atta·gene

SIGNAL TRANSDUCTION • TRANSCRIPTION FACTORS • DRUG DISCOVERY

ATTAGRAPH Reader ™
Users Manual

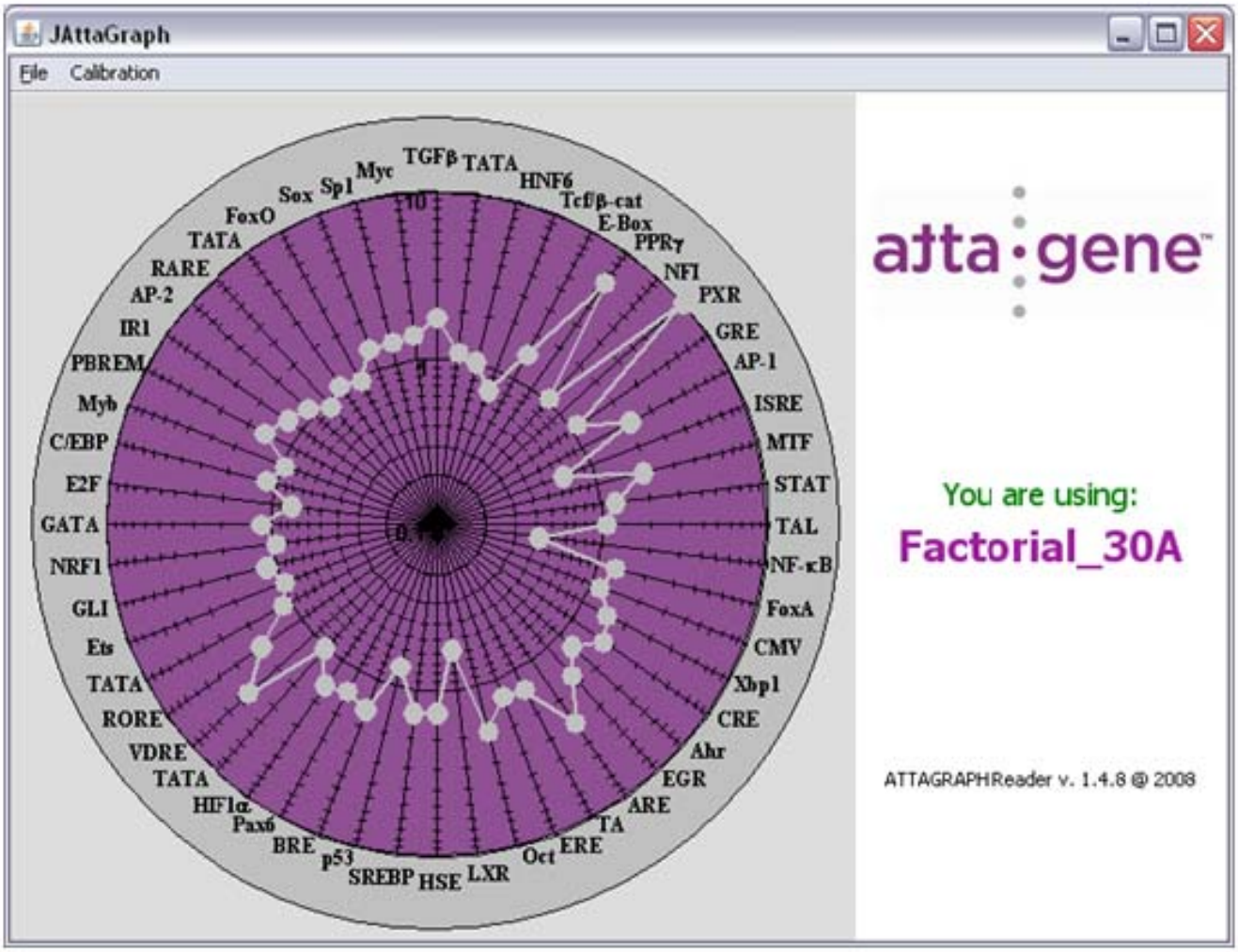

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Table 1: **Toolbar Glossary**

 Shows reporter and standard peaks data in tabular format

 Show radar graph of TF's activities

 Expand the vertical scale of the profile

 Expand the horizontal scale of the profile

 Reset the horizontal and vertical scales of the profile

 Select or deselect to show or hide the Blue (reporter) peaks

 Select or deselect to show or hide the red (size standard) peaks

 Change between use of logarithm scale or linear scale

 Change between TF's activity or relative peak values

Attagraph Reader ™ software is required for analysis of the Factorial ™ data. The software is provided as a part of the Factorial30™ transcription reporter system. It may be linked from our webpage, at Attagene.com.

Note: Please read the end user license agreement in appendix B of manual. One will see this agreement during initialization of software.

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Analysis of Data:

ATTAGRAPH Reader™ is a computer program that analyzes capillary electrophoresis fragment analysis files generated by the FACTORIAL™ assay. The essence of the FACTORIAL™ technology is a library of uniformly constructed Reporter Transcription Units (RTUs). Analysis of FACTORIAL™ data with ATTAGRAPH Reader™ will allow the user to determine the activity of each RTU and compare it to the RTU activity after a desired treatment. In order to analyze this data and interpret the results of the FACTORIAL™ assay you must:

- **Calibrate the software to a specific capillary electrophoresis machine**
- **Open FACTORIAL™ data files, in the form of raw .fsa files, for analysis**
- **Analyze the FACTORIAL™ data**
- **Export the results to an Excel spreadsheet for further analysis**
- **Save data as an ATG file or a CSV file, for storage and retrieval**

ATTAGRAPH ™ will only work properly if supplied with **raw** .fsa files from an ABI capillary sequencer. Some sequencing facilities may have a policy of altering / analyzing the fragment analysis files; if this is the case, please inform the sequencing facility that only raw .fsa files without previous analysis are required for this software.

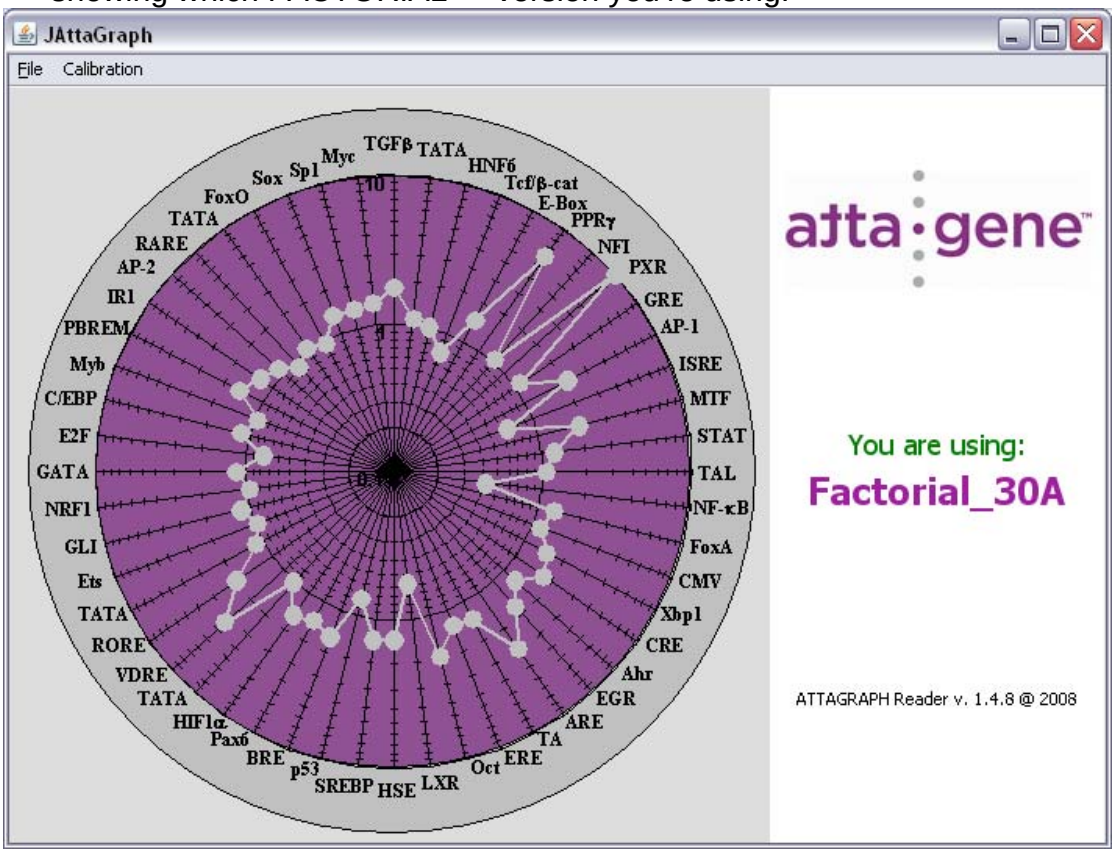
Computer Requirements:

- **PC or Mac**
- **Windows 98 or later operating system**
- **Internet access to connect to the software**
- **Java 4.5 or greater to run the software**

We can also provide fragment analysis as a service at our facility near the Research Triangle Park, NC. This is for clients who don't have access to ABI sequencers, or for clients that wish to take advantage of this part of the assay as a service. Please refer to our assay manual for instructions on this process.

