Department of Forensic Science

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FORENSIC BIOLOGY PROCEDURES MANUAL

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PLEXOR® HY QUANTITATION OF DNA

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1 PLEXOR® HY QUANTITATION OF DNA USING THE BIOMEK™ NX^P AUTOMATION WORKSTATION

Small differences in the well formation or depth of the well can cause the tips to "bottom out". When the tips are in contact with the bottom of the well, a vacuum can form and affect the accuracy of pipetting. Therefore, any substitution of plates or consumables must meet the specifications of the specific products listed below.

Gloves must be worn at all times when performing the Plexor® HY System reactions because the introduction of nucleases that could occur from un-gloved handling can interfere with the reaction.

The standard curve used for the Plexor® HY System ranges from 0.0016 ng/µL to 25.0 ng/µL. The concentration of the extracted DNA sample is extrapolated by plotting as a log of the standard curve.

1.1 Equipment

- Stratagene Mx3005P™ Quantitative PCR instrument
- Biomek® NX^P Automation Workstation

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1.2 Materials

NOTE: If any specific catalog or part number listed below is not available for purchase, an ***equivalent*** replacement must be obtained.

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- 96-well qPCR Plate – VWR Cat#82050-698
- Optically Clear Strip Caps – Phenix Research P/N 101100-082 or Greiner P/N 373250
- Optional: Optically clear film – VWR Cat#82050-994
- Micro Amp strip tubes (in strips of 8 tubes)– ABI Cat# N801-0580
- Black PCR support base (96 well) – ABI Cat# N801-0531
- P20 Tips – aerosol resistant – Beckman Cat# 379506
- P50 Tips – aerosol resistant – Beckman Cat# A21586
- Quarter module reservoir – Beckman Cat# 372788

1.3 Reagents

- Plexor® HY System kit (Promega Cat# DC1000 = 800 determinations or Promega Cat# DC1001 = 200 determinations) (stored at -20ºC) which contains:
  - Plexor® HY 2X Master Mix
  - Plexor® HY 20X Primer/IPC Mix
  - Nuclease free water

  NOTE: Type I water is considered nuclease free and may be used instead of the amplification grade water that comes with the Plexor® HY kit.

- 5X AmpSolution™ Reagent (Component of the Casework Direct Kit, Custom – Promega Cat# AX4560, Individual Part # DM123A)

- Plexor® HY Genomic DNA Standard (50 ng/µL) – stored at 4ºC after initial thawing.

  NOTE: The genomic standard must be stored at 4ºC after thawing. Freezing and thawing can affect accuracy of the standard.

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1.4 Providing Samples for Quantitation That Were Isolated/Purified Independently of the Samples in the Current Extraction/Quantitation/Amplification Batch of Samples

1.4.1 If a sample is provided for quantitation, normalization wizard amplification set up and the 1.5 mL tube transfer, the entire extract will be provided to the robot operator in a strip tube. The sample should be placed in the tube in the strip that corresponds to the appropriate well of the 96 deep square well plate.

1.4.2 If a sample is provided for quantitation and the 1.5 mL tube transfer only, the entire extract will be provided to the robot operator in a strip tube. The sample should be placed in the tube in the strip that corresponds to the appropriate well of the 96 deep square well plate.

1.4.3 If a sample is provided for quantitation only:

1.4.3.1 The entire extract or a large enough portion of the extract to allow the robot to pipet 2 µL accurately (typically 10 µL) may be provided to the operator in a strip tube. The sample should be placed in the tube in the strip that corresponds to the appropriate well of the 96 deep square well plate.

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OR

1.4.3.2 2 µL of the extract may be loaded directly into the appropriate well of the Plexor® HY plate once the robot operator has completed the plate set up in 1.6.19, prior to covering the plate with optically clear strip caps or film.

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1.4.4 These manually added samples will be added to the populatable worksheets, if not already done so, to ensure that sufficient qPCR master mix is created and pipetted to the appropriate sample wells.

1.5 Providing Samples That Were Extracted Using Casework Direct (CD) for Screening Purposes for Quantitation

1.5.1 The entire CD extract will be provided in a strip tube.

1.5.2 The sample will be placed in the tube in the strip that corresponds to the appropriate well of the Quantitation of Casework Direct Extracts worksheet.

1.6 Starting the Stratagene Mx3005P™ Quantitative PCR instrument

1.6.1 Turn on the Stratagene Mx3005P™ Quantitative PCR instrument by using the power button located on the back left side of the unit.

1.6.2 Turn on the computer.

1.6.3 Open the Mx3005P™ software "MxPro" and select "SYBR Green (with Dissociation Curve)" from the New Experiment Options window. In the same window, check the box "Turn lamp on for warm-up?" Select "OK". The lamp requires ~20 minutes to warm up before running the assay. The software can be configured and the plate set up on the Biomek® NX^P Automation Workstation while the lamp is warming up. If the New Experiment window does not open automatically, it can be found under File→New→Open "SYBR Green (with Dissociation Curve)" Experiment.

1.6.4 The software will ask "Do you wish to use SYBR Green Plate Setup from the active set "plexor"?" Click "yes" to load the Plexor® HY assay default plate setup.

1.6.5 The software will ask "Do you wish to use SYBR Green Thermal Profile Setup from the active set "plexor"?" Click "yes" to load the Plexor® HY assay default thermal profile.

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1.7 Biomek® NX^P Automation Workstation Operating Procedure

1.7.1 Remove the Plexor® HY System reagents from the -20ºC freezer and allow them to thaw prior to use. All reagents should be thawed and well mixed prior to use.

NOTE: Reagents used for the Plexor® HY System come prepared for use. It is critical for optimal performance that the reagents thaw completely and are well mixed prior to use. Unused reagents may be re-frozen at -20ºC, except the genomic DNA standard, which **MUST** be stored at 4ºC after the first use.

1.7.2 Turn on the computer, if not already on.

1.7.3 If the Biomek® NX^P Automation Workstation is not on, push in the power button and, once fully booted up, click on the desktop shortcut for the Biomek NX software.

NOTE: The "Home All Axes" command will need to be run if the Biomek NX Software was closed after a previous method. Refer to Chapter 4 in the Forensic Biology Procedures Manual, Extraction of DNA for the procedure.

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1.7.4 Prior to starting the Plexor® method, ensure that the sample information is entered into the first worksheet ("Extraction") of the associated populatable worksheets and click on the NX button.

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1.7.5 A text file will be created which is a worklist, called Run Sheet, to be imported into the Plexor® setup method. This file should be created at another workstation and saved and transported to the Biomek® NX^P robot computer. The Run Sheet file then needs to be saved to the desktop of the Biomek® NX^P robot computer. When prompted that another file named Run Sheet is there and would you like to replace it with the current Run Sheet file, click Yes.

1.7.6 To prepare the Plexor Set Up Method, in the NX software click on File→ Open→ look in "Plexor" project folder→ open "Plexor Set Up" method.

1.7.7 In the method, click Finish (red arrow in figure below 1.6.8). Clicking on the Finish line of the method causes the method to be auto-checked and will also import the Run Sheet from the desktop.

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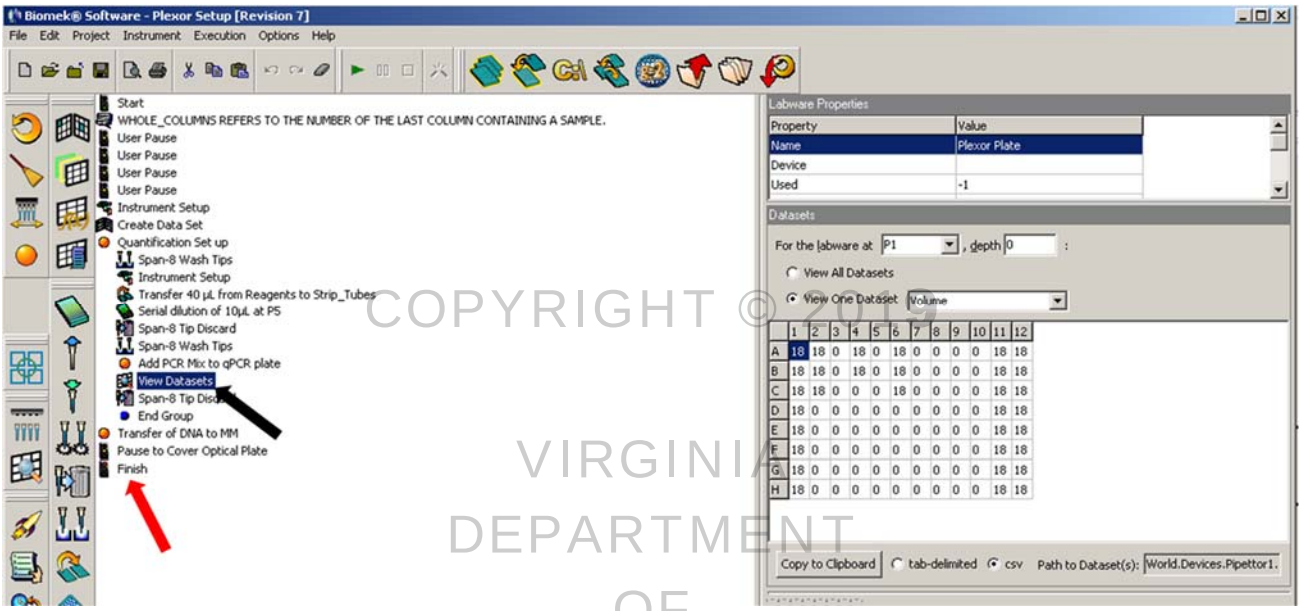
1.7.8 To ensure that the proper Run Sheet will be imported into the Plexor® setup method, double click "Quantification Set Up" of the Plexor setup method, and then click on the "View Datasets" line (black arrow in figure below). To view the appropriate dataset, on the right side of the screen click on the Plexor Plate to highlight (if it has not been already) and select "View One Dataset". If not already designated in the drop-down window, select "Volume". Check to ensure that the Run Sheet is populated with the appropriate samples in the correct pattern.



