14 Plexor HY Quantification

The Promega Plexor HY kit contains all necessary reagents for the simultaneous real-time PCR amplification, detection, and quantification of human autosomal and human male DNA in a sample. The results can be used to determine the presence of male DNA in samples with overwhelming female DNA.

The DNA quantification assay combines three separate assays:

- A mulitcopy human autosomal DNA assay
- A mulitcopy human male DNA assay
- An internal PCR control assay

The kit works with the Applied Biosystems 7500 Real-Time PCR System and SDS Software v1.2.3 for data collection. Data analysis is accomplished by exporting the data to the Plexor Analysis Software v1.5.6.7.

Safety
Body fluids, tissues, and extracts may contain infective agents. Use universal precautions during evidence handling. Follow instructions for reagent preparation. Gloves should be worn during testing. Clothing may protect unbroken skin; broken skin should be covered.

Kit Contents and Storage
The Plexor HY kit comes in two sizes, 200 and 800 reactions with a 20 µL reaction volume. Store the entire kit at -15°C to -25°C upon receipt. Store the male genomic DNA standard at 2°C to 8°C after first thaw, the remaining kit should continued to be stored at -15°C to -25°C.

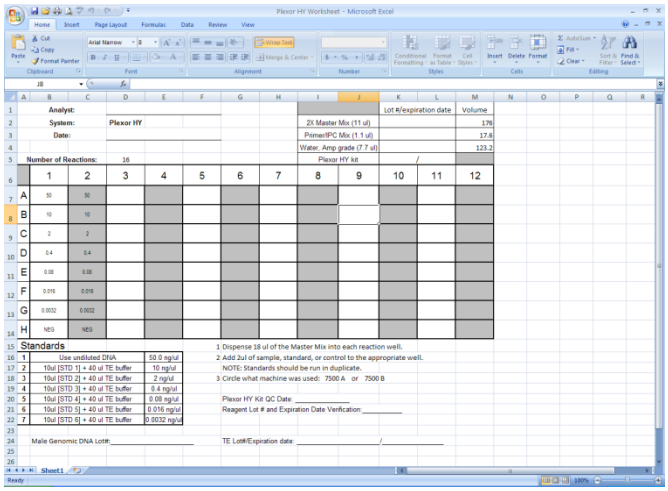
Plexor® HY System 200 reactions
Product Size Cat. # DC1001
Includes:
- 2 × 1ml Plexor® HY 2X Master Mix
- 200µl Plexor® HY 20X Primer/IPC Mix
- 150µl Plexor® HY Male Genomic DNA Standard, 50ng/µl
- 2 × 1.25ml Water, Amplification Grade

Plexor® HY System 800 reactions
Product Size Cat. # DC1000
Includes:
- 8 × 1ml Plexor® HY 2X Master Mix
- 4× 200µl Plexor® HY 20X Primer/IPC Mix
- 3 × 150µl Plexor® HY Male Genomic DNA Standard, 50ng/µl
- 5 × 1.25ml Water, Amplification Grade

NOTE: Keep Primer/IPC Mix and Master Mix protected from direct exposure to light. Excessive exposure to light may affect the fluorescent dyes.

Creating a Plexor HY worksheet

1. Open the "Plexor HY Worksheet" template (an Excel spreadsheet).
2. Type samples into the sample sheet. Ensure each standard is present and in duplicate.
3. Print the file for documentation in the case file(s).



4. Save the excel file with initials and date of run (i.e. AB092012)
5. Close excel.

Preparing the DNA Quantification Standards

Make a standard curve dilution series consisting of:

Standard	ng/µL
Male Genomic DNA Standard	50
B	10
C	2
D	0.4
E	0.08
F	0.016
G	0.0032

To create this series of standards, perform the following tasks:

