surface of the tube caps will be avoided.

DNA samples will be added to the amplification tubes or amplification plate AFTER the PowerPlex® Fusion master mix has been added to all tubes or all wells used in the plate. Each tube will be capped after sample DNA is added before proceeding to the next sample tube. Pipette tips will be changed after the addition of each DNA sample before proceeding to the next sample tube. The negative control should be processed and/or capped last, as this provides a control check for contamination during PCR set up.

1.1 Equipment

- Thermal Cycler - 9700
- Pipettes - 2 µL, 10 µL, 20 µL, 100 µL and/or 200 µL, 1000 µL
- 8- Channel Pipette – Range 0.5 µL to 10 µL
- Repeater pipette
- Biological Safety Hood
- Microcentrifuge tube racks

1.2 Materials

- Sterile ART pipette tips - 2 µL, 10 µL, 20 µL, 100 µL and/or 200 µL, 1000 µL
- Microcentrifuge tubes
- Kimwipes
- Gloves
- Sterile 0.2 mL tubes (amp tubes)
- 96 well amplification plates (TempPlate^(R) III P/N 1402-9700 or similar)
- Strip caps
- Adhesive sealing foil – VWR P/N 60941-112
- Compression pad (ABI P/N 4312639 or similar)

1.3 Reagents

- Sterile Type I Water
- PowerPlex® Fusion System amplification kit

1.4 PowerPlex® Fusion System Amplification Procedure for the 9700 Thermal Cycler

1.4.1 Amplification Parameters for the 9700 Thermal Cyclers:

- 1.4.1.1 Turn on the 9700 thermal cycler. If a program for PowerPlex® Fusion amplification has not already been stored in the thermal cycler, program as follows:

96⁰ C for 1 minute, then:

94⁰ C for 10 seconds

59⁰ C for 1 minute

72⁰ C for 30 seconds

For 28 cycles, then:

60⁰ C for 10 minutes

4⁰ C soak

The program must be run with Max mode as the ramp speed. The ramp speed is set after the thermal cycling run is started. The “Select Method Options” screen appears. Select “Max” for the ramp speed, and enter the reaction volume (13 µL).

This program may be saved as a “User File” for later use.

- 1.4.2 Transfer the PowerPlex® Fusion System amplification reagents to the designated "clean" area and allow the PowerPlex® Fusion 5X Master Mix, the PowerPlex® Fusion 5X Primer Pair Mix and the PowerPlex® Fusion Amplification Grade water (if using), to thaw completely at room temperature.

NOTE: The 2800M Control DNA (positive control) is stored at 2–10°C upon receipt.

- 1.4.3 Place the required number of labeled sterile amp tubes or 96 well amplification plate (including one label for entire plate indicating the plate name) in a rack.
- 1.4.4 Vortex the PowerPlex® Fusion 5X Master Mix and the PowerPlex® Fusion 5X Primer Pair Mix for approximately 15 seconds. Do not centrifuge after mixing.
- 1.4.5 Calculate the required volume of each PCR amplification component to prepare a master mix by multiplying the volume by the number of samples. This should include reagent blanks, positive and negative amplification controls and additional reaction volumes (depending on the number of samples being amplified) to compensate for any pipetting variation. The PCR master mix should be prepared in a clean microcentrifuge tube.

Half Reaction Master Mix:

- 2.5 µL PowerPlex® Fusion 5X Master Mix
- 2.5 µL PowerPlex® Fusion 5X Primer Pair Mix
- 2.5 µL PowerPlex® Fusion Amplification grade Water or Sterile Type I water

- 1.4.6 Add 7.5 µL of PCR master mix to each labeled sterile amp tube or well of the 96 well plate using sterile ART tips.

NOTE: Each PowerPlex® Fusion System amplification is performed in a final volume of 12.5 µL. Exactly 5 µL has been allocated for the sample DNA.