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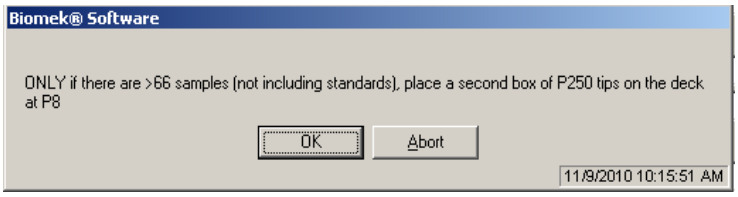
Checking the Plexor setup method to ensure that the plate specific Run Sheet has been imported. The red arrow points to the line of method that states "Finish". The black arrow points to the View Datasets line. The Dataset is shown on the right part of the screen.

1.7.9 Click on the Start line of the method and then the green arrow to initiate the method.

1.7.10 The first pop up window prompts the user to enter a value for "Whole Columns". This refers to the last column containing DNA samples and does not refer to the standards. For example, if you extracted 45 samples that were loaded throughout columns 1-8, then the value for Whole Columns would be 8. Once you enter this value, click "OK". A prompt will pop up immediately afterwards explaining what Whole Columns means. Click "OK".

NOTE: If samples need to be manually added, then the column(s) that will contain the manually pipetted samples in strip tubes must be included in the Whole Columns number unless the column(s) of samples can be inserted into an empty column before the last column of samples on the associated extraction run. The manually added sample names should be typed into the populatable worksheets to ensure that sufficient qPCR master mix is created and pipetted to the appropriate sample wells.

1.7.11 The next prompt will be as shown below. Follow the direction and click OK.



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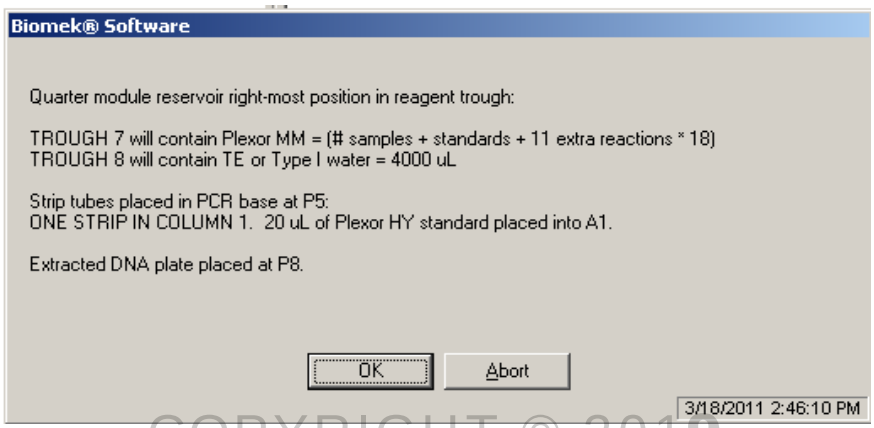
1.7.12 The following prompt will pop up directing the user as to where to place the Plexor® HY System master mix and the Type I water, and the Plexor® HY standard.



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1.7.13 Prepare the Plexor® HY System master mix in a 1.5 or 2.0 mL tube as described below:

Reagent	Casework Runs (*Not* Casework Direct Extract Runs)	Casework Direct (CD) Screening Extract Runs
Plexor® 2X Master Mix	10 µL per reaction	10 µL per reaction
Nuclease-free (or Type I) H_2O	7 µL per reaction	3 µL per reaction
20X Primer/IPC Mix	1 µL per reaction	1 µL per reaction
5X AmpSolution™ Reagent	N/A	4 µL per reaction

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NOTE: The 5X AmpSolution™ Reagent may be turbid after thawing or storage at 2-10°C. If turbidity occurs, warm the reagent briefly at 37°C and vortex until clear.

Prepare sufficient reaction mix for the desired number of reactions, making certain that the two columns of standards (16) are included as well as 11 excess reactions. An appropriate number of additional reactions (typically 2) may be added to this number, if desired. Vortex well. For small runs (fewer than 16 samples), use 16 for the number of samples (for a total of 45 reactions) to ensure the master mix covers the bottom of the reservoir.

1.7.14 Place a quarter module reservoir divided by length on the right hand side of a reservoir frame using the rightmost slot. Place the Plexor® HY master mix in the trough compartment to the left (#7). Make certain to dispense the master mix so that it is evenly distributed along the bottom of the reservoir. Remove any bubbles generated. Place the appropriate volume (~4 mL) or greater of Type I water or TE-4 into the trough on the right side (#8).

1.7.15 Continuing to follow the directions in the figure in 1.6.12, remove the Plexor® HY standard from the 4ºC refrigerator and vortex on high briefly before dispensing 30 µL of the standard into the first tube of a strip of amplification tubes. It must be dispensed into an end tube with no bubbles in the bottom of the tube and the strip placed into a black PCR support base in the first column with the tube containing the standard in the A (top) row. Once this is completed, click OK.

1.7.16 A prompt will then pop up asking if you verified that the Run Sheet has been properly imported into the method. Click OK if the Run Sheet was verified. If not, click Abort and begin the method again at 1.6.7.

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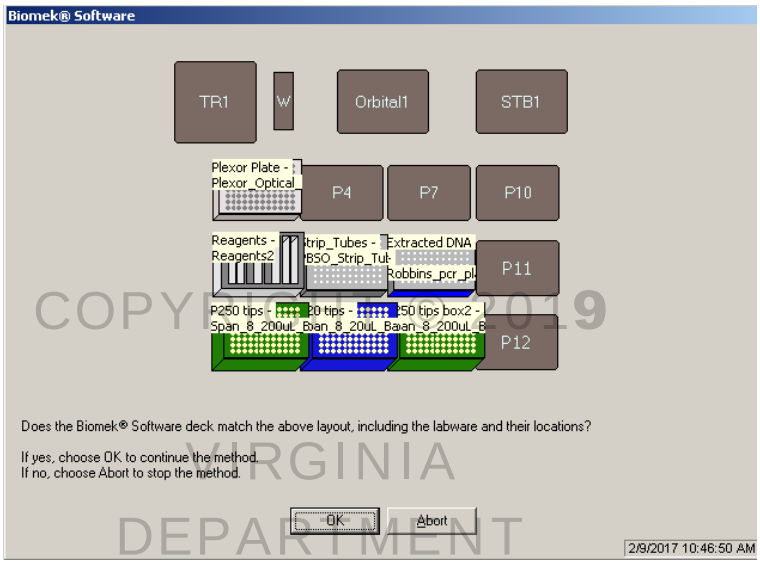
1.7.17 Prompt showing the deck layout will appear as shown below. At this time place all required items in their designated positions. Place a qPCR plate in a black PCR support base at P1, the reagent trough with the reservoirs on the right hand side at P2, a clean box of P250 tips at P3, the strip tube with Plexor® HY standard in well 1A at P5, a clean box of P20 tips at P6, and the extracted DNA samples at P8. A second box of P250 tips at P9 are only needed if >64 extracted samples are prepared. Click "OK" to run the method.



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1.7.18 Once the method completes, cover the strip tubes containing the isolated DNA with a piece of parafilm or something similar in weight to prevent evaporation of the samples.

NOTE: These samples will be uncovered and used during the Normalization and PCR Set Up method.

1.7.19 Remove the qPCR plate at deck position P1. Carefully seal the plate with optically clear strip caps or film. If using film, try to center the film over the plate and press down carefully all around the plate to ensure that the seal is tight. Avoid touching the wells of the plate and film.

1.7.20 Immediately take the plate to the qPCR instrument room and spin briefly in a table top centrifuge to drive any bubbles in the bottoms of the wells to the top.

NOTE: If the run cannot be started immediately, the plate should be stored at room temperature away from light (such as in a drawer), to prevent potential deleterious effects on the reactions. The plate should not be stored (prior to running) for longer than two hours. Short wait times (less than two hours, at room temperature) are permissible, as the reactions utilize a hot-start polymerase. The reaction plate should be stored out of the light to prevent potential photo-bleaching that could negatively affect the sensitivity of the assay.

1.8 Stratagene Mx3005P™ Operating Procedure

1.8.1 Place the 96 well qPCR plate in the Stratagene Mx3005P™ thermal cycler. Note that the plate holder in the thermal cycler opens like a waffle maker. The plate should be under the black top.

1.8.2 Program the Stratagene Mx3005P™ for thermal cycling.

1.8.2.1 Select the Plate Setup tab.

1.8.2.2 Select the wells that will NOT be used and choose "blank" as the well type.

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1.8.2.3 Select the Thermal Profile Setup tab. The default thermal cycling conditions should be programmed for the Plexor® HY assay as shown below:

Step	Temperature	Time	Number of Cycles
Initial denaturation:	95°C	2 minutes	1 cycle
Denaturation:	95°C	5 seconds	38 cycles
Annealing and extension:	60°C	40 seconds	
Melt temperature curve:	65°C initial temperature with 0.6°C increase each cycle, 40-second hold each cycle		48 cycles

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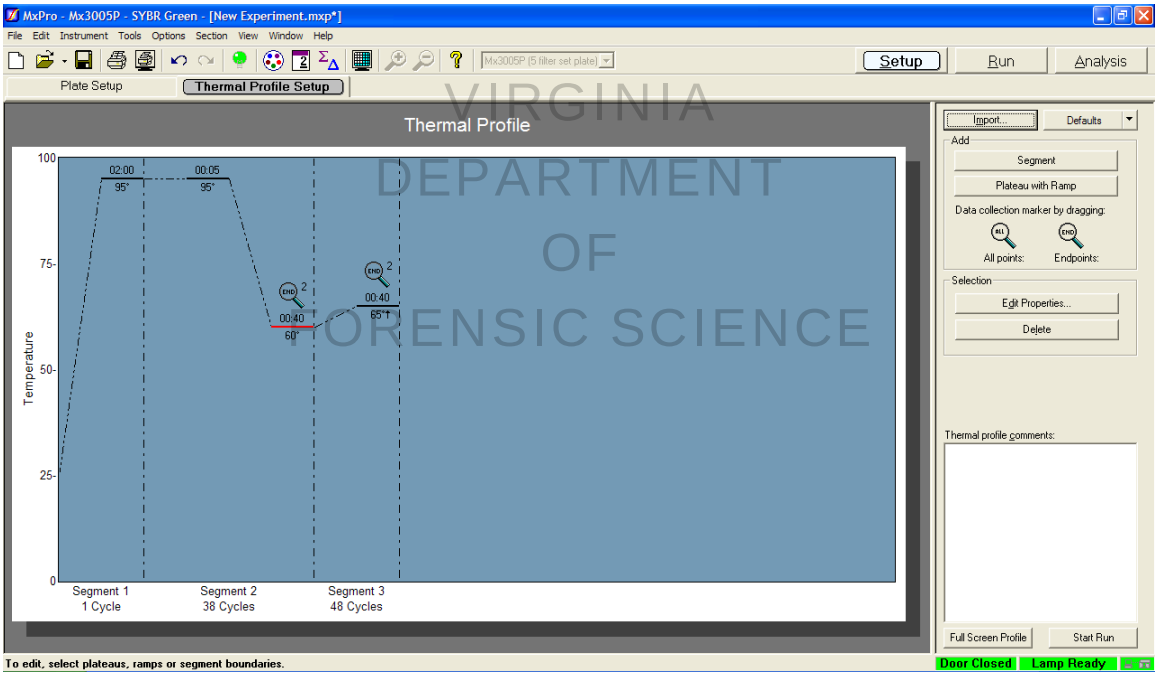
Figure obtained from the Plexor® HY System Technical Manual



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1.8.3 If optically clear caps were used to seal the wells in the plate, use the default filter gain settings.