I. Installation and Registration of ATTAGRAPH™:

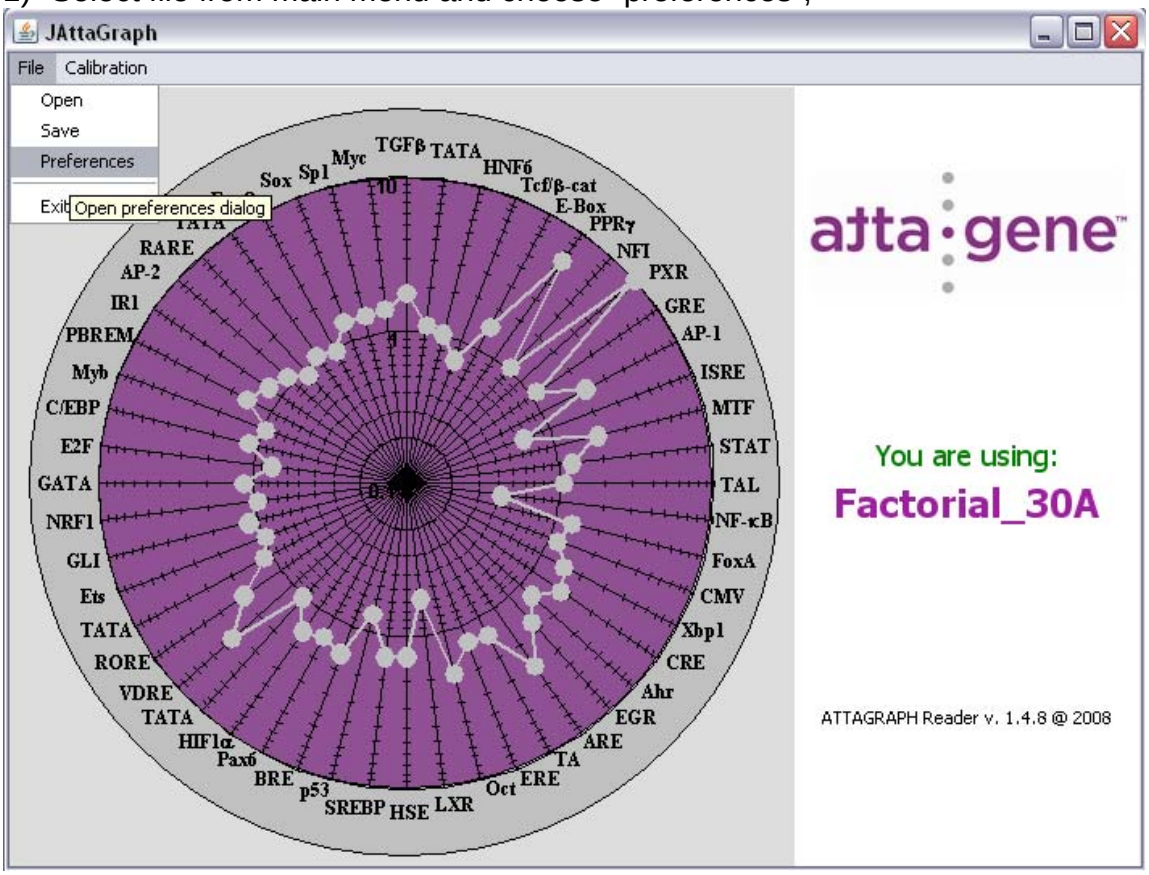
1) Connect to the internet and proceed to our webpage at Attagene.com;
2) Click on link to connect with ATTAGRAPH ™ software, you will need to register and log into the software,
3) Once you see the security pop-up message, you will need to select run.
4) Once the program has opened, the following screen-shot should appear, showing which FACTORIAL ™ version you're using.



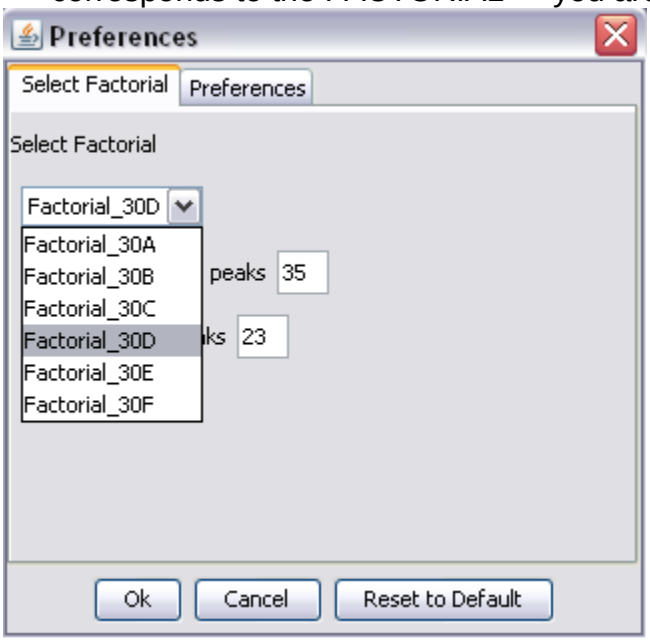
If this screen does not appear then reconnect to the internet and follow the instructions from above. If problems persist please contact info@attagene.com or by phone at 888-721-2121:

II. To change the FACTORIAL ™ version:

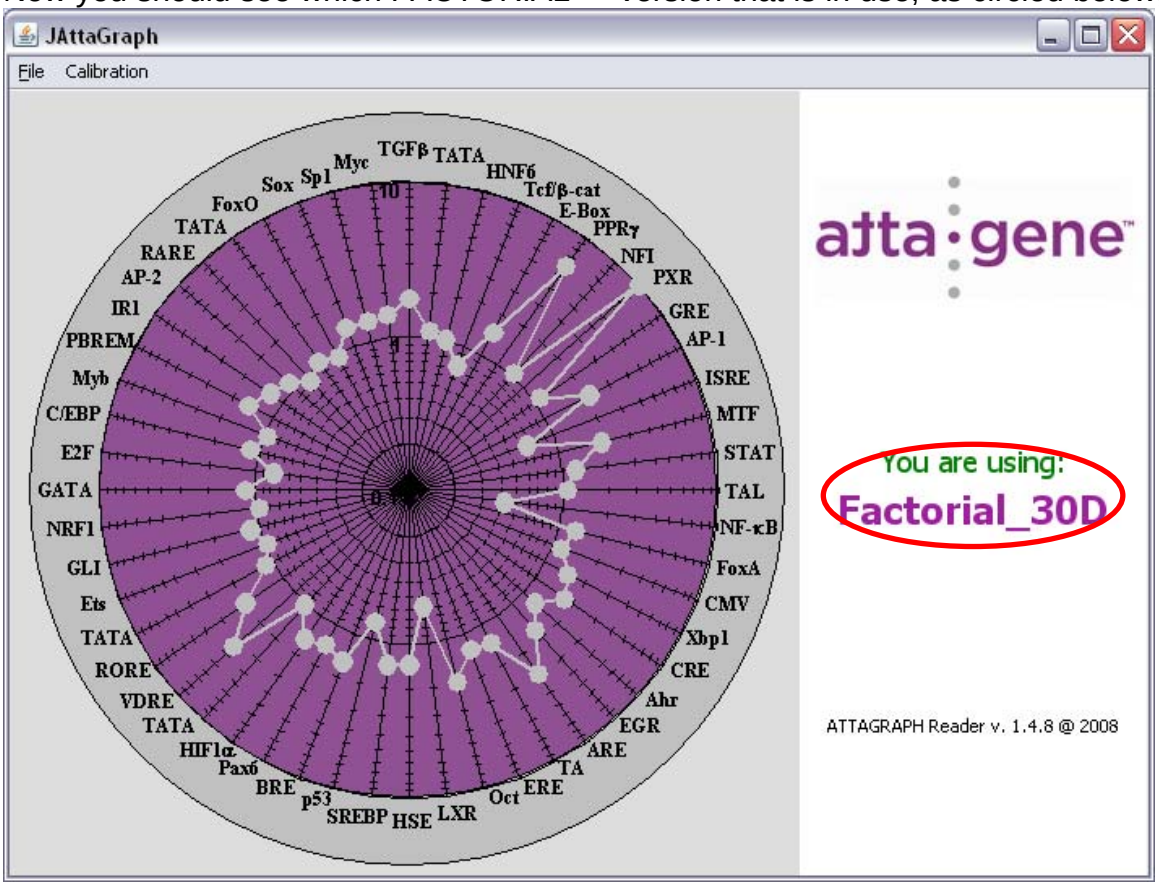
1) Select file from main menu and choose “preferences”;



2) From the preferences pop-up menu, choose the drop down menu that corresponds to the FACTORIAL ™ you are using; then click “ok”.



Now you should see which FACTORIAL ™ version that is in use, as circled below;



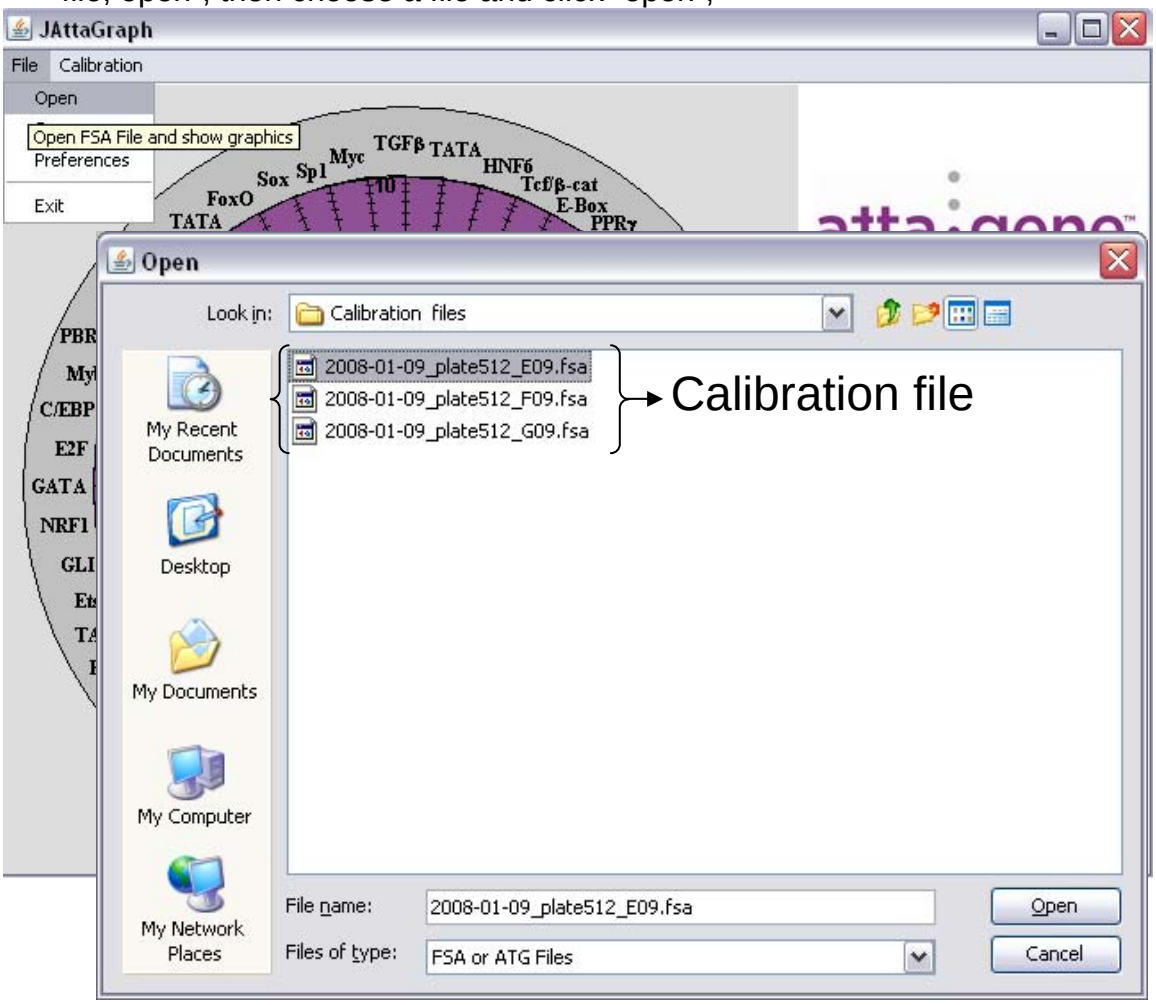
III. Calibration of the ATTAGRAPH ™ Software

