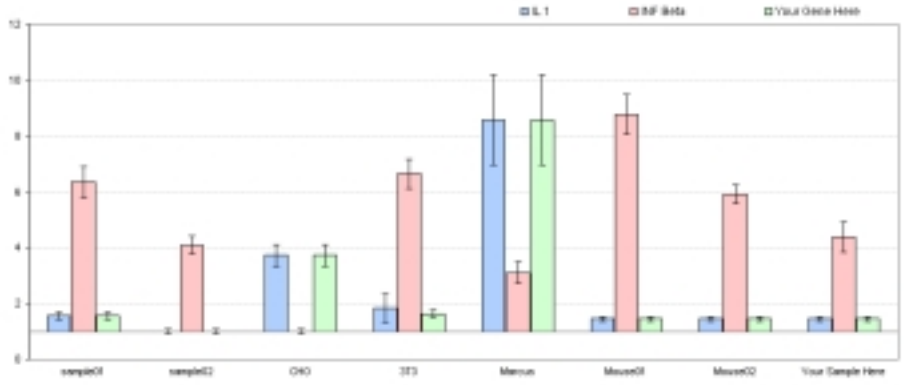
Gene Expression Macro™
Version 1.1

Instructions



Bio-Rad Gene Expression Macro Users Guide

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Bio-Rad Amplification Products . . . . . http://www.bio-rad.com/amplification/

Introduction

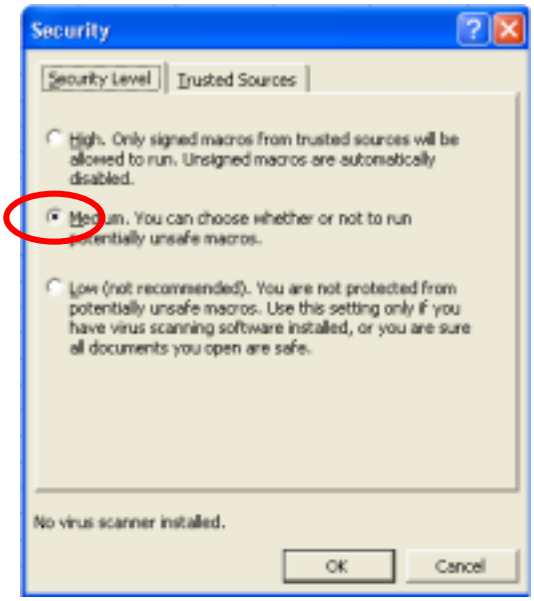
The Gene Expression Macro is an application built using Microsoft Excel.

There are several “quick ref” tabs located in the macro that can be used to guide you through the use of the macro. You can access them by clicking on these tabs or by clicking on the “HELP” button located on each tab. They can be used to familiarize yourself with the macro.

Opening the application:

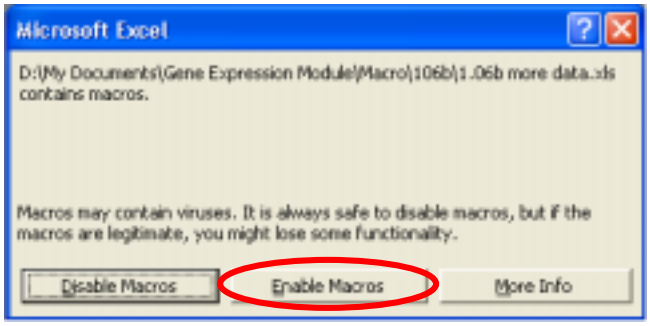
First enable medium level macro security in Microsoft Excel. Open a new Excel document and go to the ‘Tools’ menu and choose the ‘Macro’ sub-menu.

The Security Dialogue Box will look like this. The macro requires you to choose medium level of security.