NOTE: The default filter gain settings are x1 for each dye except x2 for CO560 and x4 for FAM.

1.8.4 If optically clear film was used to seal the plate, select Instrument→ Filter Set Gain Settings. In the dialog box that opens, the filter settings should be changed to x1 for each dye except FAM, which should be x2.

1.8.5 The run can now be initiated by selecting “Start Run”. The run should be launched immediately after the qPCR plate is placed into the instrument. Select “Turn lamp OFF at end of run”. The computer will prompt the user to designate a file name in which the data will be saved. This is typically the date followed by the operator’s initials.

1.8.6 Once the run is over, remove the qPCR plate containing the Plexor® reactions from the thermal cycler and discard into the biohazard waste.

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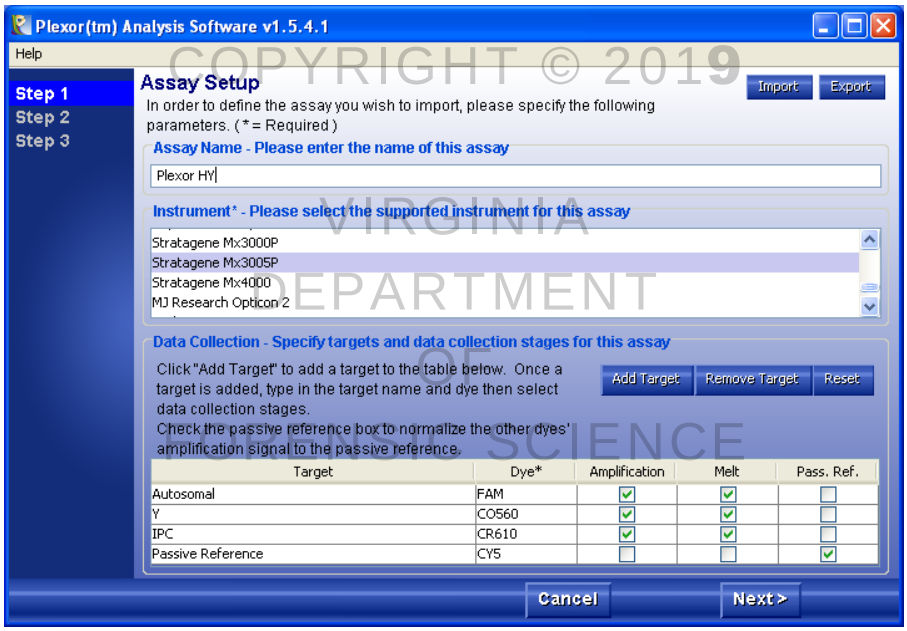
1.9 Data Analysis

1.9.1 To analyze the data, prior to import into the Plexor® software, in the Stratagene data collection program, click “File”and “Export Instrument Data”. The export function provides a drop-down window. From that window select, “Export Instrument Data to text file” and “Format 3 – Grouped by wells”. A dialog box will appear asking for a file name to save the file. Choose the same name as was used for the Stratagene file, which is typically the date followed by the operator’s initials. This will not overwrite the Stratagene data collection file (raw data) as this will be a *.txt file.

1.9.2 Open the Plexor® Analysis Desktop program. Choose “File”, “Import New Run”. Type “Plexor HY” in the Assay Name box. The parameters should be as shown below. Then click “Next>”.

NOTE: If the setup does not look like that shown below, click “Import” in the upper right hand corner of this box and browse to the assay setup template called “plexor.atp”.



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NOTE: The order of the dyes shown in the “Data Collection” box is not important, although the boxes selected for each dye are important.

1.9.3 At the “Run Info” dialog box, fill in the Experiment Title with the robot run name. Also fill in the operator name. The date should be set correctly, if the computer’s date is incorrect. Notebook ID and Reagent ID may be left blank. The Notes field may be used to document which Stratagene instrument is used, if multiple instruments are available.

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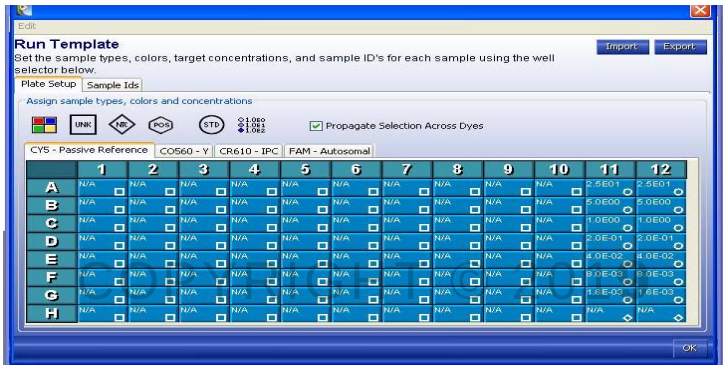
1.9.4 At the File Import screen, click "Browse" and find the *.txt file that was generated in which the raw data was saved. Click "Run Template". In the "Run Template" dialog box shown below, click on the "Plate Setup" tab and choose "Import" and select "Plexor_runtemplate5.rtp". Double click on template or select "open". Click "OK" in the bottom right hand corner of the dialog box.

NOTE: The standard curves and no template controls (columns 11 and 12) are indicated by the circles and diamonds, respectively.



1.9.5 The Plexor® Analysis settings can be imported from the file "Plexor_analysistemplate.ntp". At the File Import screen, under "Advanced Options", click "Analysis Template". In the "Analysis Defaults" dialog box, choose "Import" and select "Plexor_analysistemplate.ntp". Double click on template or select "open". Click "OK" in the bottom right hand corner of the dialog box.

1.9.6 The defaults are used for all settings, as shown below, except the expected melt temperature (T_m). The approximate T_m values should be: A = 81.5ºC, Y = 82.7ºC, and IPC = 82ºC. These melt temperatures are imported when you select the Plexor® analysis template. Click "OK". The T_m value can be adjusted manually by dragging the vertical bar to the left or right to center the T_m (see red arrow in figure in 1.8.9).

1.9.7 Click "Finish" to see the analyzed data.

1.9.8 The sample IDs can now be imported from the RunSampleNames.txt file. Open the Populatable Worksheet and select the "Sample Names" Tab. Copy the sample names from the "sample names" grid (the bottom-most grid) by selecting the appropriate wells and pressing <Ctrl-C>. Do not copy column or

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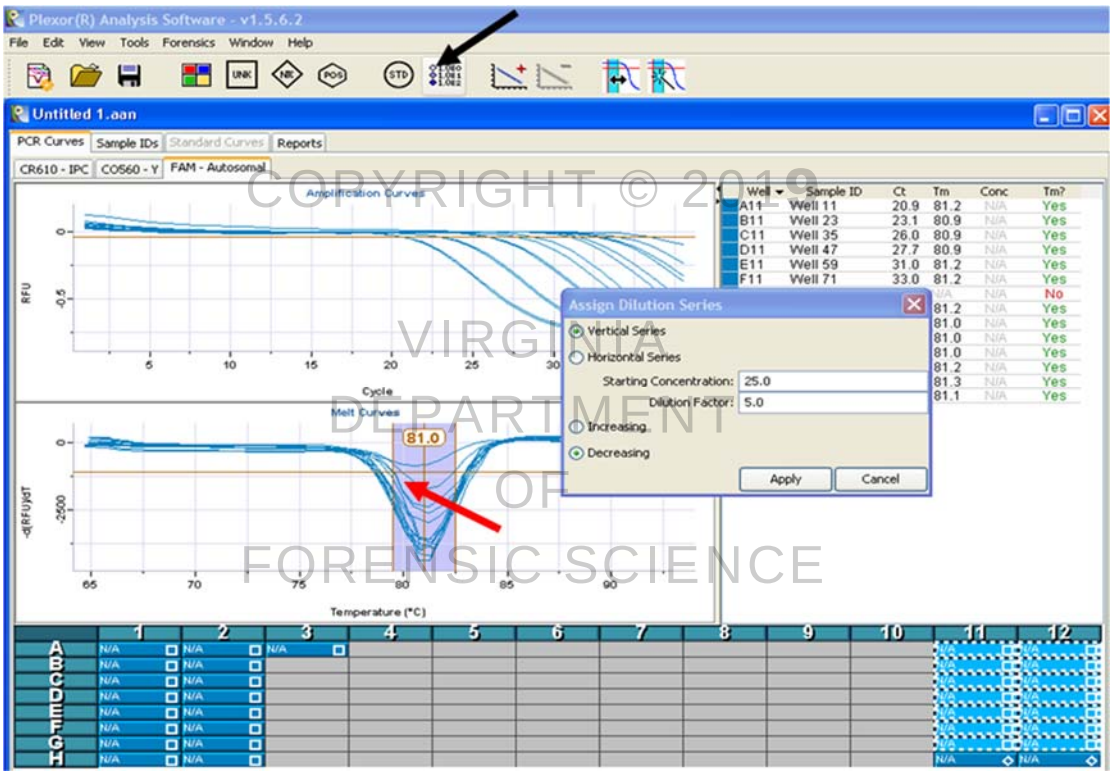
row headers (i.e., A, B, C...or 1, 2, 3...). In the Plexor Analysis software, in the Sample IDs tab, click on Well 1 and paste the sample names from the populatable worksheet into the Sample IDs Template by pressing <Ctrl-T>.