After initializing and registering the ATTAGRAPH Reader™ software, FACTORIAL™ data can be analyzed. Before the experimental data can be correctly processed the ATTAGRAPH Reader™ software should be calibrated. This calibration will allow the software to correctly interpret the FACTORIAL™ data generated by the capillary electrophoresis machine.

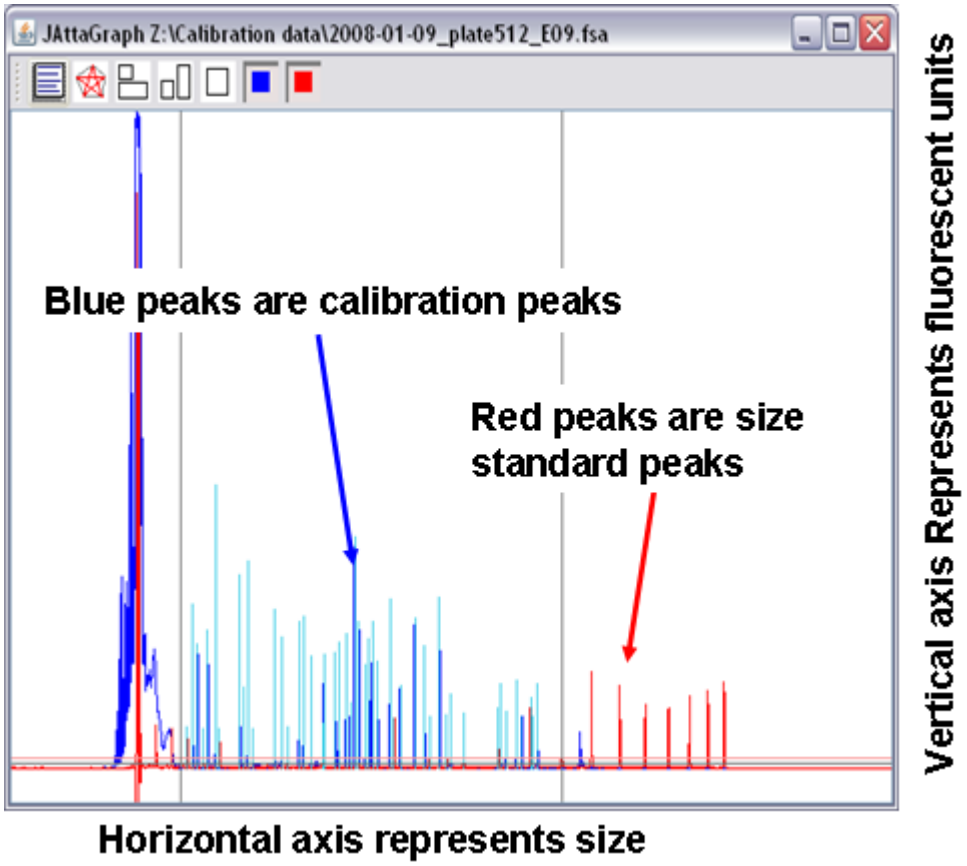
Note: Currently our software only reads runs generated from ABI capillary electrophoresis machines.

Note: calibration must be performed to compensate for the differences intrinsic to a specific capillary electrophoresis machines with regards to sizing of the DNA fragments.

1) To calibrate the ATTAGRAPH Reader™ software you need to load a file from one of the calibration standards that were run in step 10 of the FACTORIAL ™ protocol. This is an .fsa file from a capillary electrophoresis run. To do so; select "file, open"; then choose a file and click "open";

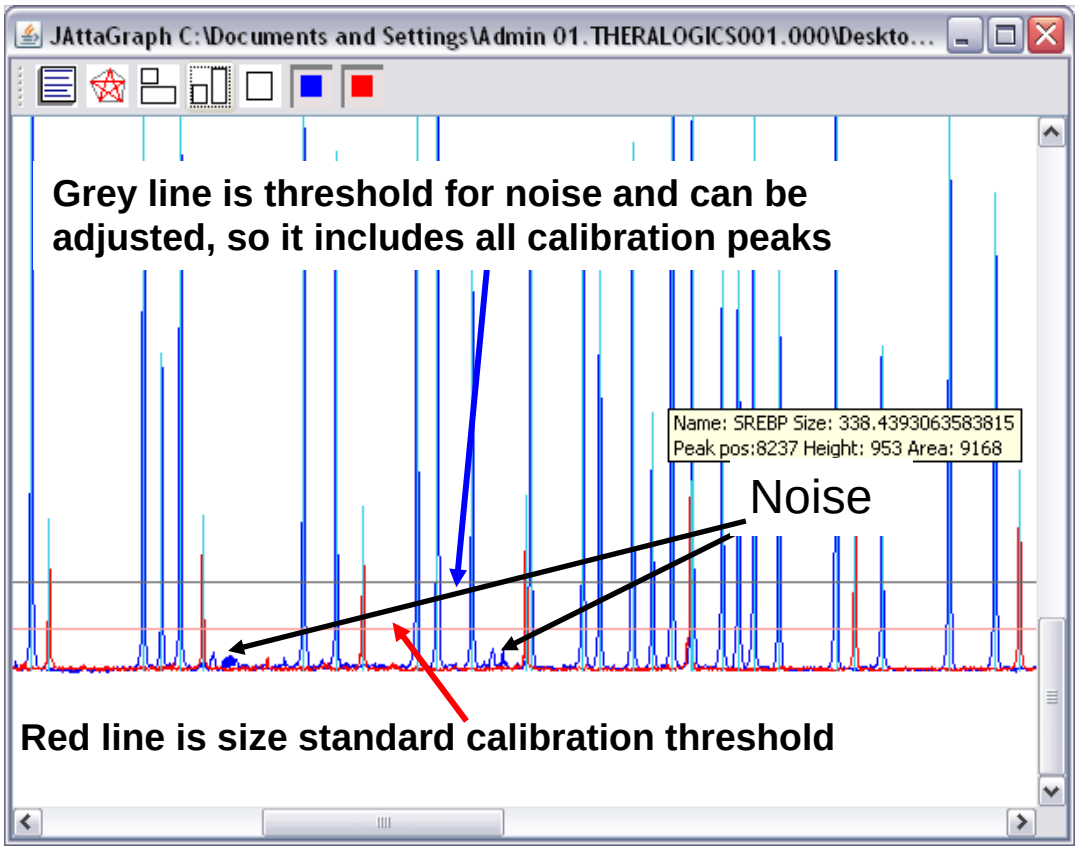


2) Once the calibration file is open, you should see a profile like the example below. The profile below shows peaks resolved according to size along the horizontal or x-axis and the heights determines the relative abundance in fluorescent units.



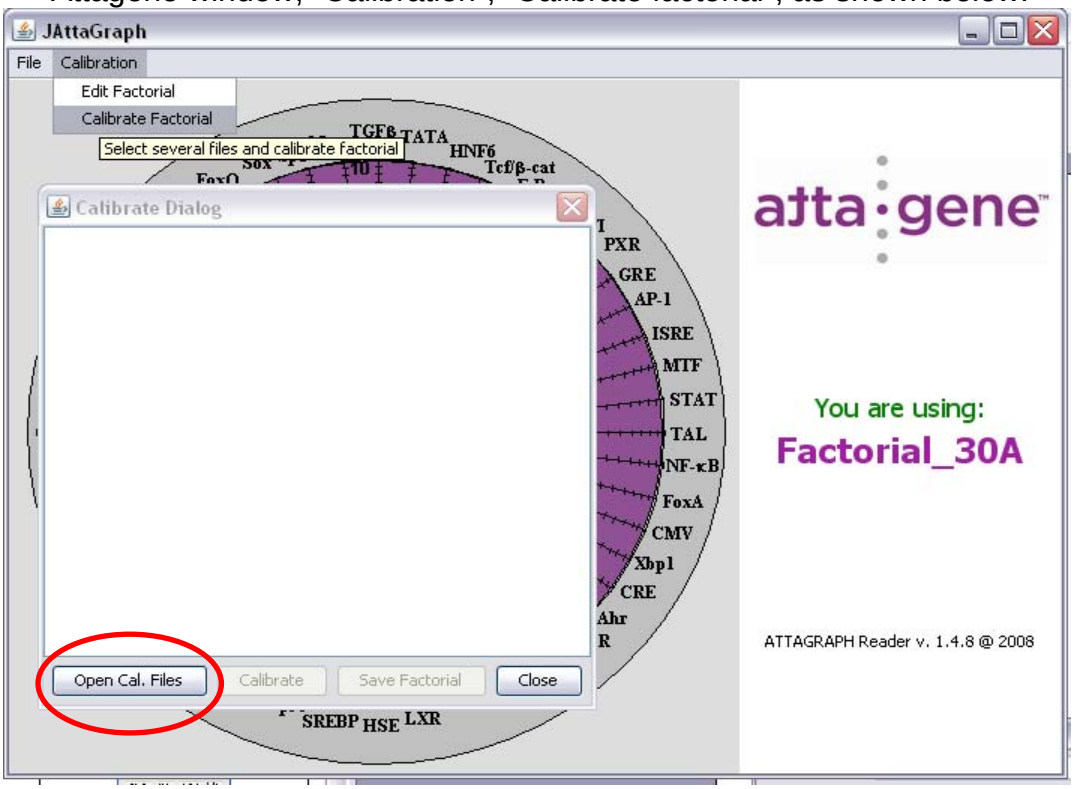
It is suggested that the calibration is completed for every capillary electrophoresis run to ensure the correct name is assigned to each reporter peak.