- 1.4.7 Prepare the sample DNA and controls as follows:

- 1.4.7.1 Open the tube containing the DNA extract or dilution of the DNA extract and carefully add 5 µL of sample DNA (0.5 ng/5 µL is, in general, the optimal target concentration, however the quantity of DNA amplified may need to be adjusted) to the appropriate labeled amp tube or well in 96-well amplification plate. The optimal target concentration range is between 0.125 ng and 0.5 ng/5 µL.
- 1.4.7.2 Positive Amplification Control: Gently mix by hand the Control DNA followed by a light tapping of the tube on the counter top to remove any liquid from the cap. Carefully add 5 µL of the Control DNA to the appropriate labeled amp tube or well in the 96 well amplification plate. The concentration of Control DNA amplified may need to be adjusted; however, unless otherwise documented, 0.5ng/5µL will be amplified.

NOTE: A stock dilution of control DNA included in the amplification kit may be prepared using sterile Type I Water or the Amplification Grade water also included in the kit.

- 1.4.7.3 Negative Amplification Control: Carefully add 5 μ L of the same diluent used to create any dilutions of associated DNA extract samples to the appropriate labeled amp tube or well in the 96 well amplification plate.
- 1.4.7.4 Reagent Blank(s): Carefully add 5 μ L of the reagent blank to the appropriate labeled amp tube or well in the 96 well amplification plate.
- 1.4.8 Place the capped amp tubes or the foil-sealed 96 well amplification plate into the thermal cycler. Push the tubes or plate down completely into the block. If a 96 well amplification plate is used, add a compression pad. Place the thermal cycler lid over the samples, and tighten.
- 1.4.9 Choose the appropriate amplification file and start the program.
- 1.4.10 When the thermal cycler prompts the user to select the total sample volume, choose 13 μ L.
NOTE: The PCR amplification program will run for approximately 1.5 hours.
- 1.4.11 After the amplification process is complete remove the tubes or 96 well amplification plate from the thermal cycler.
- 1.4.12 The amplified samples should be stored only in the post-amplification area of the laboratory and in the dark at -20 $^{\circ}$ C.

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2 NORMALIZATION AND PCR SET UP USING THE BIOMEK® NX^P AUTOMATION WORKSTATION

The Normalization and PCR Setup method is designed to import DNA quantitation data (ng/μL) and utilize that information to determine the method of DNA normalization. The method, created by Promega Corp. using the Beckman software and Visual Basic scripting, directs the Biomek® NX^P to perform a DNA normalization procedure that will accommodate each specific sample. It can perform a direct transfer of DNA samples to the STR master mix (undiluted), a small dilution in the pipette tip itself, a dilution in a single plate, or a serial dilution using two plates. To further increase the one-plate and two-plate dilution factors, the robot can pair either one or two plate dilutions with an in-tip dilution. When the entire process is complete, a set of PCR reactions will be prepared, with the DNA input quantity normalized to the target unless it fails to meet the target concentration, in which case the robot will add the maximum volume (5 μL) to the STR amplification reaction.

2.1 Equipment

- Biomek® NX^P Automation Workstation
- Biomek® Span-8 Tool
- Thermal cycler
- Biological Safety Hood
- Pipettes - 2 μL, 10 μL, 20 μL, 100 μL and/or 200 μL, 1000 μL

2.2 Materials

- Black PCR support base (96 well) – ABI Cat# N801-0531
- MicroAmp® tubes (attached caps) – USA Scientific # 1402-8100 (clear) or #1402-8108 (multi- colored)
- 96 well qPCR Plate – Greiner P/N 652260
- Adhesive sealing foil – VWR P/N 60941-112
- Modular reservoir half module 150 mL - Beckman Catalog #372786
- 96 well normalization plate - Innovative Microplate Catalog # S30026
- P250 Tips–aerosol barrier - Beckman Catalog # 379503
- P50 tips – aerosol resistant - Beckman Catalog # A21586
- P20 tips – aerosol resistant - Beckman Catalog # 379506
- Microcentrifuge tubes, 1.5 mL with unattached or attached lids
- Thermal cycler plate
- Sterile ART pipette tips - 2 μL, 10 μL, 20 μL, 100 μL and/or 200 μL, 1000 μL