1. Label 6 microcentrifuge tubes B, C, D, E, F, and G.
2. Dispense 40 µL of TE into the six microcentrifuge tubes.

3. Vortex the Male Genomic DNA Standard for 3 to 5 seconds
    a. If this is the first use of the standard, thaw the DNA.
4. Prepare Standards B through G:
    a. Pipette 10 µL of the Male Genomic DNA Standard to the tube for Standard B.
    b. Mix the dilution thoroughly
    c. Using a new pipette tip, add 10 µL of the prepared standard to the tube for the next standard (e.g. 10 µL of Standard B into the tube for Standard C; the resulting mixture will contain 10 µL of the previous standard and 40 µL of TE)
    d. Mix the dilution thoroughly
    e. Repeat steps 4c and 4d until the dilution series is complete

NOTE: Any unused standard can be labeled with the analyst's initials and date of creation (e.g. ABC010101) and stored at 2°C to 8°C for up to two weeks.

Reaction and Plate Setup Procedure

1. Allow the Plexor HY kit components to thaw to room temperature. Mix the 2X Master Mix and 20X Primer/IPC Mix by brief vortexing.
2. Make a set of positive standards as described in the Previous section (entitled "Preparing the DNA Quantification Standards").
3. Prepare a Master Mix consisting of the following reagents and volumes:
    a. The “Plexor HY Worksheet” will include the necessary volumes for the master mix
    b. The worksheet includes a correction factor for pipetting loss

Component	Volume Per Reaction (µL)
Plexor HY 2X Master Mix	11
Plexor HY 20X Primer/IPC Mix	1.1
Water, Amplification Grade	7.7

4. Vortex the Master Mix for 3 to 5 seconds, then briefly centrifuge the tube.
5. Dispense 18 µL of the Master Mix into each reaction well.
    a. Make sure the plate is correctly oriented, A1 will be to the top left
6. Add 2 µL of each sample, standard, or control (TE for negative control) into the applicable wells.
7. Seal the reaction plate with the adhesive cover.
8. Centrifuge the plate at ~3000 rpm for about 20 seconds in a plate centrifuge with plate holders to remove any bubbles.

9. Load the plate into the plate holder of the 7500. Ensure that the plate is correctly aligned in the holder.

Push the tray door to open it.

Insert the plate into the plate holder.

Load standard 96-well plates with the notched A12 position at the top-right of the tray.

Close the tray door.

Starting the 7500 Real-Time PCR System

Overview

Starting the 7500 Real-Time PCR System involves:

1. Starting the Computer, if not already on
2. Powering on the Instrument
3. Starting the 7500 SDS Software

Starting the Computer

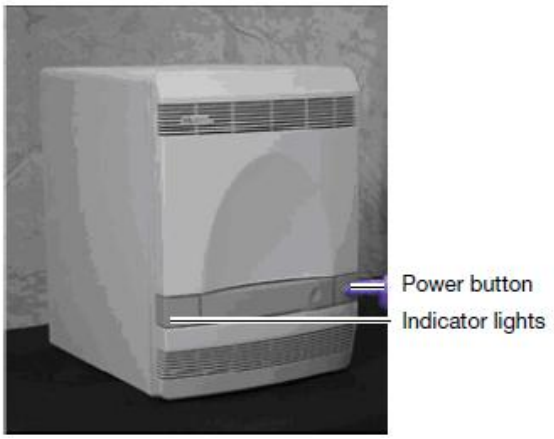
1. Open the laptop computer by pushing in the front center button, holding it, and lifting the lid.
2. Press the power button on the computer.
3. Enter the username associated with the computer, if applicable.
4. If required, type the corresponding password in the password field.

NOTE: Wait for the computer to finish starting up before powering on the 7500 instrument.

Powering on the Instrument

1. Press the power button on the lower right front of the 7500 instrument.
2. The indicator lights on the lower left of the front panel cycle through a power on sequence.
3. When the green power indicator is lit (not flashing), communication is established between the computer and the instrument.