1.9.9 The current version of the software may not import the standards properly from the template, so they must be defined. Define the standard curves by highlighting the wells of the two columns of standards under the PCR Curves tab, but not the No Template Control wells (see figure below). Click on the standard series definition button, indicated by the black arrow in the figure below and the inset window shown will appear. Be certain that the most concentrated standard is defined as 25 ng/µL and that the series is a vertical series, decreasing by a factor of five. Click "Apply".



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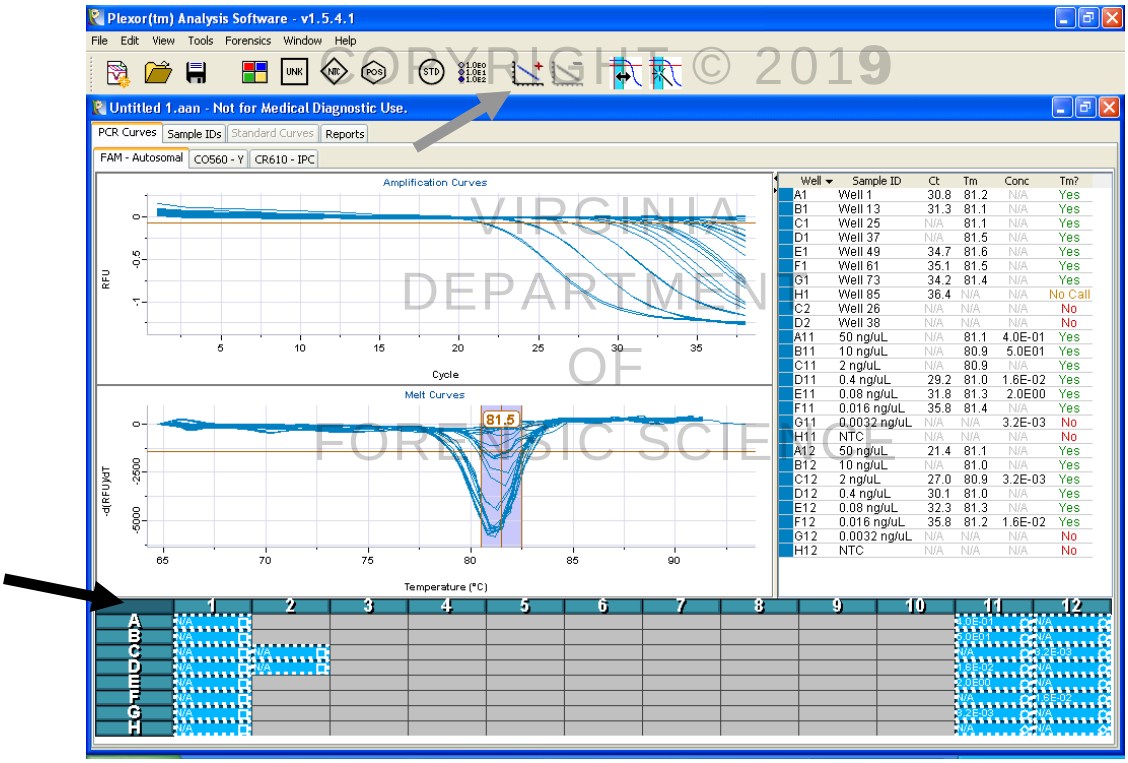
1.9.10 In the "PCR curves" tab, click on the box in the upper left hand corner of the plate map (indicated by the black arrow in the figure below) to select the entire plate of samples. This will highlight the entire plate. Be sure you are in the FAM tab, for the autosomal quantitation data. Choose "Edit", "Add Standard Curve" or click on the corresponding icon in the toolbar, as indicated with a gray arrow in the figure below. This creates a standard curve for the dye selected and quantitates the unknowns for that dye as well.

1.9.11 The software will prompt the user that the generation of a new standard curve will replace the existing standard curve. Click OK.

1.9.12 Click on the tab for CO560 – Y and repeat to create a standard curve for the Y amplicon. Again, click OK.

NOTE: No standard curve is necessary for the IPC.



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1.9.13 Evaluate the standard curves by selecting the Standard Curves tab. Assess the curves for the autosomal and Y DNA quantitation separately by selecting each of the tabs separately (FAM – Autosomal and CO560 – Y). The linearity (r^2) and efficiency (eff) of both curves should be assessed. Each standard curve should have an r^2 value of 0.98 or above to be acceptable. Evaluate the curve generated to determine if any outlier data points need to be de-selected to improve the quality of the extrapolated curve generated. Up to 3 data points can be de-selected per column of standard for a total of 6 data points, when necessary, to produce a linear standard curve.

NOTE: This includes data points in the standard curves (A11-G11 and A12-G12) that are not detected. The software does not include any undetected data points when constructing the standard curve. The de-selection of data points from either standard curve must be indicated on the report. See Appendix A for a detailed description of Plexor® troubleshooting methods, including how to de-select data points from a standard curve. The efficiency is usually 100% ± 15%.

NOTE: The baseline regions are computed and selected automatically. Should the baseline of any specific amplification plot be improperly estimated, it can be adjusted manually. Each well can

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be highlighted individually to inspect the amplification plot and adjust the baseline. Refer to Appendix A for direction on when baseline adjustment is needed and how to perform it.

1.9.14 Generation of the Forensics Report and Sample Details Report

A copy of either both the Forensics Report (prepared in 1.8.14.1) and the Sample Details Report (prepared in 1.8.14.2) **OR** just the quantitation report (Form 210-F504, prepared in 1.8.15 and 1.8.16) will be printed for inclusion in the batch paperwork.

1.9.14.1 Generation of the Forensics Report

1.9.14.1.1 To generate a report ("Forensics Report") of the quantitation data, click "Forensics" and "Set Normalization and IPC parameters". A check mark should appear near the top of the dialog box so that the report is limited to the concentration data and C_T only. The default parameters are correct and should read that the autosomal dye is FAM and the Y-STR dye is CO560. The IPC dye is CR610 and the default to flag an improper (inhibited) amplification for the IPC is 2 C_Ts.

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1.9.14.1.2 The forensics report should contain the following columns: Sample Name, Location, Sample Type, [Auto], [Y], and [Auto]/[Y]. To change the columns shown, right-click on the table (not on the column headings) and choose "Change columns shown" from the drop-down menu. The columns shown are arranged by dye in the subsequent window. To rearrange the order of the columns shown on the Sample Details report, click and drag the column header horizontally to the desired location.

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1.9.14.1.3 To print this report, select the entire table and click on the printer icon in the upper right hand corner of the window. Printing from the "File" drop down menu will only print a screenshot. If the Forensics Report does not indicate the r^2 value for the two standard curves, this should be added by hand and the notation initialed.

1.9.14.1.4 The Forensics report indicates the sample concentrations (in ng/ µL) as well as the ratio of autosomal to Y DNA in a sample. This ratio of autosomal to Y DNA may be utilized by an examiner, along with other pertinent case information, to assist in sample routing.

NOTE: A result of "N/A" in the concentration column indicates that no DNA (either autosomal, Y, or both, depending on the location of the N/A result) was detected. "N/A" may be reported for a sample's ratio of autosomal/male DNA concentration if no Y DNA is detected in the sample.

1.9.14.2 Generation of the Sample Details Report.

1.9.14.2.1 In the Plexor® Analysis software, navigate to the Sample Details tab. Make sure that only the correct columns are showing and in the correct order (Location, Sample ID, CR610 Ct, CO560 Conc, CO560 NS Call, FAM Conc, FAM NS Call). To change the columns shown, right-click on the table (not on the column headings) and choose "Change columns shown" from the drop-down menu. The columns shown are arranged by dye in the subsequent window. To rearrange the order of the columns shown on the Sample Details report, click and drag the column header horizontally to the desired location.

1.9.14.2.2 Sort the samples into column order (if desired) by clicking on the top of the "Location" column.

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1.9.14.2.3 Highlight all of the samples and standards. Click on the printer icon in the upper right corner to print the report.

1.9.15 Generation of the Quantitation Report (Form 210-F504)

1.9.15.1 In the Plexor® Analysis software, navigate to the Reports tab →Sample Details tab. Make sure that only the correct columns are showing and in the correct order (Location, Sample ID, CR610 Ct, CO560 Conc, CO560 NS Call, FAM Conc, FAM NS Call). To change the columns shown, right-click on the table (not on the column headings) and choose "Change columns shown" from the drop-down menu. The columns shown are arranged by dye in the subsequent window. To rearrange the order of the columns shown on the Sample Details report, click and drag the column header horizontally to the desired location.

1.9.15.2 Sort the samples into column order (if desired) by clicking on the top of the "Location" column.

1.9.15.3 Click on the icon in the upper right corner that is called the "Export Selected" icon as indicated below:

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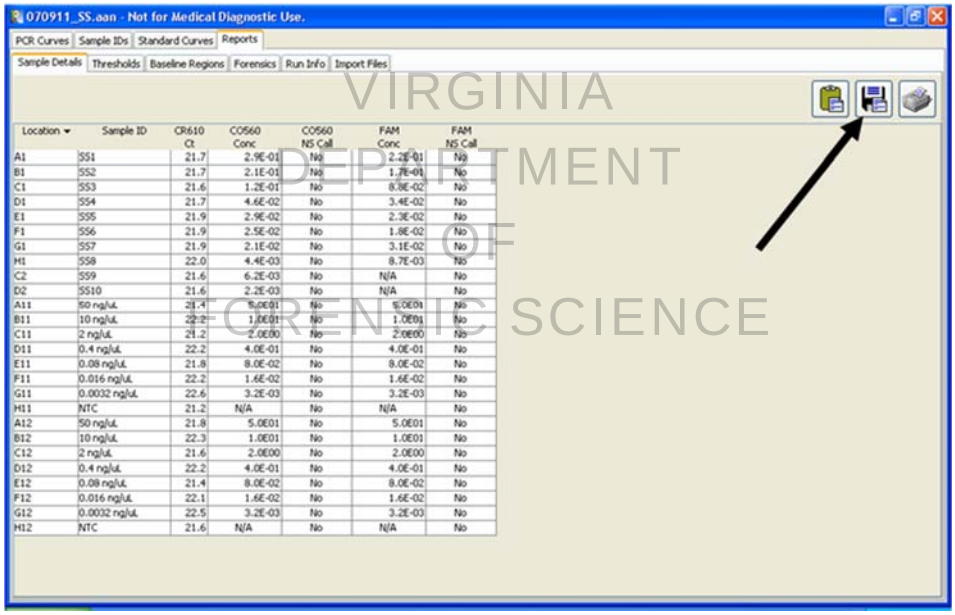