2.3 Reagents

- Sterile Type I Water
- PowerPlex® Fusion System amplification kit

2.4 Preparation for and Using the Normalization Wizard

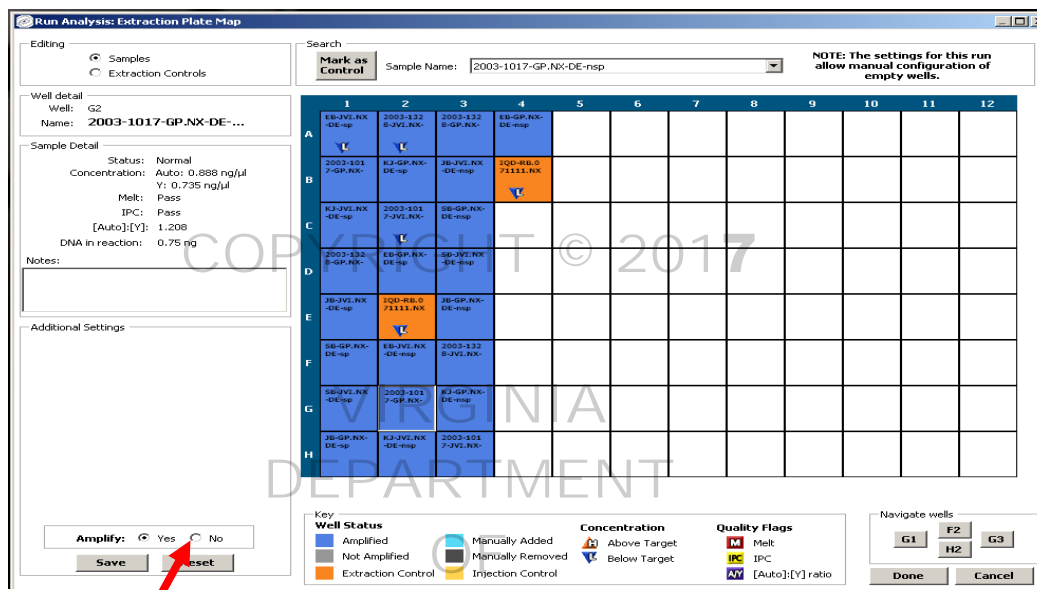
NOTE: The tool and the Biomek® NX^P robot deck must be wiped off with a solution that will remove/degrade the DNA prior to running the Normalization Wizard method.

- 2.4.1 Open the project folder containing the Normalization method. If the Biomek® NX^P software was closed, follow the steps outlined in the Forensic Biology Procedures Manual, Extraction of DNA for homing all axes prior to initiating a method.
- 2.4.2 Select the Normalization method and click on the green arrow to initiate the method. Prompts will pop up asking the user to enter the first tip that can be used for the P250 tips. Enter the value and click OK. Another prompt will pop up asking the user to enter the first tip that can be used for the P50 tips. Enter the value and click OK.