NOTE: If the green power-on indicator is flashing or the red error indicator is lit, see the Applied Biosystems *7300/7500/7500 Fast Real-Time PCR System Installation and Maintenance Guide* (PN 4347828)



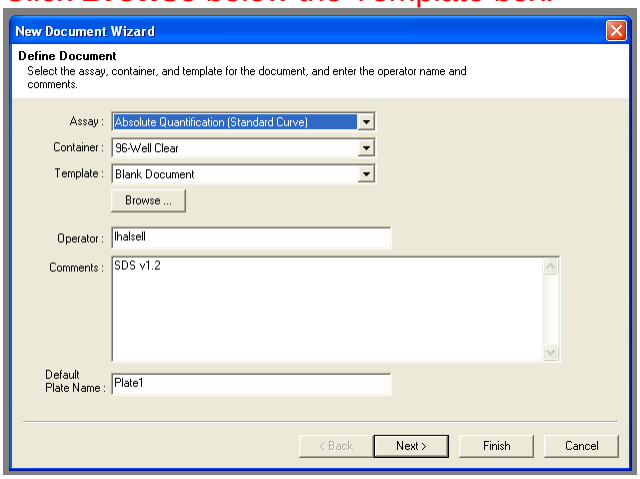
Starting the 7500 SDS Software

1. Start the **7500 System Software**. Double click on the software icon.
2.

3. The software starts and displays the word "Disconnected" in the status bar on the bottom-right corner. The status changes to "Connected" only after the New Document Wizard is completed, the software is initialized, and the software is connected to the 7500 instrument. If the connection is successful, the software displays “Connected” in the status bar on the bottom-right corner.

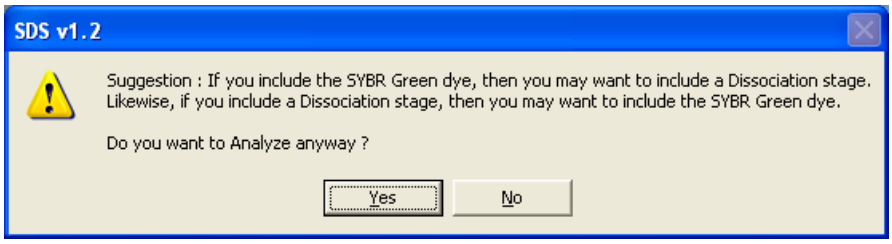
Creating a Plate Document and Starting the Run

1. Click **File > New**
2. Click **Browse** below the Template box.

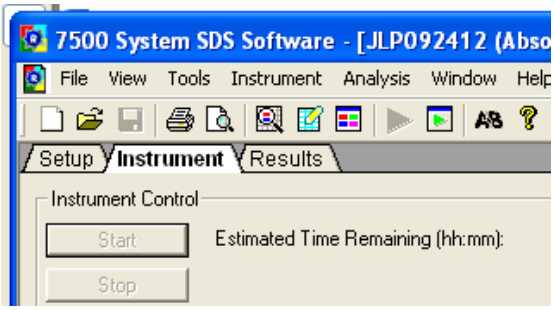


3. Navigate to the Desktop and select the “Plexor HY” template
4. Click **Open** (or double-click the template file)

NOTE: The following box will appear any time you open, close, save, or analyze the file. Always click yes to navigate pass the box.



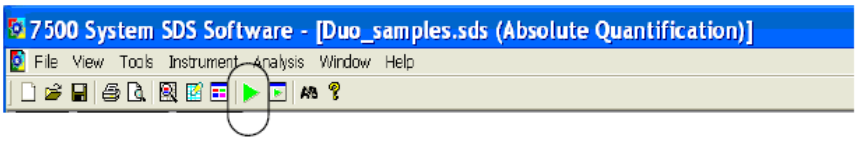
5. Click **File > Save As**
6. Name run with initials and date (i.e. AB092012)
7. Save the file to the Desktop
8. Click on the Instruments tab and click **Start** to start the run.