Now open the macro:

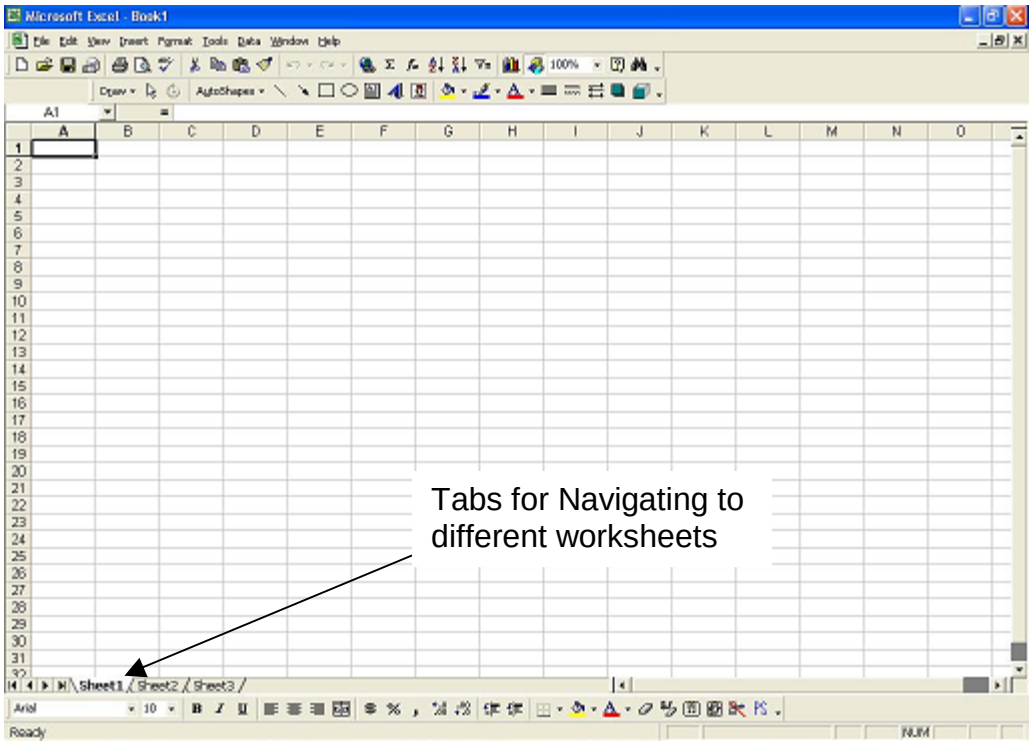
1. Double click on the genex.xls icon.
2. A dialogue box will appear asking you if you would like to enable macros
3. Choose “Enable Macros” (this is not the default choice)
4. Use the “Save As” function under the file menu to save the macro using a file name of your choosing.
5. Save your data frequently during use.



Excel Workbook Organization and Features

If you are familiar with Microsoft Excel you may want to skip this section.

When you open Microsoft Excel you will be presented with what is referred to as a workbook. A workbook is a series of spreadsheets. Each spreadsheet contains information that may or may not relate to information on other spreadsheets in the workbook. You can navigate to the various spreadsheets using the tabs located on the bottom of the interface.



The Gene Expression Macro is an Excel workbook with specialized functions that allow you to analyze your data. Editing your plate setup in the macro will be similar to normal Excel editing however all other functions are very specialized to this macro.

How the Macro Works

For each sample in your experiment you must indicate the gene being measured, and the sample's name or identity. You can designate replicates by naming wells with the same identifier. Any identifiers from the iCycler iQ® or the MyiQ™ software will be imported and placed in the plate setup in the macro.

You may or may not choose to express your results relative to a control sample (calibrator). This can be a different sample for each gene although the macro gives you the option of making the control sample the same for all genes. All other samples will be compared to this control. The control will have an expression level of "1" and is equivalent to the "calibrator" described in Livak et. al. Methods, 25, 402-408, 2001.

Gene Expression Analysis Methodology

The calculations in this spreadsheet are outlined in the methodology tab of the macro. They are derived from the algorithms outlined by Vandesompele et al. (*Genome Biology 2002, 3:research0034.1-0034.11)* and from the geNorm manual and associated sample calculations which are available on the web site *http://medgen.ugent.be/~jvdesomp/genorm/*. This web site is an invaluable guide to researchers who are interested in better understanding the issues surrounding the use of reference genes (housekeeping genes) for evaluating expression levels.

The calculations allow you to analyze results using multiple reference genes. This often results in more consistent results and more confidence in the conclusions drawn from your experiments.

As demonstrated in the example calculations on this web page this algorithm does take efficiencies of each assay (primer set) into account.