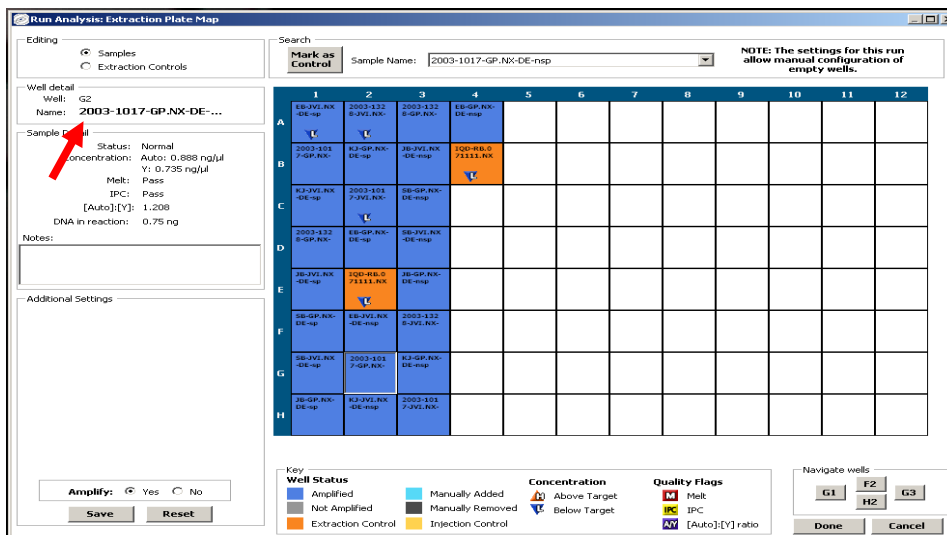
2 Normalization and PCR Setup using the Biomek® NX^P Automation Workstation

- 2.4.3 A new prompt will pop up asking the user to enter a value for manual master mix preparation. The default is False. The “False” value means that the method will create the STR master mix on the deck. If a True value is entered, this indicates to the method that the STR master mix will be created off-deck and a single tube placed into the rack for dispensing into the amplification tubes.
- 2.4.4 The next prompt to pop up asks the user to enter a value for amplifying in tubes or a plate. The default is 1 (tubes) and, if selected, the samples and master mix will be dispensed into MicroAmp® tubes in black PCR support bases at positions P9 and P12 on the deck. If 2 is selected, the samples and master mix will be dispensed into a qPCR plate for amplification which should be placed in a black PCR support base in position P9 on the deck.
- 2.4.5 The next window to pop open will be for entry of plate specific information into the STR Normalization Manager Interface.
- 2.4.6 Enter the appropriate user name and password. When the next window pops up, enter the run or analysis name, which will typically be the date and the operator’s initials. Select the Fusion amplification kit template from the drop-down window for analysis template. Click Next.
- 2.4.7 Two windows will pop up asking the user to first select an extraction control template and then select an injection control template. Be sure VA DFS Extraction Controls template is selected in the extraction control template window. The injection controls template may contain multiple choices. The template that has “fixed controls” in its name will automatically place a single positive and a single negative control into the plate map in column 12. If additional positive or negative controls are desired, then choose the appropriate injection control template. Click next after selecting the control template.
- 2.4.8 The next window to pop up will instruct the user to import the tab delineated file created from the Plexor® HY data for the run. Use the browse function to locate the file and click Next.
- 2.4.9 The next window to open up will have a summary of the number of samples included in that run. Click on the Review Extraction Plates in the upper right in order to view detailed information about the plate of samples.

2.4.10 The Plexor® HY quantitation data can be reviewed for each well individually by clicking on the well. The detailed information shows up on the left-hand side of the screen as shown in the figure below. It is at this step that manually added samples are included (such as those samples that were manually extracted and quantitated). See 2.4.11 for additional instruction on manually adding samples to a plate. Samples can also be excluded from normalization and STR setup at this point by highlighting the sample and selecting “No” where it says Amplify (red arrow in Figure below). Click Save. If no samples need to be added to the plate, click Done and go to 2.4.12. Otherwise go to step 2.4.11 for instruction on adding samples to the run.