3) You will need to zoom in and check the threshold line and make sure it overlaps all calibration peaks to distinguish from background or noise peaks. You can adjust this line by hovering over the grey line, “click and hold”, then move up or down.



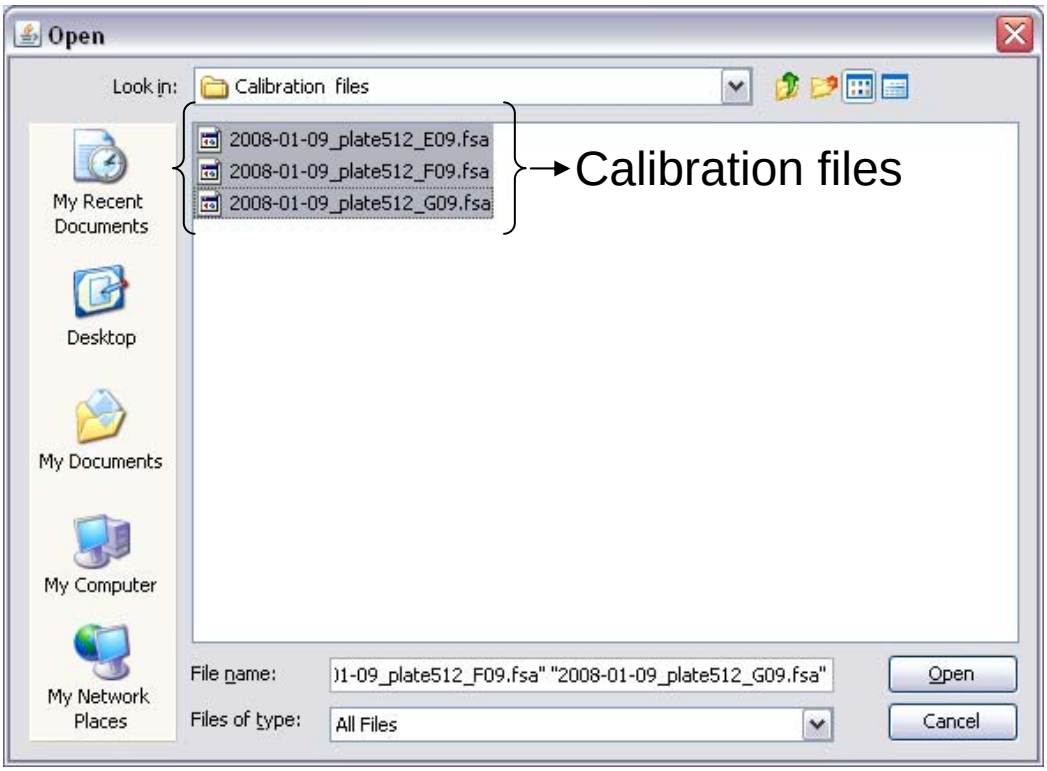
Note: a) The red line is the size standard threshold; it can be adjusted from the advanced options but is not recommended.
b) The grey line is the TF threshold and can be adjusted by clicking and dragging up or down in the window.

4) Leave the above reporter file window open and then select from the main frame Attagene window; “Calibration”, “Calibrate factorial”, as shown below.

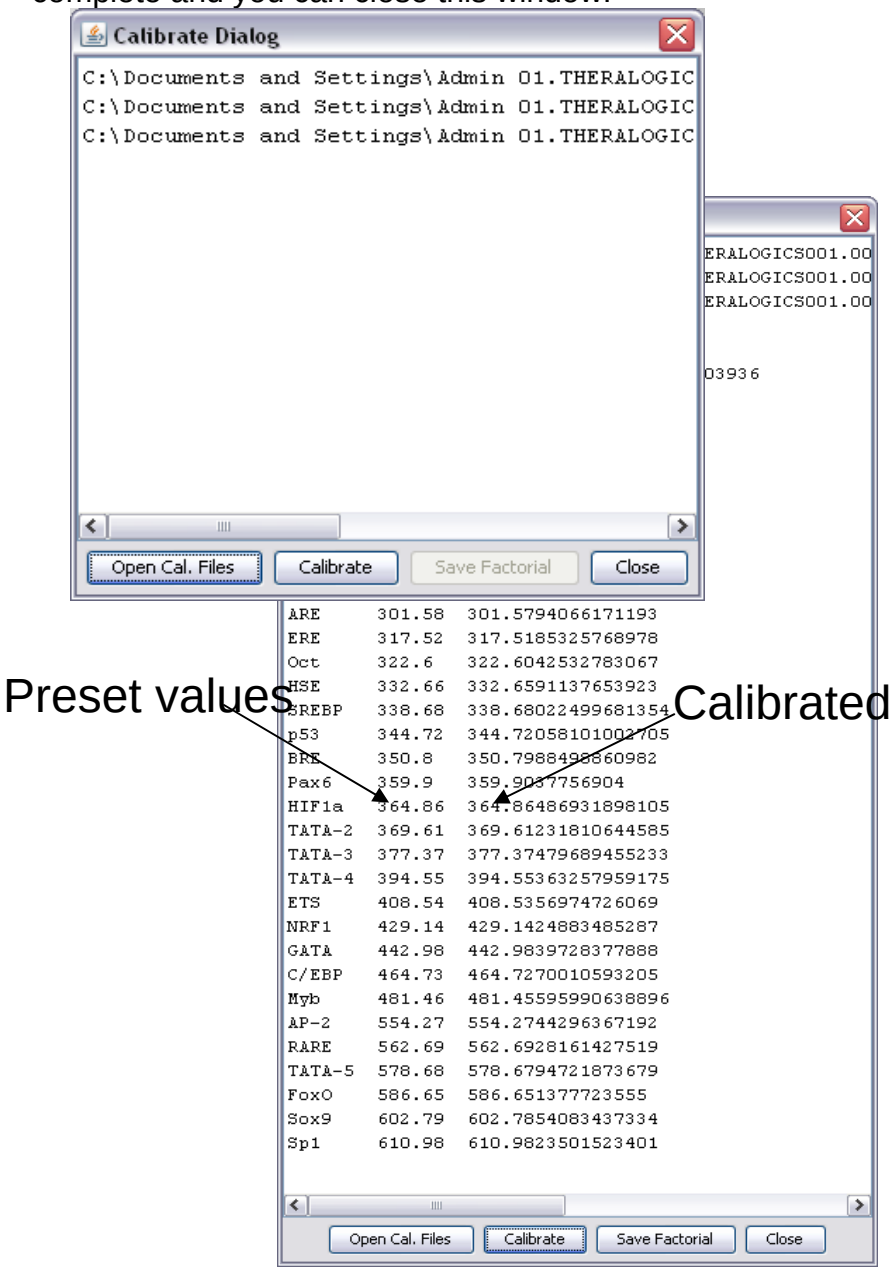


5) Once you select calibrate factorial, you will see a pop-up window that will allow you to select which files you want to use for calibration. Click on the “open Cal. Files” button, which is shown circled above.

6) From this window you can browse and choose which calibration files that you wish to use. They should be from the current run that corresponds to the data that has been generated. Search for the appropriate folder that holds the calibration files, using the ctrl or shift key to select at least three files; then choose “open”.