Other Methods of Expression Evaluation

All the methods discussed build upon the work of Livak et al (Methods, 25, 402-408, 2001) and are rearrangements of the same equation. If you assume efficiencies of 100% (or 2) you can simplify either the modification made by Pfaffl or Vandesompele to the equation outlined in the Livak paper. Each has built upon the work outlined in previous groups' publications.

The Pfaffl Modification

Like the Pffafl modification of the $\Delta\Delta C_T$ equation (Pfaffl et al, Nucleic Acids Research, 2001, Vol. 29, No 9 00, pg. 2002-2007) this macro allows you to take efficiencies of your individual genes into account. To exactly reproduce the results you would obtain using the Pfaffl model you must define a control sample (calibrator sample in the publication). The macro will express all values relative to this sample. The control sample will be given a value of 1.

The $\Delta\Delta C_T$ equation

If you choose to ignore differences in efficiencies or prefer the $\Delta\Delta C_T$ approach (Livak et al.) leave all efficiencies at 100%. The calculations will be the same as those achieved using the $\Delta\Delta C_T$ approach, provided you identify a control sample (calibrator) in your import tab.

Using the Macro

The following will guide you through the use of the tool.

Recommended Workflow

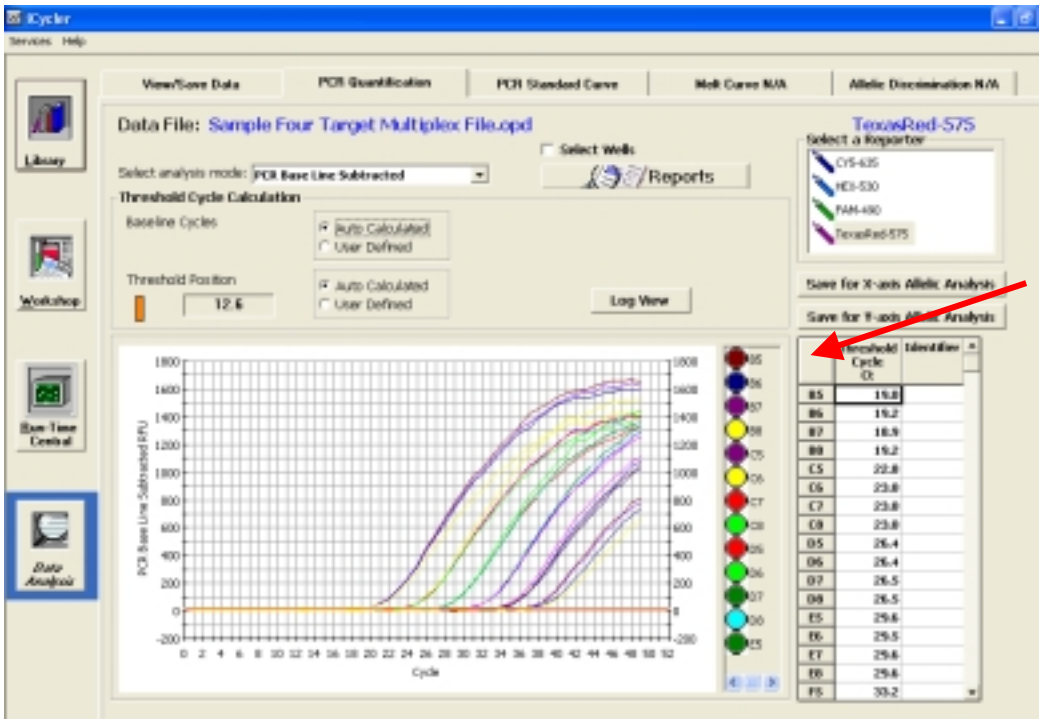
1. Import all data into import tabs
2. In one tab only
    a. Assign gene names and roles (reference gene)
    b. Assign sample identifiers if necessary
    c. **Optional.** Assign control sample if you want fold expression expressed relative to a particular sample.
3. If you have multiplex data use “copy types and identifiers to all other import tabs” button to copy sample information to all tabs
4. Assign genes in other import tabs
5. Press analyze button.

Note: Calculations require that at least one gene be designated a reference gene.