Location	Sample ID	CR610 Ct	CO560 Conc	CO560 NS Call	FAM Conc	FAM NS Call
A1	SS1	21.7	2.9E-01	No	2.2E-01	No
B1	SS2	21.7	2.1E-01	No	1.7E-01	No
C1	SS3	21.6	1.2E-01	No	8.8E-02	No
D1	SS4	21.7	4.6E-02	No	3.4E-02	No
E1	SS5	21.9	2.9E-02	No	2.3E-02	No
F1	SS6	21.9	2.5E-02	No	1.8E-02	No
G1	SS7	21.9	2.1E-02	No	3.1E-02	No
H1	SS8	22.0	4.4E-03	No	8.7E-03	No
C2	SS9	21.6	6.2E-03	No	N/A	No
D2	SS10	21.6	2.2E-03	No	N/A	No
A11	50 ng/uL	21.4	5.0E01	No	5.0E01	No
B11	10 ng/uL	22.2	1.0E01	No	1.0E01	No
C11	2 ng/uL	21.2	2.0E00	No	2.0E00	No
D11	0.4 ng/uL	22.2	4.0E-01	No	4.0E-01	No
E11	0.08 ng/uL	21.8	8.0E-02	No	8.0E-02	No
F11	0.016 ng/uL	22.2	1.6E-02	No	1.6E-02	No
G11	0.0032 ng/uL	22.6	3.2E-03	No	3.2E-03	No
H11	NTC	21.2	N/A	No	N/A	No
A12	50 ng/uL	21.8	5.0E01	No	5.0E01	No
B12	10 ng/uL	22.3	1.0E01	No	1.0E01	No
C12	2 ng/uL	21.6	2.0E00	No	2.0E00	No
D12	0.4 ng/uL	22.2	4.0E-01	No	4.0E-01	No
E12	0.08 ng/uL	21.4	8.0E-02	No	8.0E-02	No
F12	0.016 ng/uL	22.1	1.6E-02	No	1.6E-02	No
G12	0.0032 ng/uL	22.5	3.2E-03	No	3.2E-03	No
H12	NTC	21.6	N/A	No	N/A	No

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1.9.15.4 Save the .tab file when prompted.

1.9.16 Printing the Quantitation Report (Form 210-F504)

1.9.16.1 Open Microsoft Excel. Choose File→ Open and browse to the file saved in a designated folder to import the tab-delimited data into Excel.

NOTE: It may be necessary to change the "Files of Type:" to "All Files" for the exported data file to be observed.

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1.9.16.2 At the prompt, choose "Finish" as shown below.

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1.9.16.3 On the quant spreadsheet, click <CTRL-p> to generate the Plexor® HY report for the casefile.

1.9.16.4 If the report does not indicate the r^2 value for the two standard curves, this should be typed into the Excel file or added by hand to the printed report.

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1.9.16.5 The report may be printed via File→ Print. The print settings may be altered using File→ Page Setup, if desired.

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1.9.17 The "CR610, Ct" column is a flag for whether inhibition of a sample may be observed via the IPC, as determined by whether the cycle at which the IPC crossed the threshold was within the expected result. The IPC should cross the threshold at a similar cycle number in each sample with a degree of tolerance between samples. If a given sample's IPC C_T is not within 2 cycles of those observed in the standard curve, this may be an indication of inhibition by the sample/sample matrix. Routinely, these samples are amplified for autosomal STRs regardless of whether inhibition is indicated. However, given this information, the examiner may choose to amplify a dilution of the sample to overcome the potential inhibition. In addition, if a sample is believed to contain PCR inhibitors, a Microcon® filter unit may be utilized to improve typing results.

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1.9.18 The "NS Call" column is an indication of whether non-specific amplification is potentially observed in the melt curve for the sample. Any melt observed outside of the expected melt window (generally 79-81 ± 1.5°C for autosomal and 81-83 ± 1.5°C for Y) and of sufficient magnitude to cross the threshold will result in a "YES" in the "NS Call" column. Routinely, these samples are amplified for autosomal STRs regardless of whether non-specific amplification is indicated. If a "YES" is observed in several samples (greater than 5 that have concentrations of greater than 50 pg/µL), this could be an indication of poor reagents or thermal cycling conditions. If this occurs, QC measures should be taken to ensure the instrument and reagents are functioning properly.

1.9.19 Saving the Plexor® Analysis File

Under File, select Save Analysis File and give the Plexor® Analysis file the same filename as used for the qPCR setup and *.txt export. This will not overwrite the previous files, as it has a *.aan extension. This file should be saved to a designated folder in the computer for future reference or use. If the Normalization Wizard will be used, the quantitation data can be exported to a file (as described in 1.9.20) that can be imported into the Normalization Wizard.

1.9.20 Export of quantitation data to a file for import into the Normalization Wizard

1.9.20.1 Under the "Forensics" drop-down window, select "IPC and Normalization Wizard Parameters" and make certain that the correct dyes are selected for each of the targets (e.g., FAM for autosomal) and then click OK. In the Plexor® Analysis Software, choose

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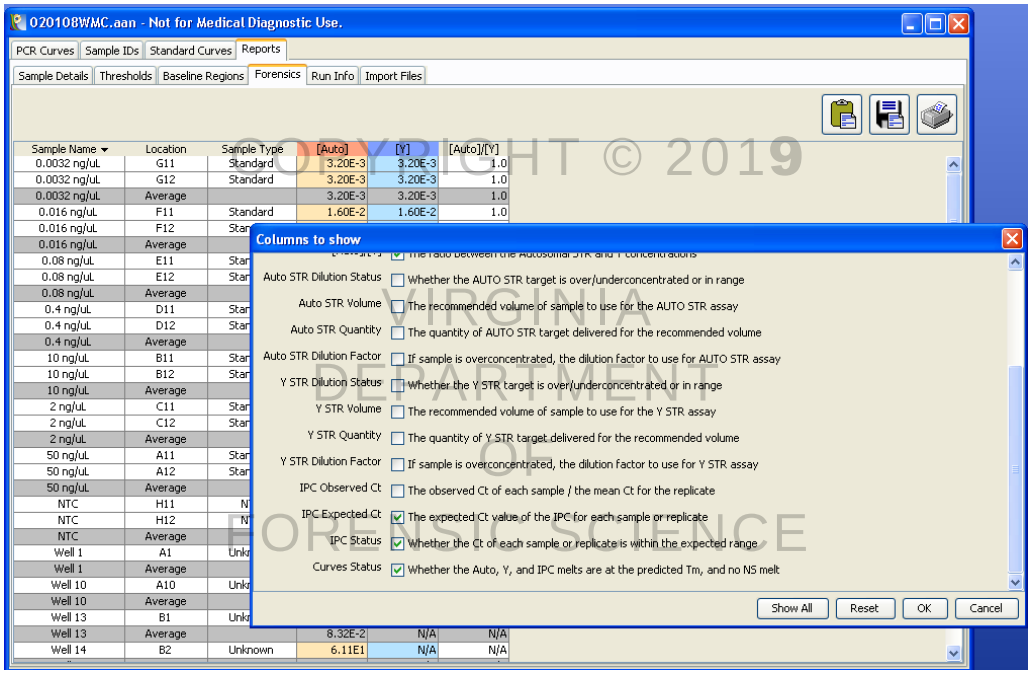
"Forensics" and "Export Concentrations in 96 Well Plate Format" and "Export FAM – Autosomal Concentrations". (If amplification setup based on the Y chromosome quantitation is desired, choose "Export CO560 – Y Chromosome Concentrations"). A dialog box will appear asking for a file name. Save this file with an appropriate file name on the computer, the network or if the Plexor® computer is directly linked to the Biomek® NX^P computer, to the appropriate designated folder on the C-drive of the Biomek® NX^P robot.

1.9.20.2 Select the Forensics tab. With that window open, right click and select the "change columns shown" option as shown below. The Sample Name, Location, Sample Type, Auto, Y and Auto/Y will already be checked. Scroll down and also check (if not already checked) the IPC Expect Ct, IPC Status, and Curves Status and then click OK.



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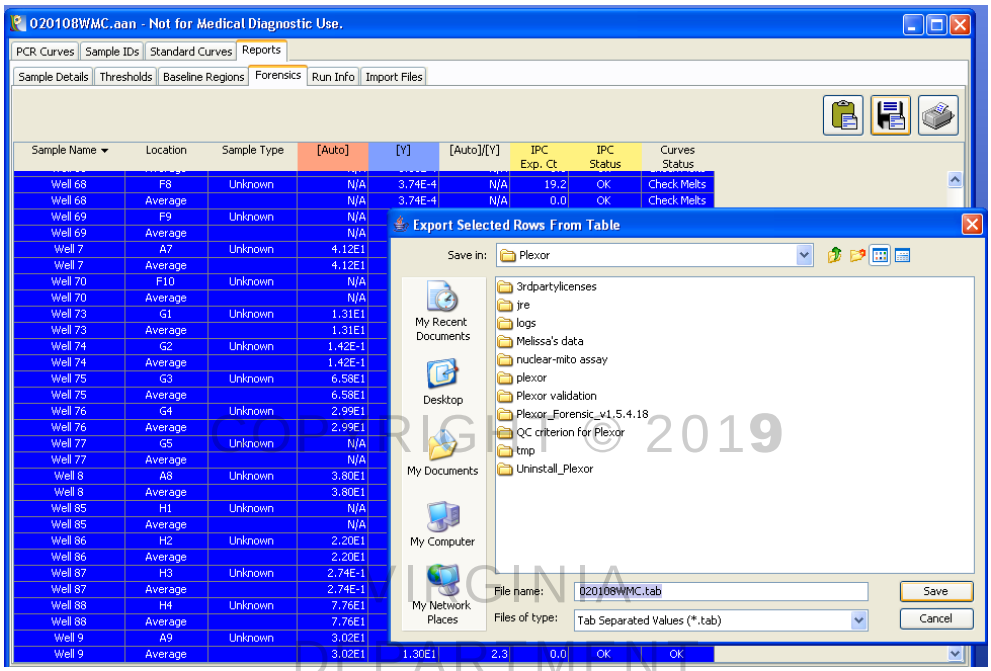
1.9.20.3 Select all of the rows and click on the "export selected icon", shown highlighted in the upper right corner of the figure below:



1.9.20.4 Save the quantitation data as a tab file in the desired location, such as the desktop or to a flash drive. This data file will need to be physically located on the Biomek® NX^P workstation computer in order to for the normalization and PCR setup method to import it.