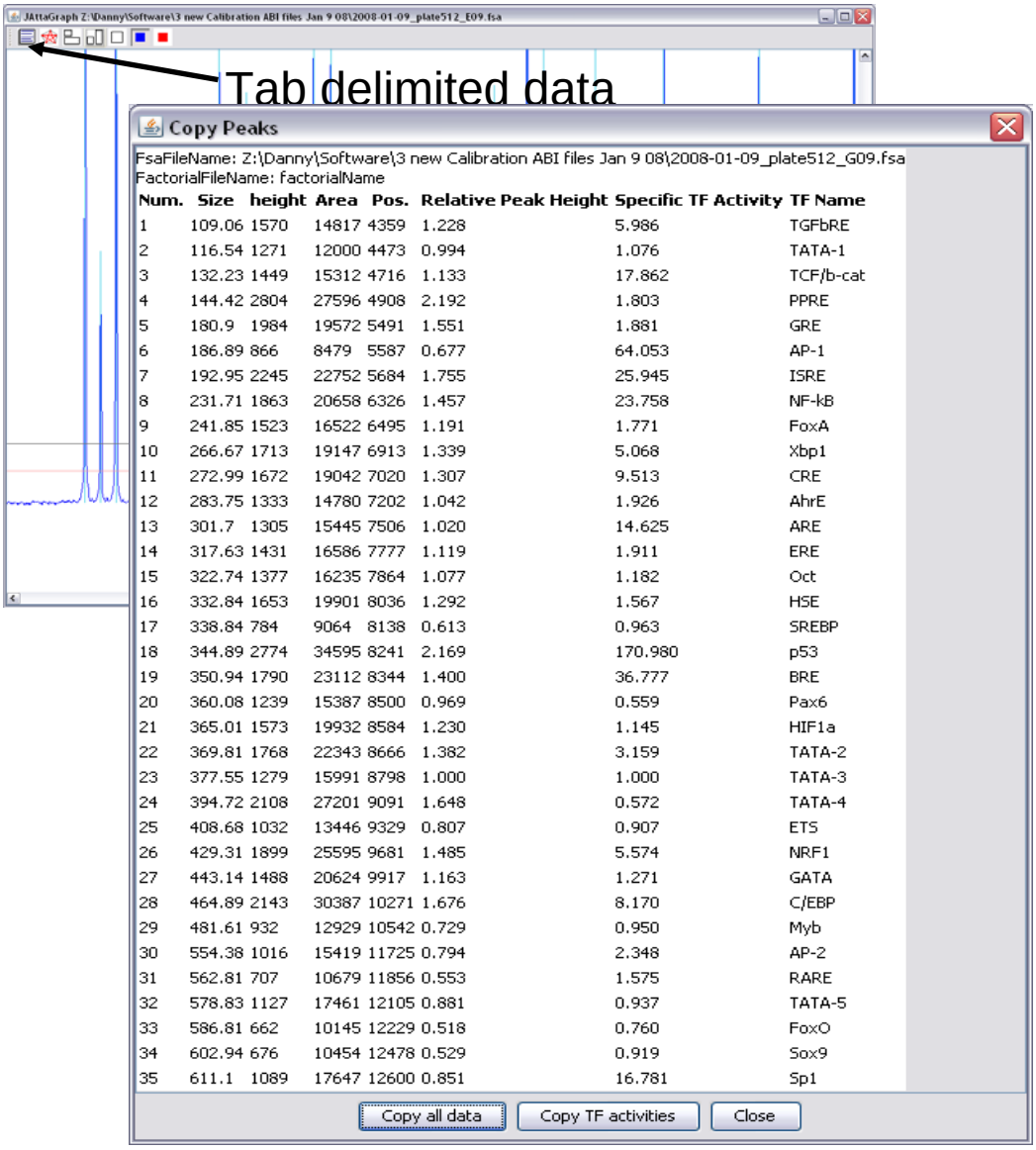


7) The following window will open and allow you to double check that the correct calibration files are present; after review, select “calibrate”. Then you will see the dialog box populated with the differences between Pre-set and calibrated values, which is shown below. After review, select “Save Factorial”. Calibration is now complete and you can close this window.



Note: The size difference between pre-set and calibrated reporter values should be no more than 3 base pairs, which corresponds to three integers in the table. For example; 364 should never go higher than 367 or lower than 361.

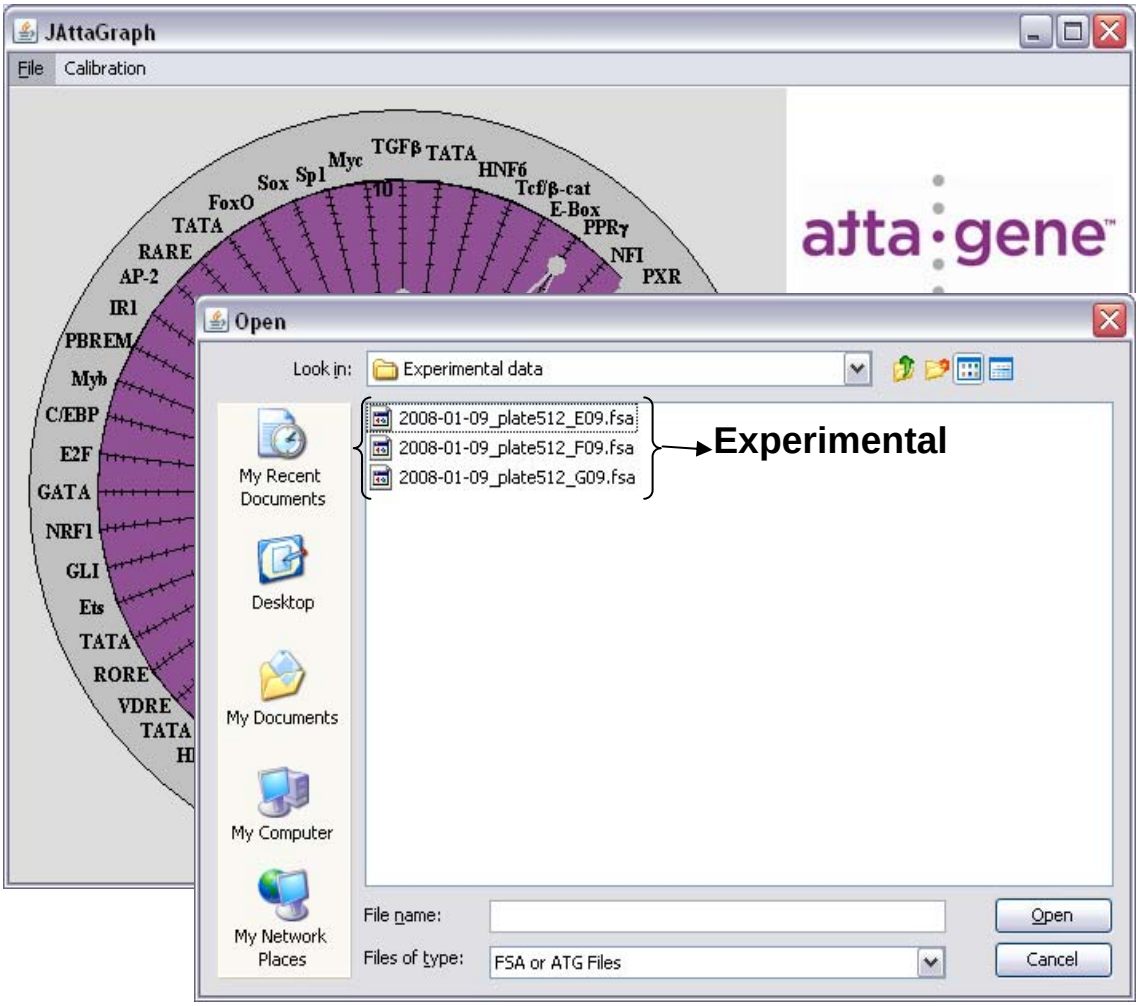
8) Open another calibration file and set grey threshold to cross all calibration (blue) peaks, then make sure there are no un-named peaks and all major peaks have positive values. You can open the tab delimited data to review these values by selecting the “show peaks data” button below.



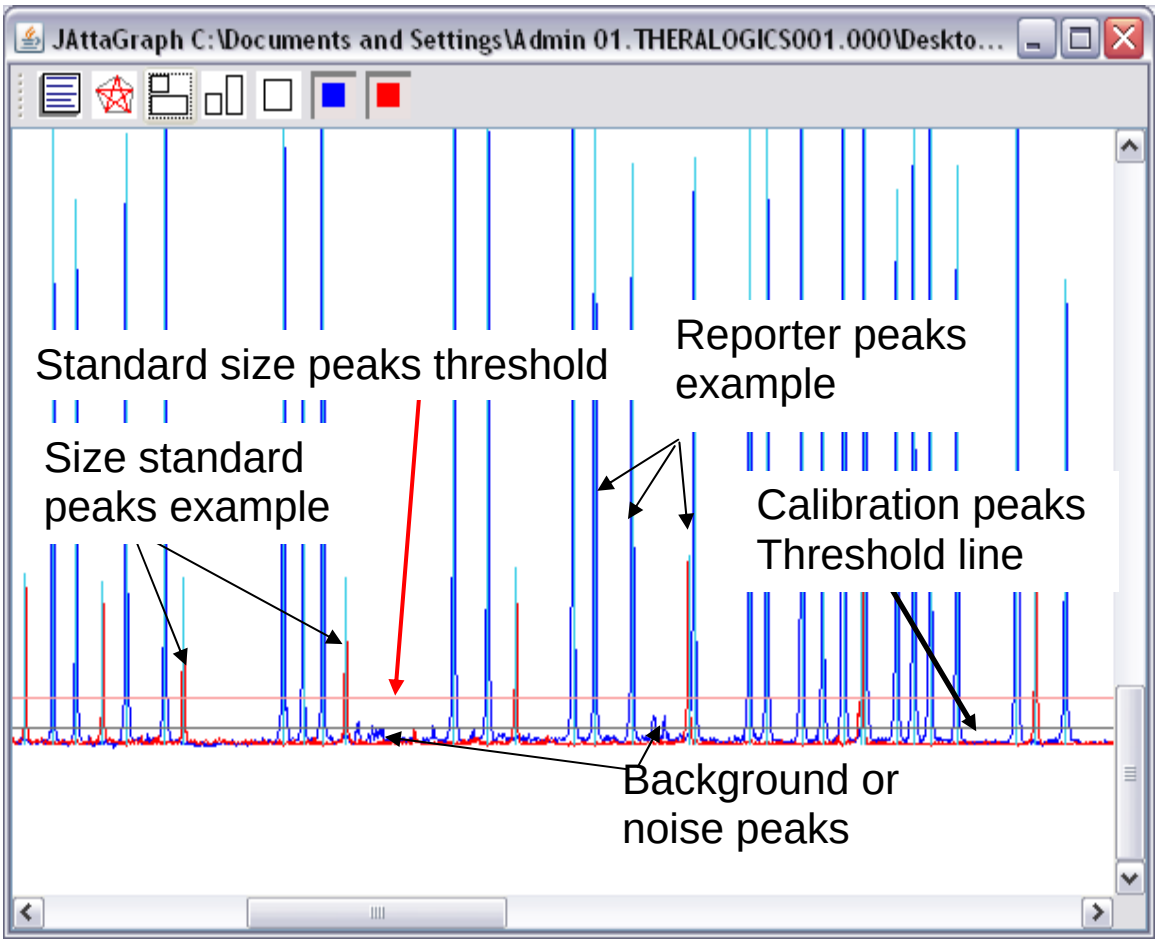
9) Once you have checked to make sure all calibration peaks have positive values and there are no un-named peaks, you may close these two windows and proceed to analyzing your experimental data.