9. Ensure that the **Estimated Time Remaining** shows a time before leaving the room.

Analyzing run and Exporting files with 7500 SDS Software

1. When the run is complete the instrument will display a successful run completion message.
2. Click okay
3. Omitting unused wells
   a. Select the unused wells according to your worksheet.
   b. With the well selected, hit Ctrl + M
   c. The omitted wells will now show an “X” through them
4. On the menu bar, select **Analysis > Analyze**.
   a. Alternatively the green arrow in the menu can be clicked



5. Exporting Files
   a. Select **File > Export > Delta Rn**
   b. Save the file with the run name and the suffix “-delta” (i.e. AB092012-delta)
      i. Save the file to the Desktop with the run file
   c. Select **File > Export > Dissociation > Raw & Derivative data**

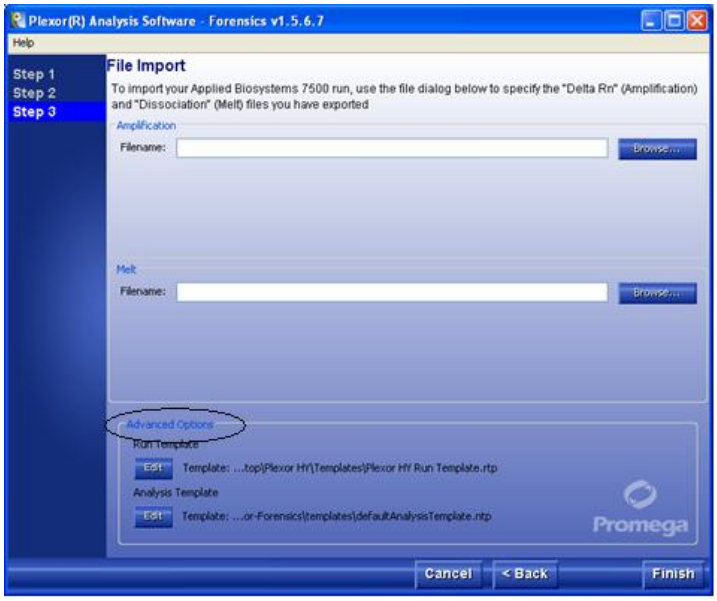
   d. Save the file with the run name and the suffix "-diss" (i.e. AB092012-diss)
      i. Save the file to the Desktop with the run file
   e. Do **NOT** open the export files. This will corrupt them for later use.
6. Close the 7500 run file and 7500 SDS software
   a. Click Yes to Save if prompted to do so.
7. Move the three saved files to the appropriate month and year folder of \\travfs01\CrimeLab\SHARED\BiologyInstruments\7500PCR\Plexor HY
8. Remove and discard the plate from the 7500
9. Turn off the 7500

Plexor HY Analysis software

Note: The following instructions are suitable on software that has been appropriately initialized

Software set up and file import

1. Start the Plexor Analysis Software
2. Select **File > Import New Run**
3. Fill in the Assay Name with the same name as the 7500 run file (i.e. AB092012)
4. Make sure that the instrument is set to **Applied Biosystems 7500 SDS v1.4 & prior**
5. Click **Next**
6. Fill in the Operator Name, initials are acceptable
   a. The remaining fields can be left blank
7. Click **Next**
8. Click the upper **Browse** button to add the Amplification file
9. Navigate to the previously saved "Delta Rn" file (i.e. AB092012-delta)
10. Select file and click open
11. Click the lower **Browse** button to add the Melt file
12. Navigate to the previously saved "Raw & Derivative data" file (i.e. AB092012-diss)
13. Select file and click open
14. Under the **Advanced Options**
    a. Click **Edit** under **Analysis Template**
    b. Select **Defaults > Reset to Default**
    c. Click **OK**



15. Click Finish
16. A window will appear with the run data

Data Analysis and Report Generation

1. Make sure the **FL-Autosomal** Tab is selected
2. With all samples selected, go to **Edit > Add Standard Curve**
    a. Alternatively you can hit the “d” key
3. Select the **CO560-Y** Tab
4. With all samples selected, go to **Edit > Add Standard Curve**
    a. Alternatively you can hit the “d” key
5. Select the **Sample Names** Tab
    a. Open the Excel Plexor HY worksheet saved previously
    b. Select and Copy the wells that were used for the run
    c. Return to the Plexor Analysis software
6. Select **Edit > Paste Sample Names from Template**
    a. Alternatively you can hit the “Ctrl + t” key
7. Click **Accept Changes** in the bottom right of the window
8. Select **Forensics > Set Normalization and IPC parameters**
    a. Do not change any settings
9. Click **Ok** on the window that pops up
    a. Give the software a minute and the window will change to the Forensic report tab under the Reports tab
10. Check that the first location is A1. If not click **Location** until it is.
    a. Click on **Location** so the samples will be sorted by location
11. Select **File > Save Analysis File**
12. Title the file with initials and run date (i.e. AB092412)
13. Save the file to
    \\travfs01\CrimeLab\SHARED\BiologyInstruments\7500PCR\Plexor HY