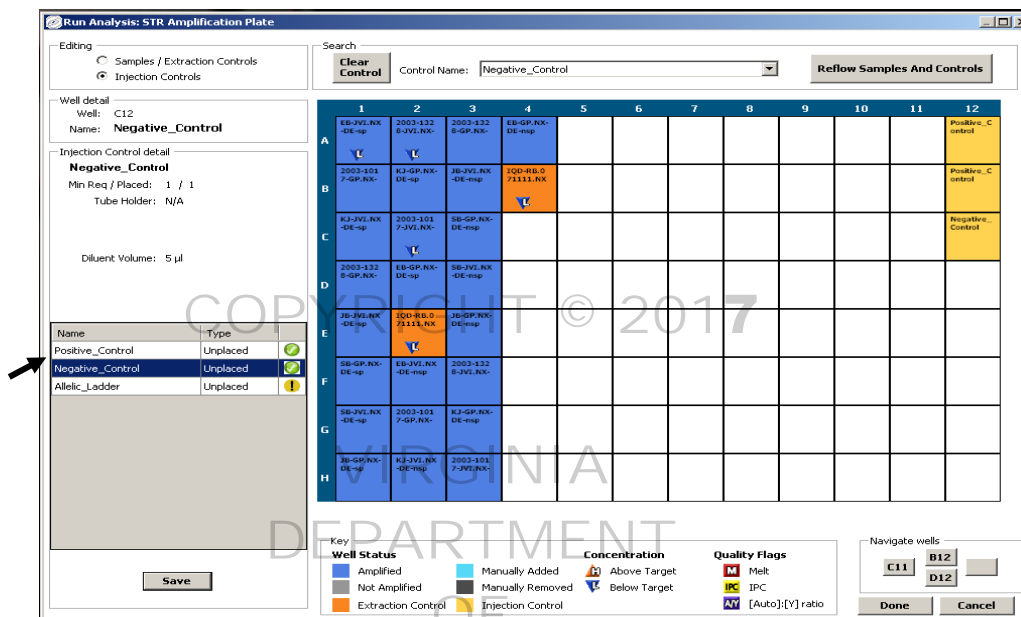
2.4.11 Click on the well where the sample has been manually added. The well detail box (red arrow) will now open a line for entering in the sample name (see the left side of Figure below). The sample detail box will now allow for concentration information also to be entered. The dilution factor will be automatically calculated unless it is specified by the user. Once the information has been entered for a sample, click on save and move onto the next well for manual addition. Once all of the information for the manual samples has been entered and saved, click Done.



2.4.12 The next window that will pop up will concern the amplification and injection controls. Only the positive and negative controls will be utilized for this step. The positive, negative and allelic ladder (if applicable)

controls require a minimum of one. To place the positive, negative and allelic ladder controls click on the Review Amplification Plate button.

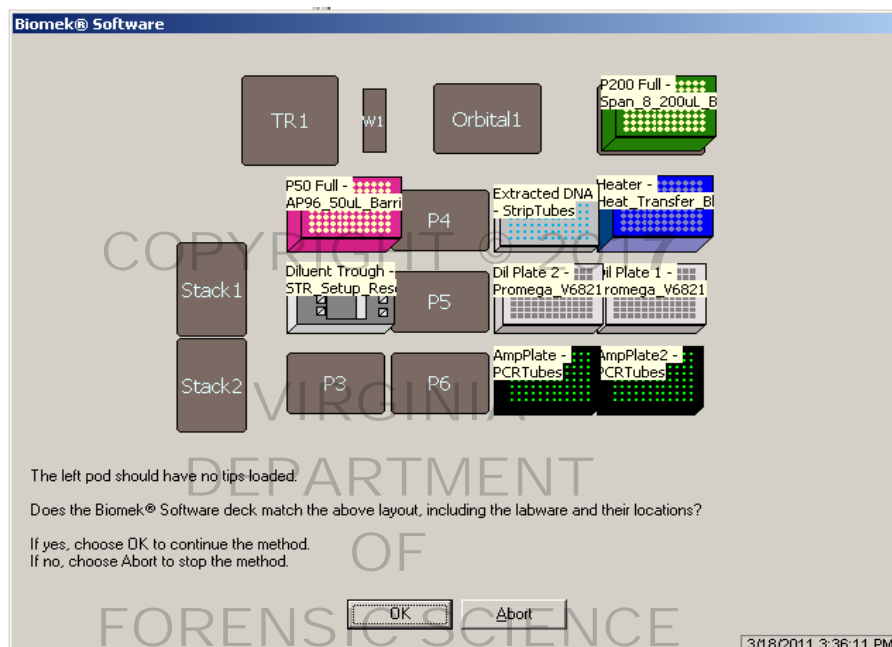
- 2.4.13 If the control template was chosen that did not have fixed controls, to add the positive, negative and allelic ladder controls (if applicable), highlight the control in the box in the figure below (black arrow) and click on the well where the control is to be placed and then click Save. Click Done when finished.