IV. Analyzing experimental data:

1) Go back to main frame window and select “file, open”, to browse for an experimental data file. Select the file you want and click “open”; as shown below.



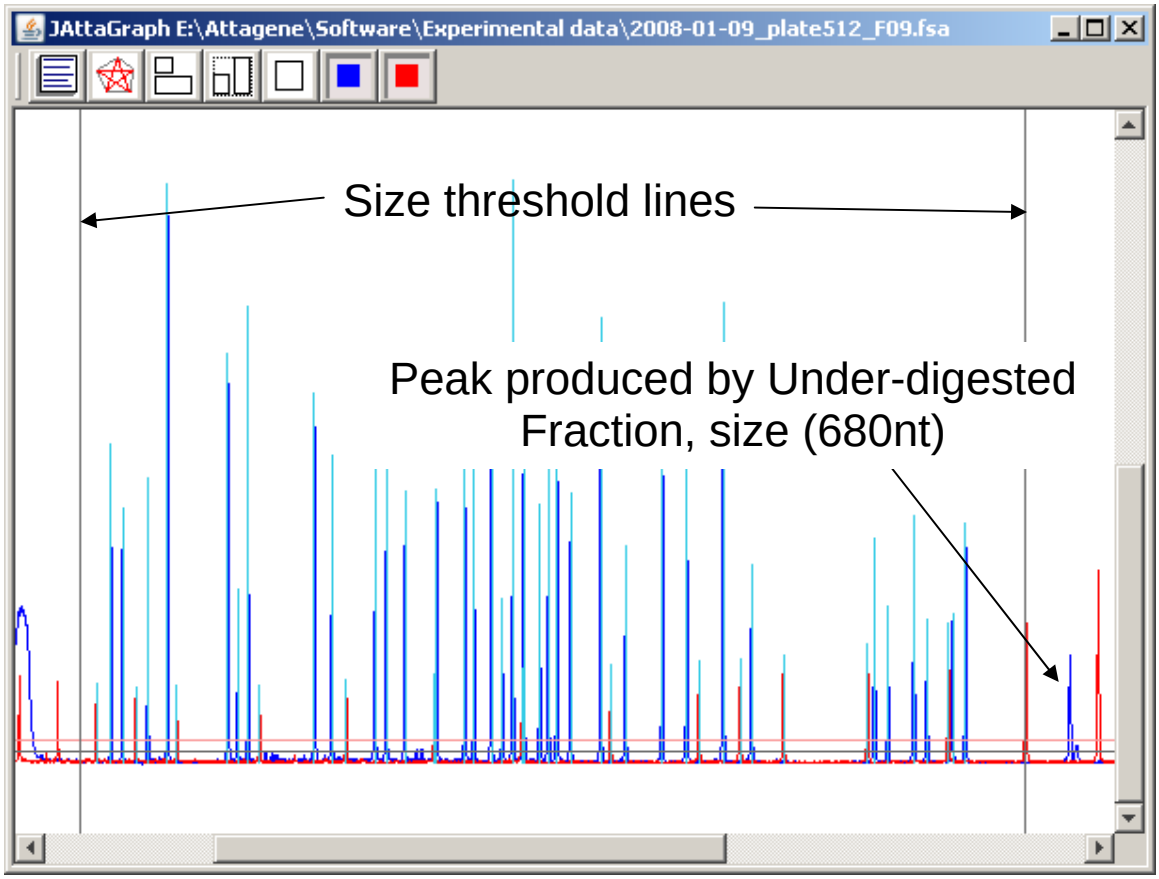
2) When data opens you will see a graph similar to the one below. This is your experimental data.



One can select or deselect the red (size standard) peaks or blue (reporter) peaks to view them separately.

3) Unlike a calibration sample, in the experimental sample some TF reporter peaks may not be present due to low corresponding TF activity. We recommend placing the threshold as low as possible; but not below 50 fluorescent units. Check the value of the lowest TF activity peak crossed by the threshold and make sure that it's not below 50 fluorescent units.

4) One should also look at the under digested fraction peak, which is the 1st blue peak after the horizontal size threshold lines. This peak should not be very high and if so one should refer to problem 2 of the trouble shooting guide. An example is below.



5) After you have checked the under-digested peak and adjusted the threshold you may extract the TF activity data.

6) To look at the tab delimited data, select the “show peaks data” button in far left corner of the tools menu. You will get another pop-up window showing the data.

Table 2
Standard size table

Standard 1	100
Standard 2	125
Standard 3	150
Standard 4	200
Standard 5	250
Standard 6	300
Standard 7	350
Standard 8	400
Standard 9	450
Standard 10	475
Standard 11	500
Standard 12	550
Standard 13	600
Standard 14	650

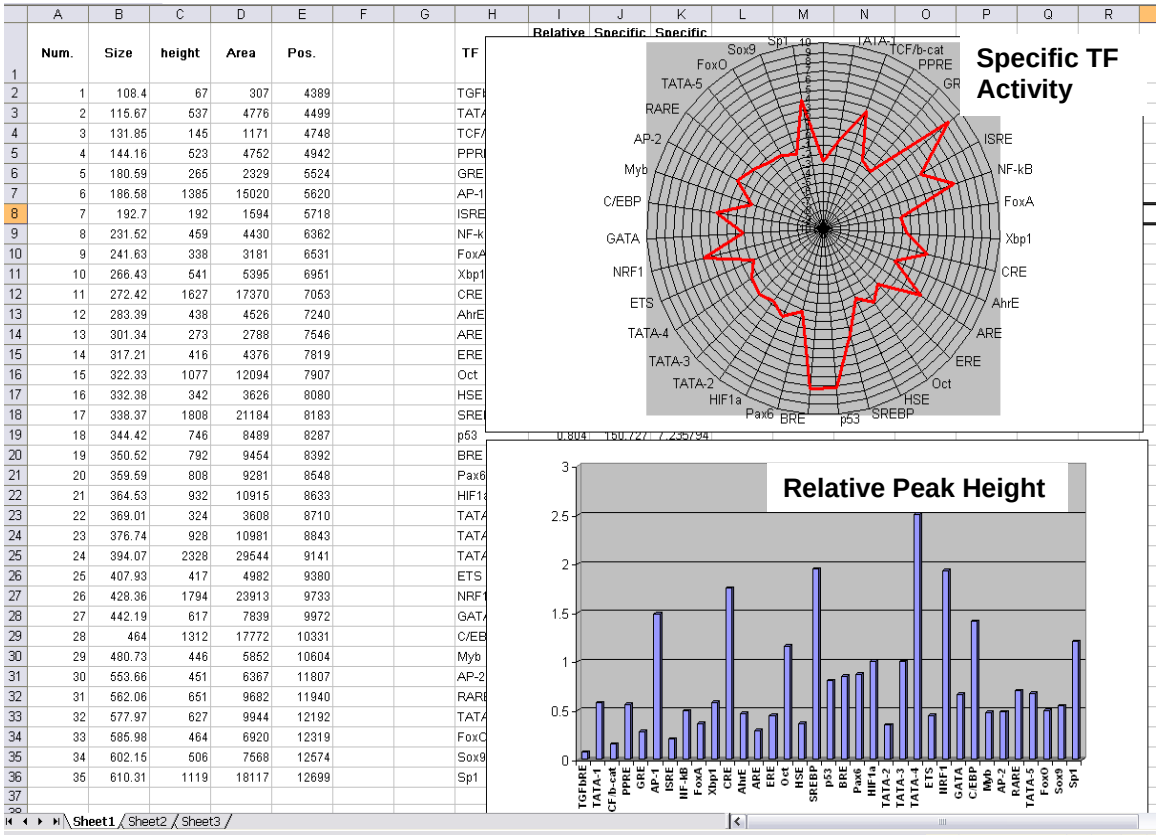
Note: There should be 35 blue (TF activity peaks) and 14 red (standard size) peaks that you should see in this view.

Note: The standard size peaks values should match the values in Table 2 above.

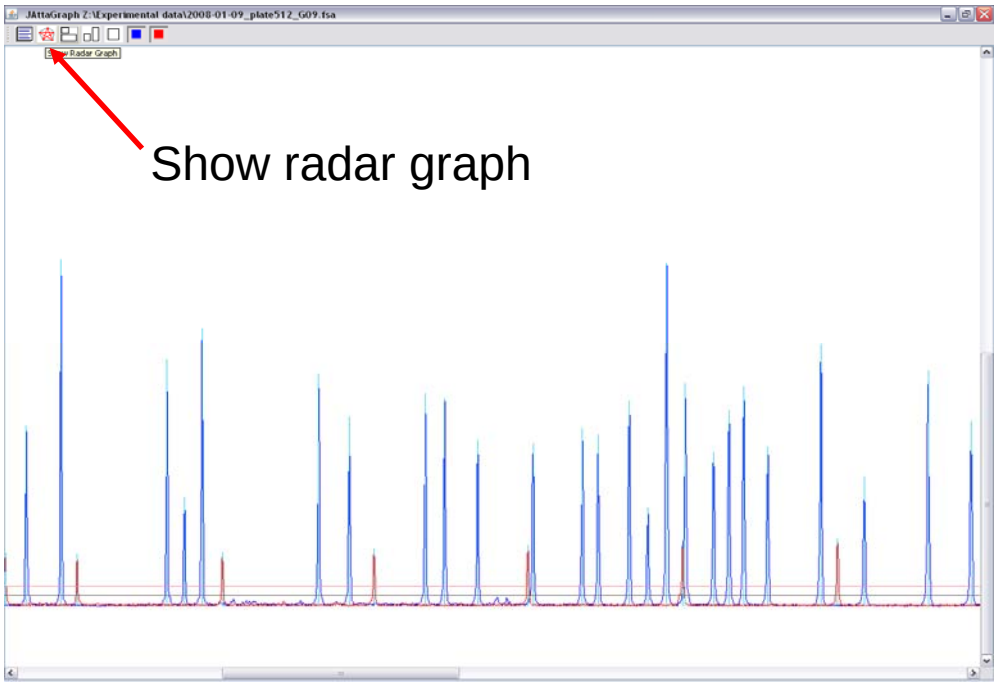
Note: If you do not see the red size standard peaks, you may have deselected them in the graph; this would be the case for blue TF activity peaks if you had deselected them as well.