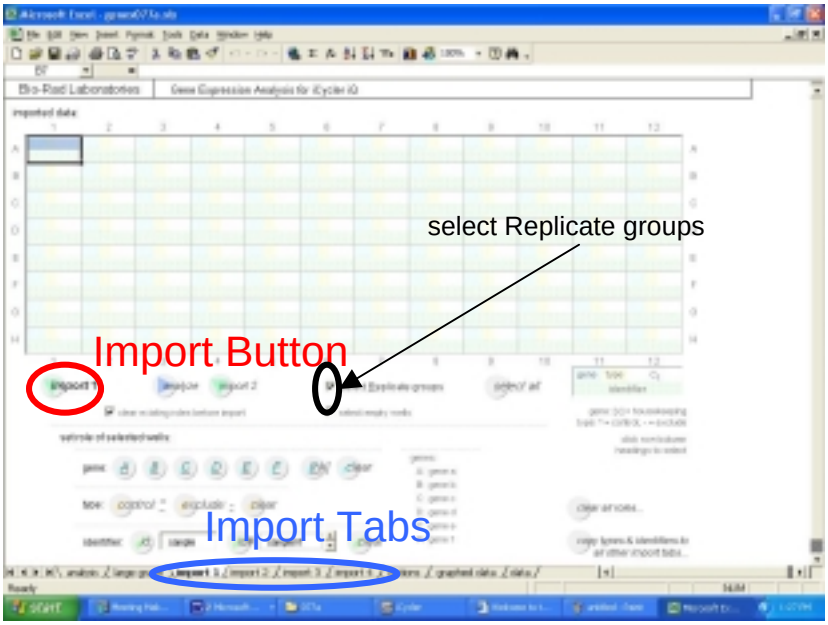
Importing Data

Data should be copied from the PCR Quantification tab in the iCycler iQ or MyiQ software. An image of this tab is shown below.

1. Click in the upper left corner of the Threshold Cycle Chart located to the right of the amplification plots.
    - Make sure the entire chart is highlighted black
    - Make sure you have optimized threshold settings if necessary
2. Press ‘Ctrl’ + ‘C’. This will copy the data to local memory.



3. Navigate to the “import 1” tab of the macro.
4. Click on the “import 1” button located to the bottom left of the plate interface.



5. Your “plate” will now be populated with the data from your experiment. Any identifiers used in your data from the iQ or MyiQ software will be applied to the appropriate well.
6. If you have more than one dye layer (multiple fluorophores) you must import the data from each fluorophore into its own tab.


Make sure the data you import matches the data you intended to use, especially when you are switching between fluorophores. In order to get the updated data it is sometimes necessary to un-highlight and then highlight the C_T chart in the iCycler iQ or MyiQ software. Click somewhere else in the chart and click in the uppers left corner again. This is only necessary in certain versions of Windows operating systems.

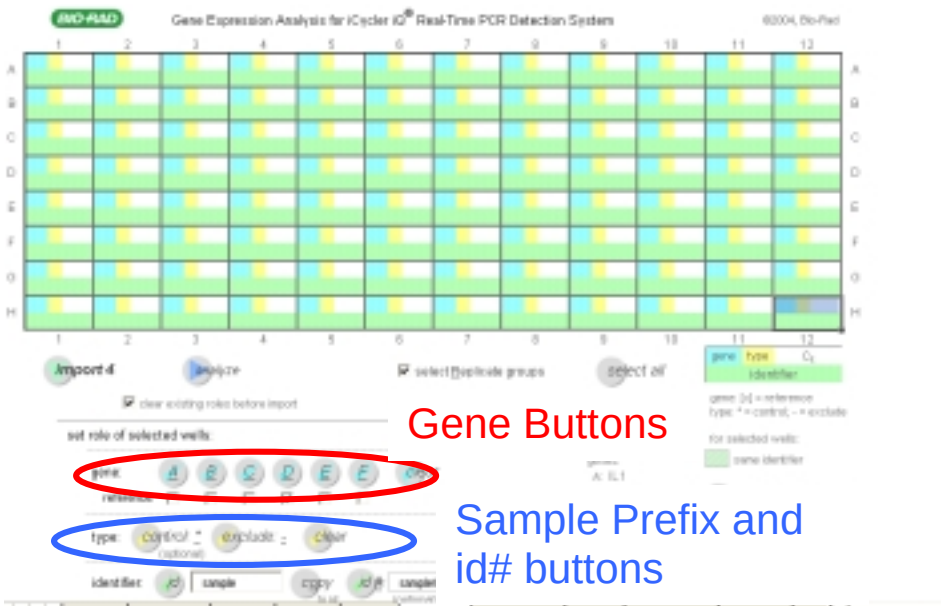
Editing samples once the data is imported

Note that the plate interface in the import tab should now be populated with C_T values as well as any identifiers if they were present in the iQ or MyiQ data set. You can edit all characteristics in this interface.