- 2.4.14 If the fixed controls were chosen, the plate setup will be shown. Click Done when finished reviewing the plate.
- 2.4.15 The next window will require entry of the login and password again to confirm the credentials. Simply enter the user name and password used at the beginning of the Normalization Manager Information entry process and then click on Finish.
- 2.4.16 The final window of the Normalization Manager will indicate that the output files have been generated and that the window will close in a number of seconds. The user can either click on the Close Now button or let it close automatically. The method will now begin using the information entered into the Normalization Manager for the method.
- 2.4.17 After the Normalization Manager closes, there will be a prompt instructing the user to place labware on the deck and the volume of diluent needed. It will indicate whether, for that particular plate of samples (with its range of DNA concentrations), one or two dilution plates will be needed. Click OK once the plates and diluent are placed on the deck in the appropriate positions. The volume indicated is the minimum volume of diluent required, so pipetting a larger volume is acceptable.
- 2.4.18 The next prompt will indicate where the boxes of tips need to be placed and if one or two boxes of P50 tips will be needed. Click OK once the tips are placed on the deck.
- 2.4.19 The next prompt will indicate in what positions in the left side of the tube holder the reagent tubes belong. Once the reagent tubes and empty tube are loaded into the rack, click OK. Ensure that each of the reagent tubes contains at least the volume indicated in the prompt, which will vary depending on the number of samples for that specific plate of samples. If manually preparing the master mix this prompt will indicate in what position the tube of master mix belongs. Position 1 is the rearmost position and Position 4 is the most forward position.

2 Normalization and PCR Setup using the Biomek® NX^P Automation Workstation

- 2.4.20 The next prompt will indicate in which position on the right side of the tube holder rack the positive control belongs. If not already prepared, prepare a dilution of the positive control so that the concentration is 0.5 ng/5 μ L unless it is necessary to utilize a different concentration. If that is necessary, then the positive control template in the Normalization Manager may need to be modified or a new one created.
- 2.4.21 The final prompt, shown in the figure below, is the deck setup and the placement of labware and tips. The deck setup needs to be confirmed prior to clicking OK to continue the method.



- 2.4.22 The method will take approximately 30 minutes for a large plate of samples.
- 2.4.23 If the normalization and PCR setup method designed to pipette into a PCR plate was selected, cover the plate with a foil seal. If the normalization PCR setup method designed to pipette into tubes was selected, cap the tubes.
- 2.4.24 Place the capped amp tubes or the foil-sealed 96 well amplification plate into the thermal cycler. Push the tubes or plate down completely into the block. If a 96 well amplification plate is used, add a compression pad. Place the thermal cycler lid over the samples, and tighten.
- 2.4.25 Choose the appropriate amplification file and start the program.
- 2.4.26 When the thermal cycler prompts the user to select the total sample volume, choose 13uL.
- NOTE:** The PCR amplification program will run for approximately 1.5 hours.
- 2.4.27 After the amplification process is complete remove the tubes or 96 well amplification plate from the thermal cycler.
- 2.4.28 The amplified samples should be stored only in the post-amplification area of the laboratory and in the dark at -20 °C.
- 2.4.29 For the case file records, print out Section 4, Sample Dilution Tracking, of the automatically stored version of that particular analysis (stored as a pdf file C:\Documents and Settings\All Users\Application Data\Promega Corporation\STR Normalization Manager\Analysis Run Performed). The printout shall include the amplification controls.