7) You can now select all data or only specific TF activities for copy and paste into Excel. To copy all data, click on, “copy all data” button, and open Excel; then select paste, or right click and paste. To copy only TF activity, click on the “copy TF activity” button and follow the same route as the copy all data. Once you are satisfied with the data you can view the data in a radar style graph within our program. Alternatively you can also do this in Excel. You can close this window when done.

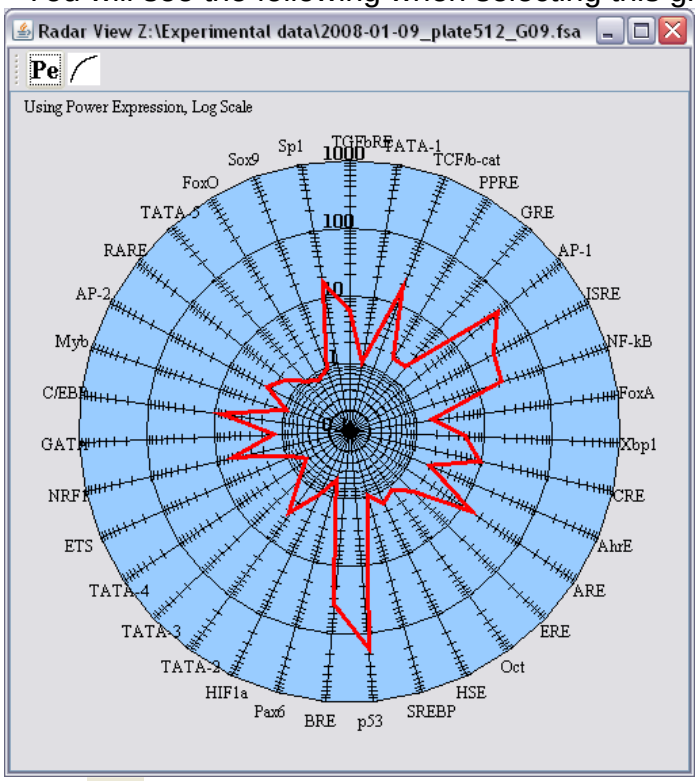
Below is an example of all data copied to Excel and radar graphs drawn of specific activity and histograms drawn of relative peak height for the 30 TF’s.



8) You can also view your data graphically in our software, to view the data in Radar graphs within our software, select the Attagraph viewer button as shown below .



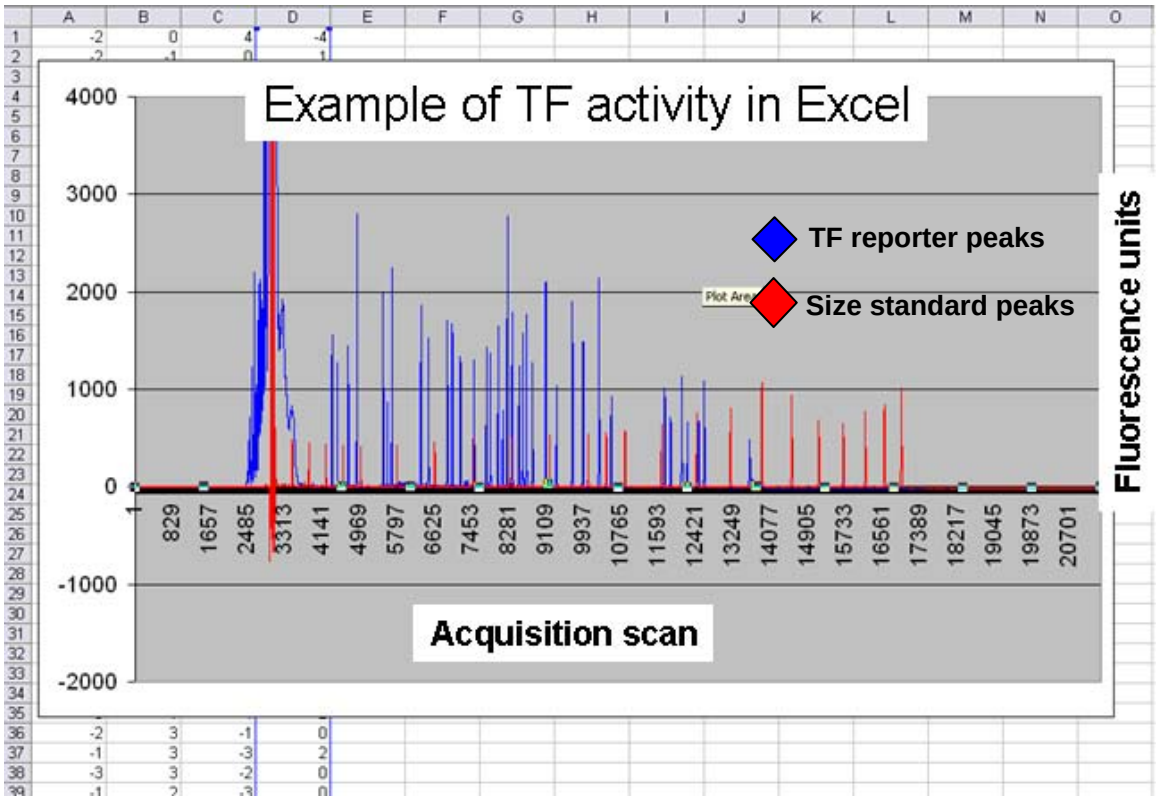
- You will see the following when selecting this graphing feature.



- The button toggles between TF activity and relative height of corresponding reporter peaks.
- The button is for scale and can be toggled from linear to log base$_{10}$.

9) Not only can you look at your graphs, but you can save the raw data in two formats,
    a. An .atg file – this is the **recommended** way to save the raw data, and it will have experimental data attributes attached, so one may look at the data later and have all relevant data from the experimental run. Once you have data saved as an atg file, you can open data without worrying about Factorial version used or calibration files. We recommend naming the .atg file the same as the .fsa file to save on confusion.
    b. A .csv file – which saves the raw capillary electrophoresis values, and could be used with other spreadsheet applications that require this type of file. This file shows the data in the raw form and has 4 columns for each of four channels, blue, green, black, and red. The blue (TF activity) is column A, while the red (size standard) is column D. Columns B and C are for other channels that are not currently used. Each scan from the capillary electrophoresis machine is assigned a fluorescent value. You can open this data in Excel and draw graphs, change scales.

Below is an example of a CSV file opened in Excel and line graphs drawn for the corresponding data.



Troubleshooting:

Problem #1: Small or No peak heights for the Reporter (blue) peaks between the vertical gray lines but your standard (red) peaks look fine.

Cause: The sample was not added into the master-mix plate before loading onto the capillary electrophoresis machine.
Solution: Rerun the samples with both the size standards and the labeled experimental sample present.

Cause: Not enough PCR product in your primer extension reaction.
Solution: Repeat the PCR (step VI) and increase the number of cycles up to 35 cycles. Make sure that you have used a Mass Ladder to correctly assess the amount of DNA from your PCR reaction.

Cause: The Primer Extension labeling reaction failed.
Solution: Make sure that the labeling reaction protocol on your thermocycler is the same profile as indicated in the protocol (Note: only 1 cycle is used).
Solution: The Primer Extension reaction was lacking a component, repeat the Primer Extension protocol.
Solution: The fluorophore conjugated oligonucleotide was stored improperly or the expiration date has passed.

Problem #2: Small or No peak heights for the Reporter (blue) peaks, except for the 680 nt peak (Full lenght) from capillary electrophoresis but your standard (red) peaks look fine.

Cause: Incomplete digestion of the Labeled DNA.
Solution: Repeat the labeling and digestion procedure. Make sure that you use the recommended incubation temperature and duration. You may increase the digestion time to at least 5 hours. Also you can run aliquot on gel; see picture below.

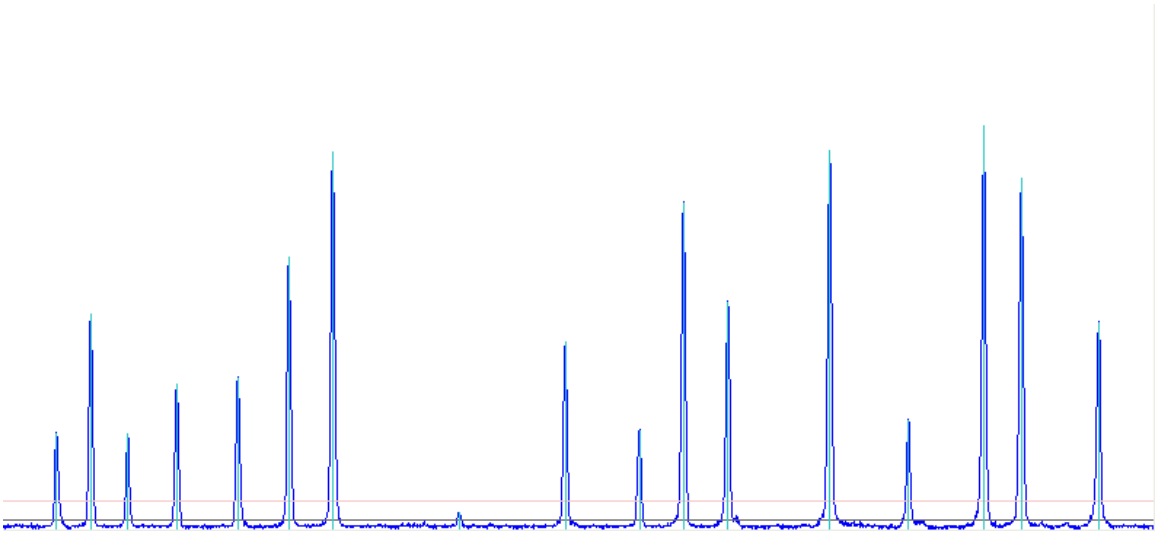
Problem #3: Many TF's have low or zero value
Note: It is common for some TF's to have low corresponding values

Cause: Blue Threshold is not correctly adjusted.
Solution: Adjust the blue threshold you must open the preference file under the File option:
Cause: Corresponding TF activity is low.
Solution: Try another cell type
Solution: Try another version of FACTORIAL.
Solution: Call us for custom design.