NOTE: There may be apparent discrepancies between the quantitation reports and the normalization wizard. These apparent discrepancies will be minor (usually 0.01 ng/µL or less) and originate from the rounding and truncating that each software program performs.

1.9.21 While still in the Stratagene data collection software, verify that the lamp has been turned off by looking at the indicator in the bottom right hand corner of the screen. Close out the Plexor® Analysis Software and Stratagene data collection software. If prompted to save the changes, click "YES". The instrument can then be turned off.

1.9.22 If the samples quantitated are Casework Direct screening extracts, once the associated data are obtained, the remaining extract volume may be discarded.

1.9.23 If the samples quantitated are casework samples (not Casework Direct screening extracts) and are not moving forward to the Normalization Wizard PCR Setup, they may be transferred to 1.5mL tubes for long term storage. Refer to the Forensic Biology Procedures Manual, DNA Amplification and 1.5mL tube transfer for long term storage.

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2 MANUAL PLEXOR® HY QUANTITATION OF DNA

Gloves must be worn at all times when performing the Plexor® HY System reactions because the introduction of nucleases that could occur from un-gloved handling can interfere with the reaction.

The standard curve used for the Plexor® HY System ranges from 0.0016 ng/µL to 25.0 ng/µL. The concentration of the extracted DNA sample is extrapolated by plotting as a log of the standard curve.

A maximum of 80 samples can be quantitated at one time in a 96-well qPCR plate, as two columns must be reserved for the standards.

2.1 Equipment

- Stratagene Mx3005P™ Quantitative PCR instrument
- Biomek® NX^P Automation Workstation

2.2 Materials

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NOTE: If any specific catalog or part number listed below is not available for purchase, an ***equivalent*** replacement must be obtained.

- 96-well qPCR Plate - Greiner P/N 652260
- Optically Clear Strip Caps – Phenix Research P/N 101100-082 or Greiner P/N 373250
- Optional: Optically clear film – VWR Cat#82050-994
- Micro Amp strip tubes (in strips of 8 tubes)– ABI Cat# N801-0580
- Black PCR support base (96 well) – ABI Cat# N801-0531
- P20 Tips – aerosol resistant – Beckman Cat# 379506
- P50 Tips – aerosol resistant – Beckman Cat# A21586
- Quarter module reservoir – Beckman Cat# 372788

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2.3 Reagents

- Plexor® HY System kit (Promega Cat# DC1000 = 800 determinations or Promega Cat# DC1001 = 200 determinations) (stored at -20ºC) which contains:
  - Plexor® HY 2X Master Mix
  - Plexor® HY 20X Primer/IPC Mix
  - Nuclease free or Type I water

  Type I water is considered nuclease free and may be used instead of the amplification grade water that comes with the Plexor® HY kit.

- 5X AmpSolution™ Reagent (Component of the Casework Direct Kit, Custom – Promega Cat# AX4560, Individual Part # DM123A)

- Plexor® HY Genomic DNA Standard (50 ng/µL) – stored at 4ºC after initial thawing.

  NOTE: The genomic standard must be stored at 4ºC after thawing. Freezing and thawing can affect accuracy of the standard.

2.4 Starting the Stratagene Mx3005P™ Quantitative PCR Instrument

2.4.1 Turn on the Stratagene Mx3005P™ Quantitative PCR instrument by using the power button located on the back left side of the unit.

2.4.2 Turn on the computer.

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2.4.3 Open the Mx3005P™ software "MxPro" and select "SYBR Green (with Dissociation Curve)" from the New Experiment Options window. In the same window, check the box "Turn lamp on for warm-up?" Select "OK". The lamp requires 20 minutes to warm up before running the assay. The software can be configured and the plate set up on the Biomek® NX^P Automation Workstation while the lamp is warming up.

2.4.4 The software will ask "Do you wish to use SYBR Green Plate Setup from the active set "plexor"?" Click "yes" to load the Plexor® HY assay default plate setup.

2.4.5 The software will ask "Do you wish to use SYBR Green Thermal Profile Setup from the active set "plexor"?" Click "yes" to load the Plexor® HY assay default thermal profile.

2.5 Programming the Stratagene Mx3005P™ for the Thermal Cycling

2.5.1 Select the Plate Setup tab.

2.5.2 Select the wells that will NOT be used and choose "blank" as the well type.

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2.5.3 Select the Thermal Profile Setup tab. The default thermal cycling conditions should be programmed for the Plexor® HY assay as shown below.

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Step	Temperature	Time	Number of Cycles
Initial denaturation:	95°C	2 minutes	1 cycle
Denaturation:	95°C	5 seconds	38 cycles
Annealing and extension:	60°C	40 seconds	
Melt temperature curve:	65°C initial temperature with 0.6°C increase each cycle, 40-second hold each cycle		48 cycles

(Figure obtained from the Plexor® HY System Technical Manual.)

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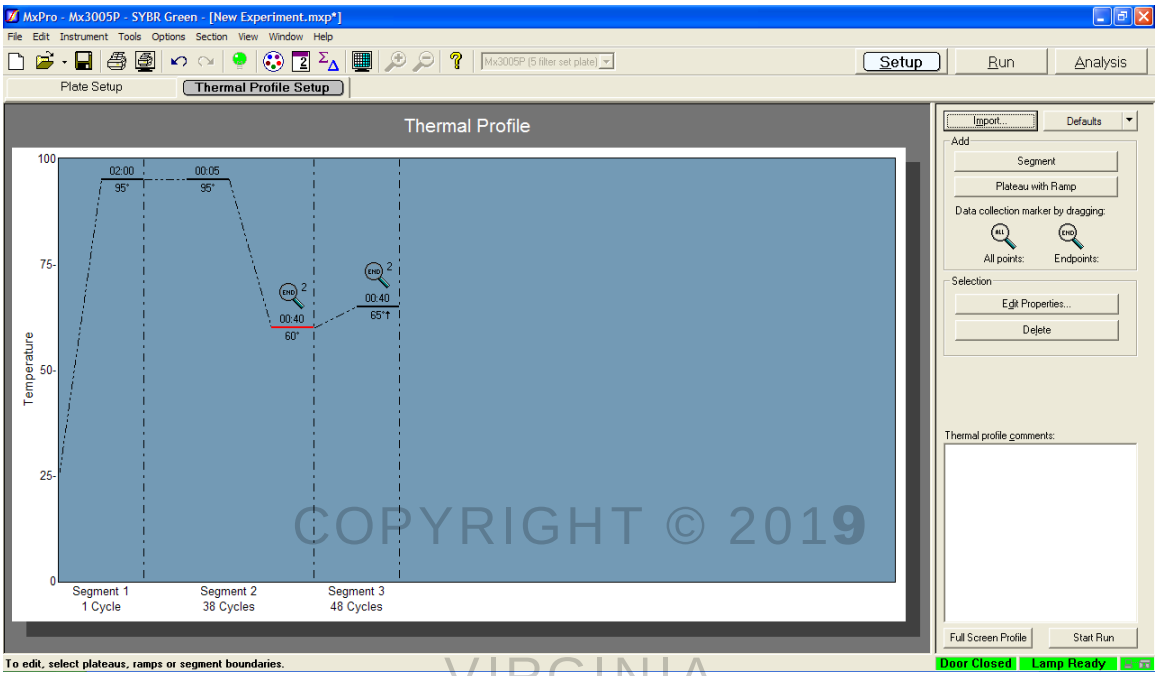

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2.5.4 If film will be used to seal the plate, select "Instrument" and "Filter Set Gain Settings". In the dialog box that opens, the filter settings should be changed to x1 for each dye except FAM, which should be x2.

2.5.5 If optically clear caps will be used, then use the default filter gain settings.

NOTE: The default filter gain settings are configured for the caps (x1 for each dye except x2 for CO560 and x4 for FAM)

2.5.6 The instrument is now ready to run the Plexor® HY System assay once the reactions are prepared in the qPCR amplification plate.

2.6 Preparation of the Standard Curve

2.6.1 After the initial use, the Plexor® human genomic DNA Standard (50 ng/µL) must be stored at 4ºC to prevent deleterious effects on the DNA due to freeze-thawing.

2.6.2 Prepare a 5X serial dilution of the DNA standard as follows:

2.6.2.1 Label seven microcentrifuge tubes B through H.

2.6.2.2 Transfer 40 µL of Type I H_2O or the amplification grade water included in the Plexor® HY kit to tubes B-H.

2.6.2.3 Vortex the Plexor® DNA standard (50ng/µL) to mix it thoroughly.

2.6.2.4 Transfer the appropriate amount of DNA to tube B as shown in Table 1, close the lid and vortex to mix thoroughly.

2.6.2.5 Repeat the DNA transfer, as shown in Table 1, followed by vortexing, for each standard C-G.

2.6.2.6 Undiluted genomic standard will be used for standard A, added directly to the Plexor® cocktail in the qPCR plate.

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2.6.2.7 The no template control, sample H, will consist of either the Type I H_2O or amplification grade water used to make the above dilutions.

DNA Standard	Concentration (ng/µL)	Volume of Type I or amp grade H_2O to add to tube	Volume of DNA to add to tube
A	50	-	-
B	10	40 µL	10 µL from 50ng/µL Plexor® DNA standard
C	2	40 µL	10 µL from Standard B
D	0.4	40 µL	10 µL from Standard C
E	0.08	40 µL	10 µL from Standard D
F	0.016	40 µL	10 µL from Standard E
G	0.0032	40 µL	10 µL from Standard F
H	0	40 µL	-

Table 1. Preparation of the Plexor® HY Standard Curve

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2.7 Preparation of Plexor® qPCR Reactions

2.7.1 Remove the Plexor® HY System reagents from the -20ºC freezer and allow them to thaw completely prior to use. All reagents should be thawed and well mixed prior to use.

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2.7.1.1 Unused Plexor® HY System reagents may be re-frozen at -20º C **EXCEPT** the genomic DNA standard which **MUST** be stored at 4º C between uses.