Hint: Completely define all samples in one import tab before proceeding to the next. All attributes other than gene name can be copied into other tabs by clicking on the “copy types and identifiers to all other import tabs” button.


Sometimes if you already have identifiers assigned in your data the macro will group samples as replicates which you do not consider replicates. Turn off the “**select Replicate groups**” check box indicated in the second figure on page 7of this guide.

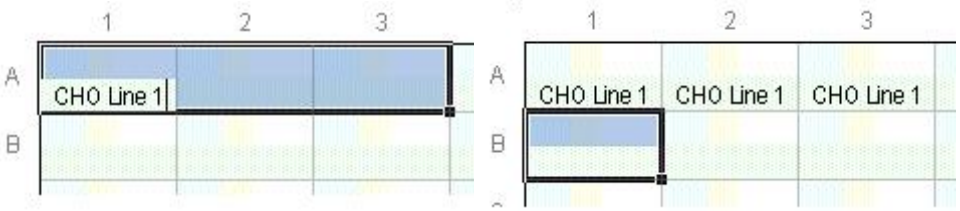
1. First assign the gene being measured in each sample. Select wells and then press the appropriate button. This is done by using the “gene” buttons located under the plate setup. You must assign a letter A-F for each gene.



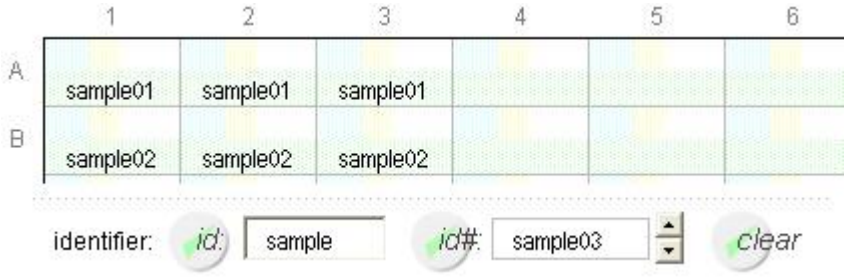
a. You can customize each gene in the “analysis” tab. E.g. you can assign a name to gene A (Gene A = Protein Kinase C)
b. You can assign these customized names prior to doing anything else by going to the analysis tab first. This is done on the analysis tab. See the figure on top of page 13.

2. Designate one or a number of genes as reference (housekeeping) gene(s). Do this by selecting the reference check box located under each gene button. This will convert the gene label in the plate to a lower-case bracketed letter (A to [a]) and highlight it gray. This indicates that this gene is a reference (housekeeping) gene.

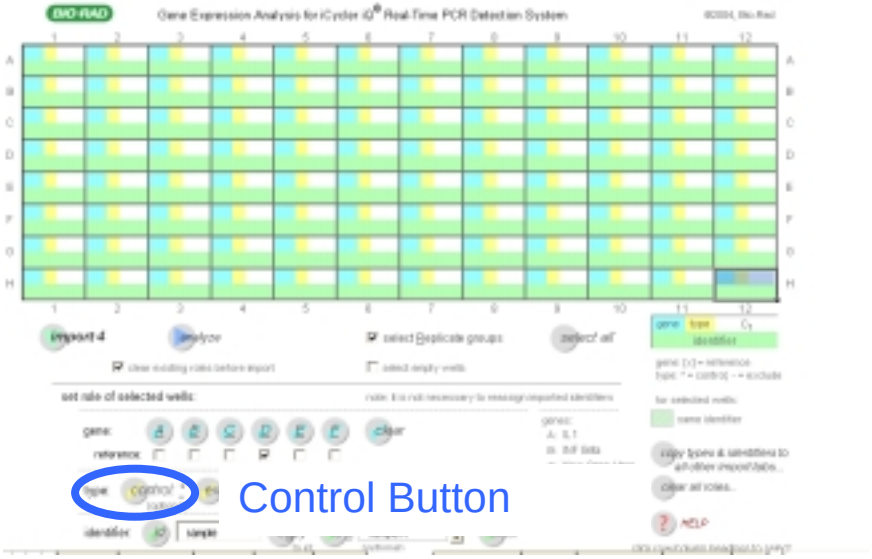
3. Now edit identifiers by selecting the well or wells you want to edit and typing the name you want to apply. When selecting multiple wells all wells will update after you hit enter or click someplace else.