3 TRANSFER OF EXTRACTED DNA FOR PERMANENT STORAGE

This is a transfer method designed to transfer DNA extracts from the PCR strip tubes into pre-labeled 1.5 mL tubes. Since sample order is maintained throughout the process (loading into 96 deep square well plate for isolation/purification, the Plexor® HY quantitation and the PCR setup), that order and separation between one examiner's set of samples and another's is maintained for the transfer method. Each Beckman 24 Microfuge tube holder holds 24-1.5 mL tubes, so in order to accommodate a plate of 80 DNA extracts, four Beckman 24 Microfuge tube holders are placed onto the deck and the pattern of sample loading maintained.

3.1 Equipment

Biomek® NX^P Automation Workstation

3.2 Materials

- Microcentrifuge tubes, 1.5 mL
- Beckman 24 Microfuge tube holders- Beckman Catalog # 373661
- 1.5 mL eppendorf tubes
- Beckman white 1.5 mL tube inserts- Beckman Catalog # 373656
- Black PCR support base (96 well) – ABI Cat# N801-0531
- P250 Tips–aerosol barrier - Beckman Catalog # 379503
- Tough tag labels for 1.5 mL tubes

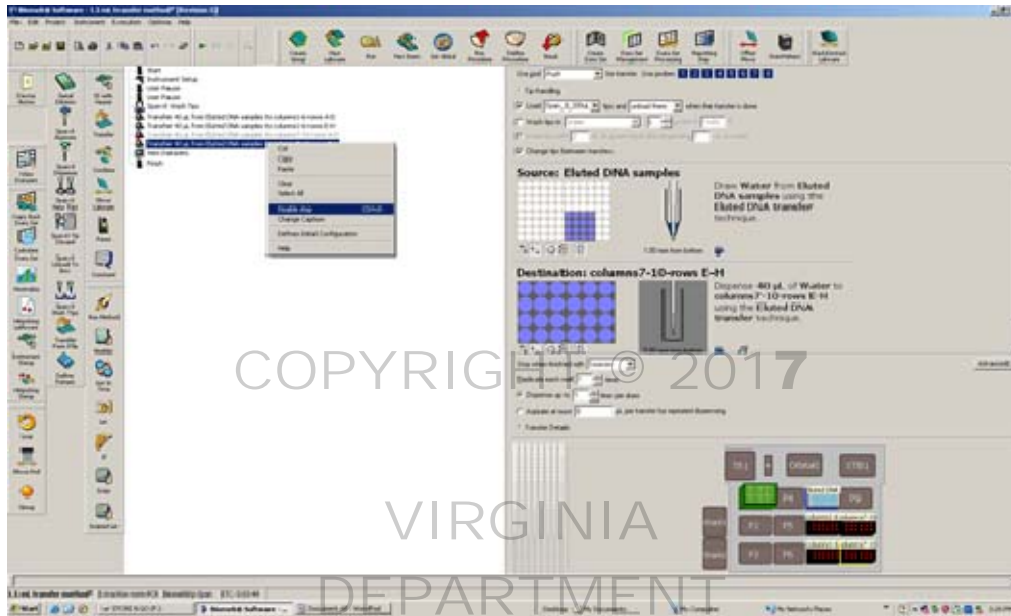
3.3 Starting the Biomek® NX^P Workstation

- 3.3.1 If the power is not already on for the robot, then turn on the power switch on the back, right-hand side of the robot and follow the procedure outlined in the Forensic Biology Procedures Manual, Extraction of DNA for homing all axes prior to initiating a method.