2.7.2 Prepare the Plexor® HY System master mix in a 1.5 or 2.0 mL tube as described below:

Reagent	**Casework Runs (*Not* Casework Direct Extract Runs)**	**Casework Direct (CD) Screening Extract Runs**
Plexor® 2X Master Mix	10 µL per reaction	10 µL per reaction
Nuclease-free (or Type I) H_2O	7 µL per reaction	3 µL per reaction
20X Primer/IPC Mix	1 µL per reaction	1 µL per reaction
5X AmpSolution™ Reagent	N/A	4 µL per reaction

NOTE: The 5X AmpSolution™ Reagent may be turbid after thawing or storage at 2-10°C. If turbidity occurs, warm the reagent briefly at 37°C and vortex until clear.

Prepare sufficient reaction mix for the desired number of reactions, making certain that the two columns of standards (16) are included as well as an appropriate number of excess reactions (approximately 3 for small runs or 6 for larger runs).

NOTES: When manually pipetting the master mix and genomic DNA into the 96-well qPCR plate, it is imperative that no bubbles be introduced. If bubbles are observed, use a clean unused pipette tip to carefully remove the bubble.

When preparing for a Casework Direct Screening Extract Run, either:

- one batch of reaction mix to include both samples and standards may be made using the Casework Direct recipe.

  OR

- a reaction mix for the samples using the Casework Direct recipe and a reaction mix for the standards using the Casework Run recipe may be made. This simply allows for the conservation of the 5X AmpSolution™ Reagent needed for the Casework Direct Screening Extracts.

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2.7.3 Transfer 18 µL of the Plexor® HY System master mix prepared in 2.7.2 into each well of the 96-well qPCR plate to contain sample or a standard curve sample. The standard curve samples will be placed in the last two columns (11 and 12) of the 96-well qPCR plate.

2.7.4 Transfer 2 µL of each sample or standard curve sample into the appropriate well of the 96-well qPCR plate. The DNA standards are to be placed in duplicate in columns 11 and 12. The undiluted Plexor® human genomic DNA Standard (2 µL) will be used for wells A11 and A12. Standard B, as prepared in 2.6.2, (2 µL) will be used for wells B11 and B12, etc. The Type I H_2O or amp grade water used to prepare the dilutions in 2.6.2 (2 µL) will be used for wells H11 and H12 and will act as no-template controls.

2.7.5 Once the entire plate is loaded with reaction mix and samples, visually examine the wells to be sure no bubbles are present. If bubbles are observed, dislodge them with a sterile, unused pipette tip per well.

2.7.6 Seal the plate with optically clear strip caps or film.

2.7.6.1 If using the optically clear film, ensure that a secure seal is made with the plate such that no evaporation of liquid will occur. In addition, be sure the filter gain settings have been adjusted in the MxPro data collection software as described in 2.5.4.

2.7.6.2 Cap the remaining samples containing the isolated DNA to prevent evaporation.

2.7.6.3 Proceed by following the procedures outlined in 1.8 (Stratagene Mx3005P™ Operating Procedure) and 1.9 (Data Analysis) of this manual.

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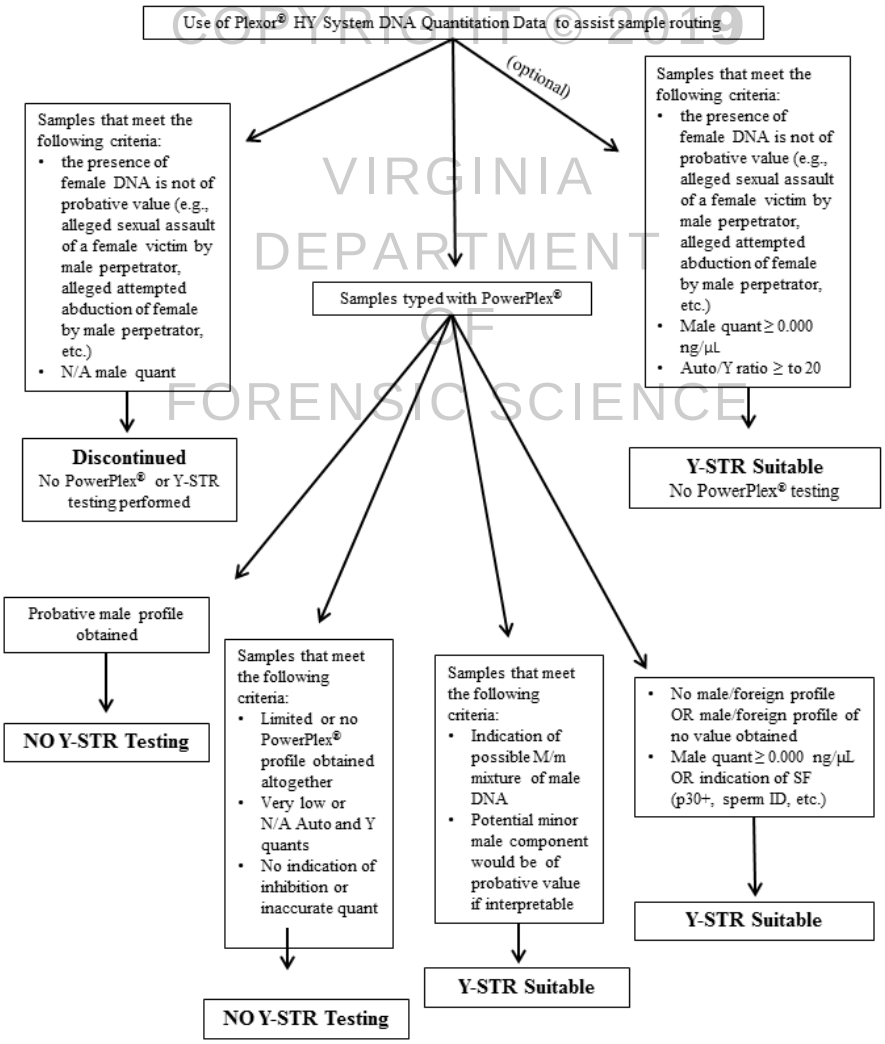
3 USING THE PLEXOR® HY QUANTITATION DATA FOR PCR-BASED TYPING DECISIONS (EXCLUDING CD SCREENING EXTRACT DATA)

Examine the Plexor® HY Quantitation System data before, during or after PowerPlex® typing of casework samples. Use the Decision Tree shown below to assist in routing samples through PowerPlex® and/or Y-STR typing.

If one fraction of a sample will be typed with PowerPlex®, both fractions will be typed with PowerPlex®.

If one fraction of a sample will provide probative results with PowerPlex® typing, the other fraction does not need to be routed to Y-STR typing.

It is preferred that a male known reference sample be available for comparison prior to Y-STR testing being conducted. In most cases for which no male known reference sample has been submitted, the examiner will report that Y-STR testing can be conducted in the future in accordance with the FB PM Report Writing.



In general, if probative information to the case is provided with the PowerPlex® results, no Y-STR testing is necessary.

If multiple samples in a case are suitable for Y-STR testing, case information may be relied upon to select which sample(s) to test, if any.

Y-STR suitability does not necessitate Y-STR testing. Examiner discretion may be used.

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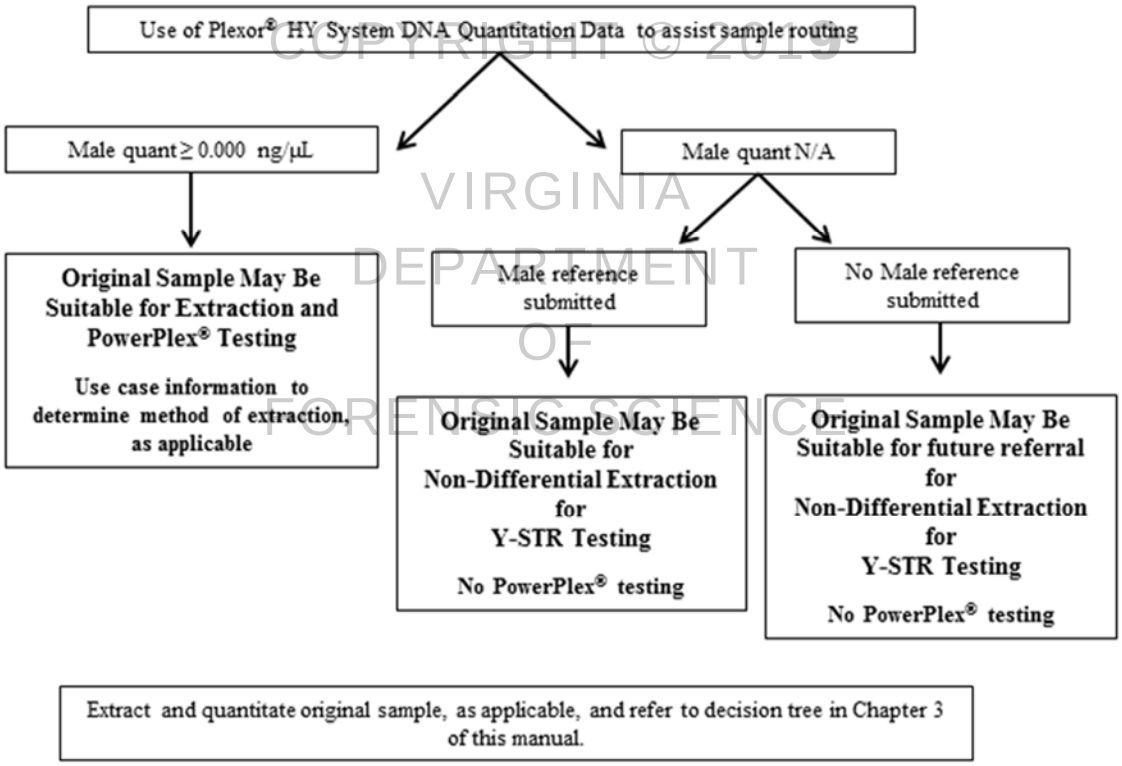
4 USING THE PLEXOR® HY QUANTITATION DATA FOR PCR-BASED TYPING DECISIONS (CD SCREENING EXTRACTS)

Examine the Plexor® HY Quantitation System data obtained for Casework Direct screening extracts. Use the Decision Tree shown below to assist in routing samples through PowerPlex® and/or Y-STR typing.