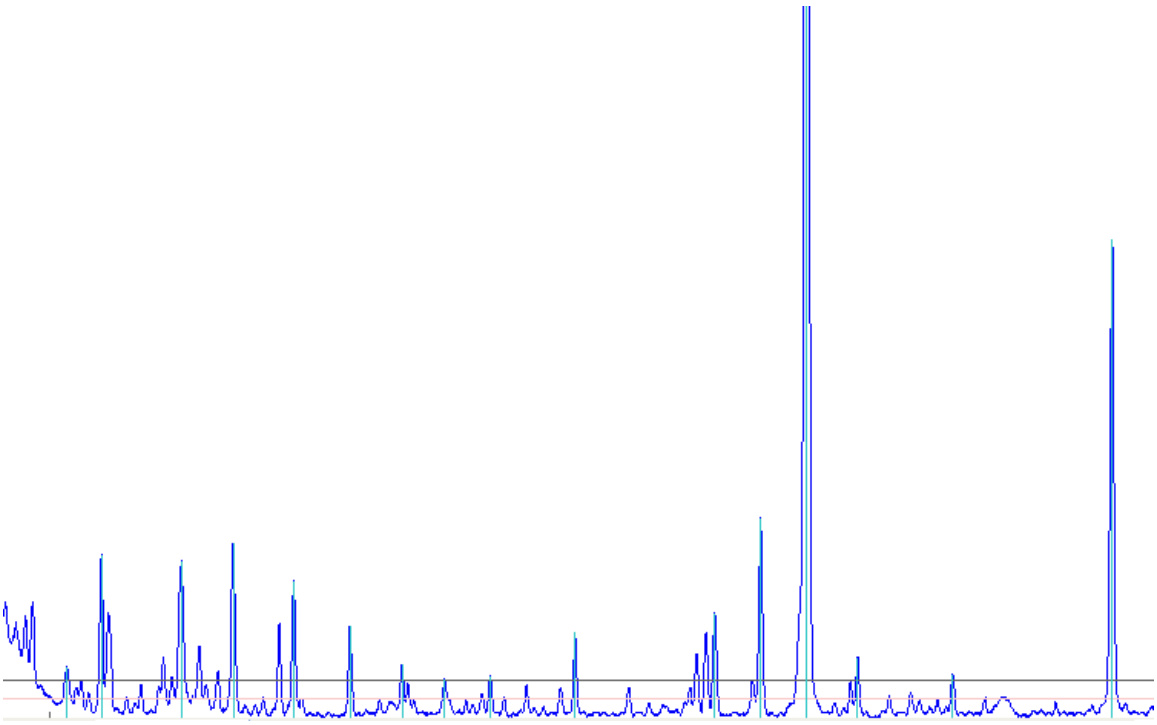
Problem #4: Blue and / or Red peaks atypical as the capillary run progresses.
Cause: 1) Capillary Array may be overused and need replacing
2) Running buffer is old
3) POP 7 is expired
Solution: Replace defective reagents or array and re-run your samples.
Cause: Instrument settings incorrect.
Solution: Use correct settings, refer to appendix A.

Problem #5: Extra peaks are seen in the capillary electrophoresis plot.

This is a normal profile:



This profile below has extra peaks.



Cause: Too much PCR product was used for the labeling reaction.
Solution: Recheck the amount of PCR product against the mass ladder. If there is too much PCR product present then use 1:20th of the reaction for labeling.

Problem #6: Some or all of the Reporter (blue) peaks from the capillary electrophoresis plot are unnamed.
Note: It is normal to have some un-named reporter peaks, this is not an issue unless most peaks are missing.
Cause: The molecular weight standards that were added to your labeled and processed DNA sample were not the standards supplied with your kit.
Solution: Rerun your samples but use only the supplied capillary electrophoresis standards.

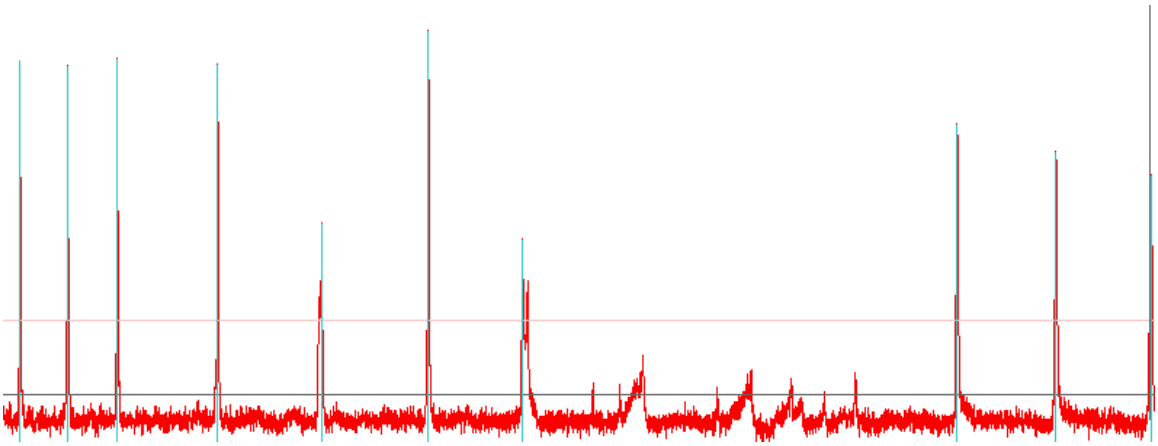
Cause: The calibration run has not been performed to help ATTAGRAPH determine where the reporter peaks migrate on your capillary electrophoresis machine.
Solution: Run the calibration standards supplied in your kit and load the .FSA file into ATTAGRAPH.

Cause: Too much PCR product was used for the labeling reaction and parasite peaks have occurred.

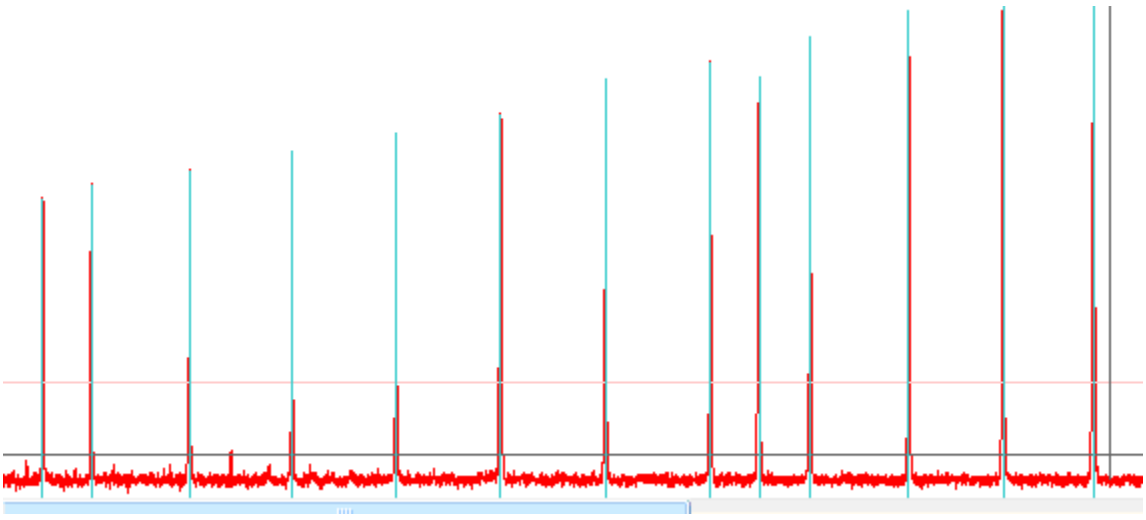
Solution: Recheck the amount of PCR product against the mass ladder. If there is too much PCR product present then use 1:20 of the reaction for the labeling reaction.

Problem #7: Size standard signal is atypical.

See representative example below:



Compared to the normal profile below



Cause: Most of the named reporter peaks are missing.
Solution: adjust threshold

Cause: Sample isn't clean
Solution: purification of PCR product is necessary, with the proteinase K step.
Solution: Replace defective reagents or array and re-run your samples.

For Technical Support please contact us at info@attagene.com or call 888-721-2121.

Appendix: A

Settings used on the ABI 3130 XL Genetic analyzer Electrophoresis machine for fragment analysis of the FACTORIAL™Reagent.

Name	Value	Range
Oven_Temperature	60	18...65 Deg. C
Poly_Fill_Vol	6500	6500...38000 steps
Current_Stability	5.0	0...2000 uAmps
PreRun_Voltage	15.0	0...15 kVolts
Pre_Run_Time	180	1...1000 sec.
Injection_Voltage	1.2	1...15 kVolts
Injection_Time	23	1...600 sec.
Voltage_Number_Of_Steps	20	1...100 nk
Voltage_Step_Interval	15	1...60 sec
Data_Delay_Time	60	1...3600 sec.
Run_Voltage	10.0	0...15 kVolts
Run_Time	3000	300...14000 sec.

Appendix: B

End user license agreement:

This End-User License Agreement ("EULA") is a legal agreement between you (either an individual or a single entity) and Attagene Inc. This software is the copyright of Attagene, Inc. and is made available for analyzing data generated during the Factorial assay. This software is not intended for use with any other application. This software can't be redistributed whatsoever, and is not for commercial use. This software is solely for use by purchaser of factorial reagent kits and not for use with any other application This software can not be redistributed in the original or modified form. Not responsible for incompatibility issues with the JAVA interface. Java is a trademark of Sun Microsystems.

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