NOTE: The 1.5 mL tubes need to be pre-labeled prior to placing into the 24 well microfuge racks using the same pattern as the samples were loaded into the 96 deep square well plate for isolation/purification.

- 3.3.2 Once the Biomek® NX^P has been homed, using either the File drop-down window or the folder icon, open the Open Method window.
- 3.3.3 Select the 1.5 mL transfer method. With the method open, click on the green arrow to start the method. The method will begin and a prompt window will open showing the deck layout for the method. Place the labware in the designated positions and click OK.
- 3.3.4 Follow the directions listed in the next pop up window. 3.3.5 will provide direction on disabling the lines of the method, if necessary. If it is necessary to disable lines of method, then the method must be aborted, the lines disabled and the method re-started.

- 3.3.5 If six columns or fewer of DNA samples will be transferred, then disable the last two transfer steps, which transfer samples in columns 7-10, rows A-D and columns 7-10, rows E-H. As shown in the Figure below, highlight the line in the method and right click. Select Disable. The line will appear grayed out and with a red X once it has been disabled.



- 3.3.6 The method will now run until completion. Once it has finished and the 1.5 mL method is being closed, the user will be asked if he/she would like to save the changes if any of the transfer steps were disabled. Click “No” so that the disabled steps will not be disabled the next time the method is opened.

4 PROCEDURES FOR DRYING EXTRACTS FOR PERMANENT STORAGE AND RESOLUBILIZATION

4.1 Equipment

- DNA concentrator/evaporator
- Microcentrifuge tubes
- Pipettes - 2 μL , 10 μL , 20 μL , 100 μL and/or 200 μL , 1000 μL

4.2 Materials

- Plastic zip closure bags or similar storage medium
- Sterile ART pipette tips - 2 μL , 10 μL , 20 μL , 100 μL and/or 200 μL , 1000 μL
- Microcentrifuge tubes and lids
- Kimwipes

4.3 Reagents

Type I water

4.4 Drying Extracts for Permanent Storage

Evidence samples and known reference samples will be dried down at a separate time and/or in a separate place. If an evaporator is used, either evidence or known references may be placed in the evaporator at one time, but not both. Two evaporators may be used simultaneously – one for evidence and one for knowns, if desired.

4.4.1 Using an evaporator to dry the extracts

- 4.4.1.1 Pulse spin the microcentrifuge tubes containing the extracts to force the liquid extract to the bottom of the tubes.
- 4.4.1.2 Open the tubes.
- 4.4.1.3 Place the tubes into the evaporator and turn on at room temperature.
- 4.4.1.4 Once the extracts are dry, close the cap or place a new cap on each tube and place into a plastic zip closure bag or similar storage medium.

4.4.2 Air drying the extracts

- 4.4.2.1 Pulse spin the microcentrifuge tubes containing the extracts to force the liquid extract to the bottom of the tubes.
- 4.4.2.2 Open the tubes.
- 4.4.2.3 Place the tubes in an appropriate location such as a drying cabinet or hood to allow the samples to air dry. The rack containing the tubes may be covered with a Kimwipe, if desired.
- 4.4.2.4 Once the extracts are dry, close the cap or place a new cap on each tube and place into a plastic zip closure bag or similar storage medium.

4.5 Resolubilizing DNA Extracts

- 4.5.1 Add a volume of Type I water to the microcentrifuge tube(s) that is consistent with the sample volume prior to drying.

4 Procedures for Drying Extracts for Permanent Storage and Resolubilization

- 4.5.1.1 If the sample is being reconstituted for further testing and the optimal amplification target will not be met by reconstituting in this amount, a smaller volume may be used as long as the associated reagent blank is treated in the same manner.
- 4.5.2 Place the tube(s) in a 56°C incubator or heat block for a minimum of 2 hours, but no more than 3 hours.
- 4.5.3 If no associated reagent blank is available, a reconstitution blank must be created with the same amount of Type I water as used for the sample(s).

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