When extracting the original sample based upon the Decision Tree below, choose the extraction method based upon case scenario (e.g., differential extraction for potential seminal fluid stains, non-differential extraction for samples extracted for possible saliva, trace DNA, etc.)

If multiple samples in a case are triaged using Casework Direct, not all samples must continue through extraction and typing. The quantitation data obtained for the Casework Direct screening extracts may be used to select the sample(s) most likely to give a useable result for further testing.



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APPENDIX A – TROUBLESHOOTING DURING PLEXOR® DATA ANALYSIS

1 Removing outlier data points from a standard curve

1.1 Evaluate the standard curves by selecting the Standard Curves tab. The curves for the autosomal and Y DNA quantitation will need to be evaluated separately by selecting each of the tabs (FAM – Autosomal and CO560 – Y). Each standard curve should have an r^2 value of 0.98 or above to be acceptable. Evaluate the curve generated to determine if any outlier data points need to be removed to improve the quality of the extrapolated curve generated. Up to 3 data points for each column of standards can be de-selected, when necessary, to produce a linear standard curve. The de-selection of data points from either standard curve must be indicated on the report. Only a Project Coordinator or previous Project Coordinator who has maintained his/her proficiency may approve the de-selection of data points.

1.2 De-selection of data points from the standard curve does not result in deletion of the data. The data for those wells are still reported on the printed reports. De-selection of the wells simply results in not using that data for the generation of the standard curve, from which concentrations of the unknowns are estimated.

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1.3 Standard Samples can be selected/de-selected for the generation of each standard curve, the autosomal and the Y, independently. That is, if a data point is an obvious outlier in the standard curve for the autosomal target, this well may be de-selected for the generation of the autosomal standard curve. If the sample is not an outlier for the Y standard curve, it may be included in the generation of the Y standard curve.

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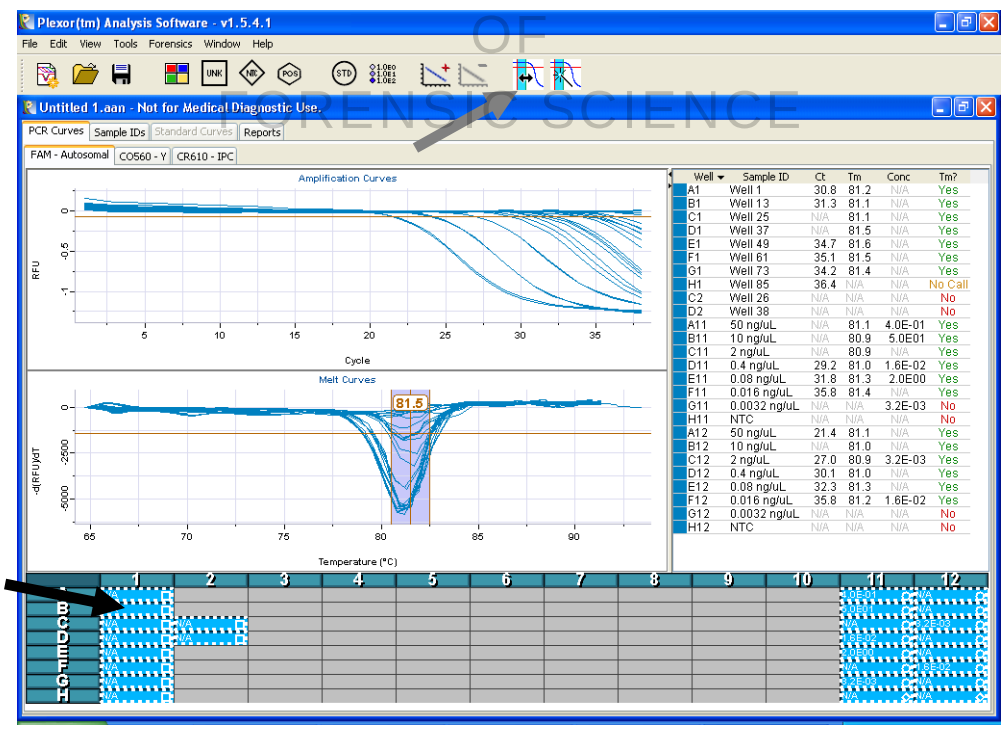
1.4 In the generation of a standard curve, all samples and standards are selected by clicking on the box indicated by the black arrow, as shown below. Note that this selection is specific to the dye selected via the tab above the data.



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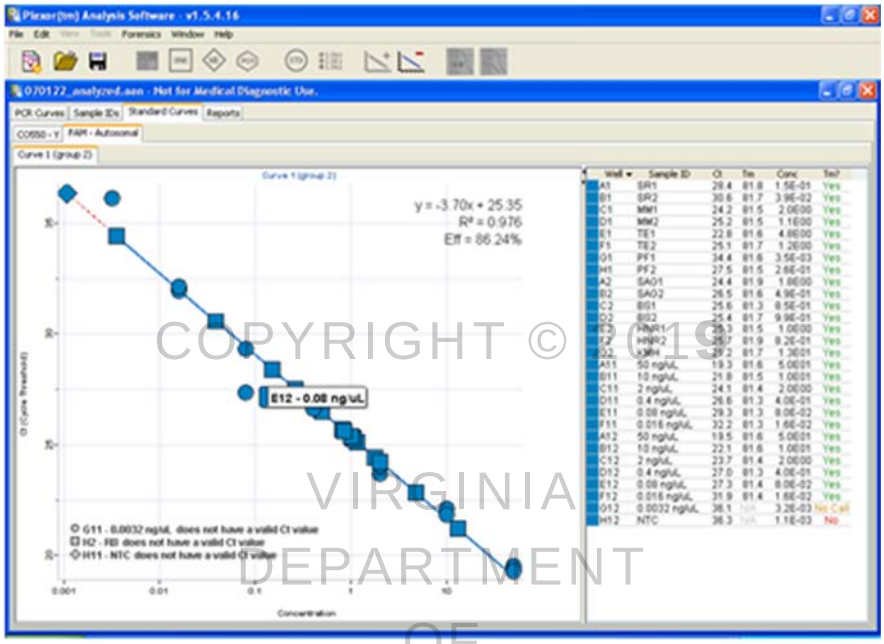
1.5 If after reviewing the standard curve, the r^2 value is lower than is acceptable, an examination of the curve may reveal what or which data points significantly deviate from the extrapolated curve. These data points can be identified by moving the mouse (arrow) over the outlier data point, which then displays the well location of the data point, as shown in the figure below. This or these data point(s) are the points which should be de-selected to improve the standard curve.



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1.6 Samples are deselected by holding down the <Ctrl> key on the keyboard while using the mouse to click on the sample of interest. This sample is then no longer highlighted.

1.7 The standard curve can then be generated in the usual manner "Edit">"Add Standard Curve" or by clicking the icon shown below:

1.8 If a standard curve was already generated for that dye, the software will prompt the user that the generation of a new standard curve will replace the existing standard curve. If this is desired, click "OK".

1.9 If de-selection of data points on the other standard curve is necessary, be sure to select the other tab (as shown in Figure 1) and repeat steps 1.1.3 – 1.1.8, above.

1.10 The de-selection of data points must be indicated on the Plexor® report(s) and initialed.

2 Adjusting a Sample's Baseline to More Optimal Values

2.1 The baseline regions are computed and selected automatically by the Plexor® Analysis Software. Should the baseline of any specific amplification plot be improperly estimated, it can be adjusted manually. Each well can be highlighted individually to inspect the amplification plot and adjust the baseline. The need to manually adjust the baseline for a sample or standard should arise rarely if ever.

2.2 The baseline of individual samples should be adjusted, if needed. First, view the amplification plots for the entire plate, be sure to select the "PCR Curves" tab, then click on the empty box at the upper left hand corner of the sample plate. Choose "Edit", "Display and manually adjust baseline regions" and click "OK" in the dialog box that appears.

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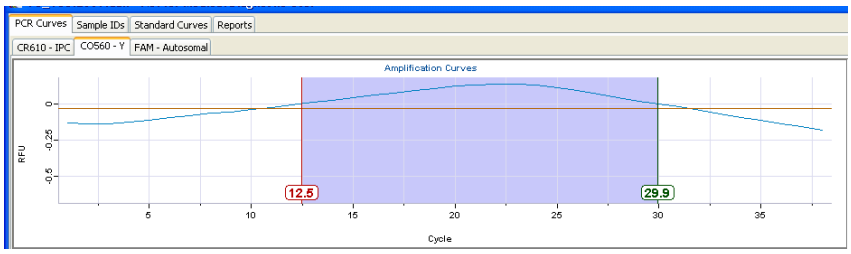
2.3 The baseline should be set in a flat region of the amplification curve before the decrease in signal, which is indicative of accumulation of PCR product. Manual adjustment of the baseline region for each sample is possible, although rarely necessary. It is possible to alternate between each dye to view the various amplification plots for each well. An improperly set baseline region can result in a skewed amplification plot, such as that shown below:



2.4 To correct the baseline region of a sample, if needed, each well can be highlighted individually. Choose "Edit", "Display and manually adjust baseline regions" and click "OK" in the dialog box that appears. The baseline can then be adjusted by dragging the lower and upper limits. The upper limit should be approximately 5 cycles before the decrease in fluorescent signal in an area where the signal is flat. The lower limit is usually set to a region that creates a flat baseline, resulting in a baseline region of approximately 6 cycles or more. An amplification plot with an appropriately-selected baseline region is shown below:

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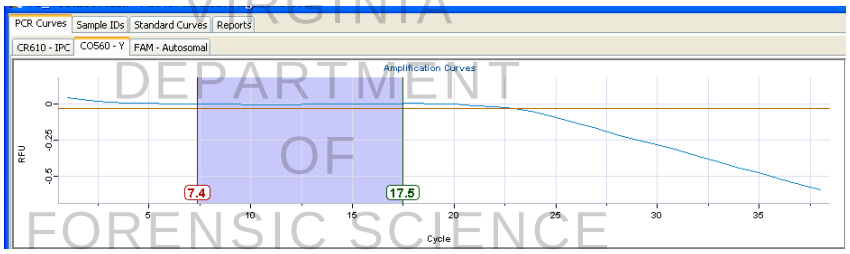

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NOTE: The software automatically recalculates the estimated DNA concentrations and standard curves affected by the altered baseline region.

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