Data interpretation

1. Standard Curves Evaluation
    a. Under the "Standard Curves" tab
        i. Click on the "FL-Autosomal" tab
            1. The Autosomal curve, the slope must be between -3.1 to -3.7 and R2 ≥ 0.990
        ii. Click on the "CO560-Y" tab
            1. The Y curve, the slope must be between -3.0 to -3.6 and R2 ≥ 0.990

    NOTE: A limited number of outlier points may be omitted from the curve to bring these values within acceptable range. If these values are not within range, the procedure must be re-run. Removal of more than 3 data points must be approved by the Technical Leader. Points can be removed by changing the sample type under the "PCR Curves" tab. Refer to Plexor HY manual for more detail.

2. Click back to the "Reports" tab, and select the "Forensic" tab.
3. Check that the **IPC Status** column is "OK" for all samples.

    NOTE: A higher value indicates the presence of inhibitors. The absence of an IPC value and a quantification value for a sample indicates either complete inhibition or a failure of the amplification reaction. If necessary a dilution may be made and run again with Plexor HY. A dilution which would theoretically contain fewer inhibitors may be quantified.

4. Check that the **Curve Status** column is "OK" for all samples.
    a. Depending on sample type (standard, non-template control, or sample) three other options may be displayed depending on sample type
        i. Check STD – displayed for samples designated as standard controls
        ii. Check NTC – displayed for samples designated as non-template controls
        iii. Check Melts – displayed for sample designated as unknowns

    NOTE: "Check" messages will only be displayed on samples that have concentration values. The message indicates that the melt curve needs to be assessed for that sample.

    b. Examine the melt curves for samples that are **NOT** "OK".
        i. Melt curves need to be assessed for both Autosomal and Y results.
    c. Melt curves can be examined under the "PCR Curves" tab. The melt curve is the bottom graph

    NOTE: Melt curves are a measure of the PCR products produced during amplification. If a sample has a low level DNA concentration, but no melt curve, then the detected fluorescence is most likely from non-specific fluorescence and not from amplifiable DNA.

d. When examining the melt curves in the “PCR Curves” tab three different calls are available in the “Tm?” column on the right side
   i. Yes – the melt curve is within the designated range
   ii. No – the melt curve is not within the designated range
   iii. No Call – the melt curve is within the designated range, but there is insufficient amplification product to cross the threshold
e. Documentation of why melt curves were not “OK” will need to be made on the report print out.

5. Return to the “Forensic” tab under the “Reports” tab
6. Check that all negative controls (including reagent blanks) have less than 5 x 10^{-3} (0.005) ng/µl of detectable DNA present. If negative control values exceed 0.005 ng/ul, then consult with the Technical Leader on available options.
7. Close the Plexor run file by **File > Close Current Analysis**.
   a. Alternatively you can click the red X in the upper right run file window.

      This is just above the printer icon.
8. Save results if asked to do so.
9. Reopen the file by File > Open Analysis File
   a. Alternatively you can click the file icon in the menu bar
10. Navigate to the saved file and select it. Click **Open**
11. Return to the “Forensic” tab under the “Reports” tab if not already there
12. Make sure all samples are selected. All samples will be highlighted blue.
    a. All samples can be selected by clicking one sample and hitting Ctrl + A
13. Select the print icon on the right
14. Under Page Setup, change the orientation to Portrait
15. Click Print
16. Close the Plexor run file and software.
    a. Save any changes if prompted to do so
17. Add any comments to the report that are necessary. Examples include:
    a. If any points were dropped from the standard curve.
    b. Results of melt curve investigations
18. Place a copy of the report containing the results in the case file.