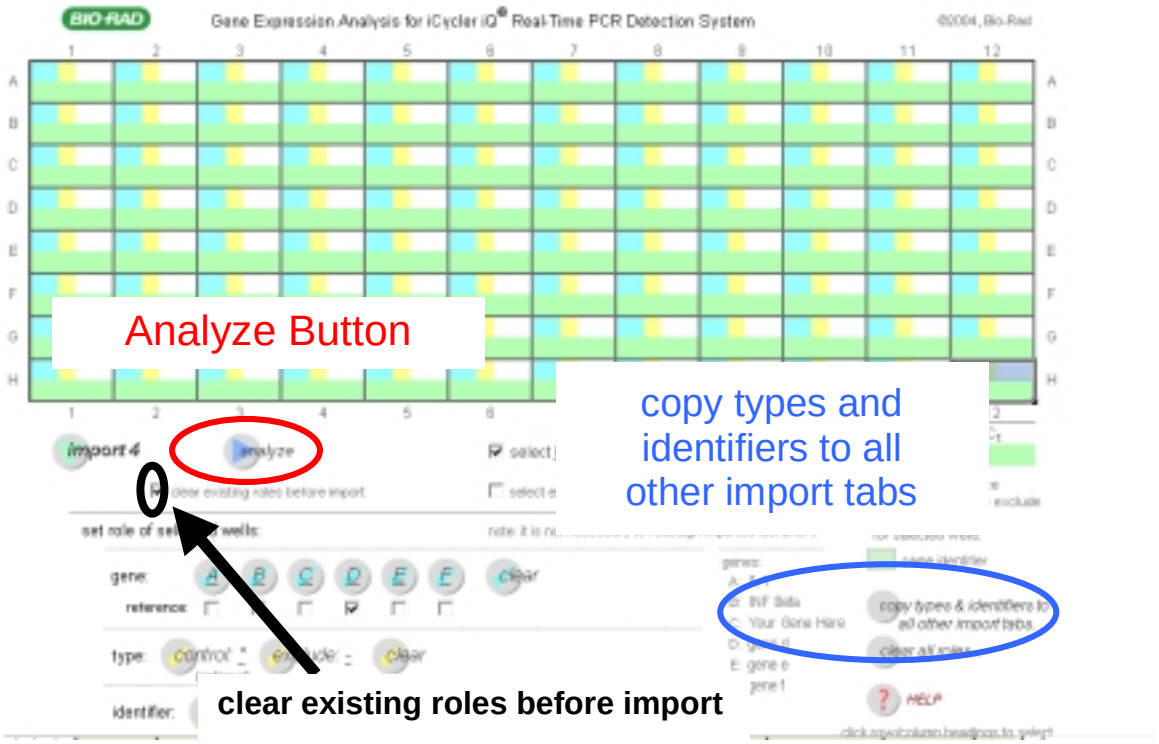
Alternately, you can select an identifier prefix like “mouse” and use the “id #” button to identify your samples as mouse 1, mouse 2, mouse 3, or you can use the default prefix of “sample”.



4. **Optional:** Choose a control sample for your analysis. Highlight the sample and press the control button. It is the sample that will have an expression value of 1 and to which all other samples will be compared.



5. If you are analyzing a multiplex experiment you should first import each additional dye layer and then copy all the identifiers and control information into the remaining tabs by pressing the "**copy types and identifiers to all other import tabs**" button. This will copy all information from the active tab to all other tabs in the workbook. You will still have to assign gene designator (A-F) and role (Reference) to the appropriate gene(s).



Analyzing Data

Once all identifiers genes and controls are assigned you can analyze the data by pressing any analyze button. This will automatically place you in the analysis screen where you can:

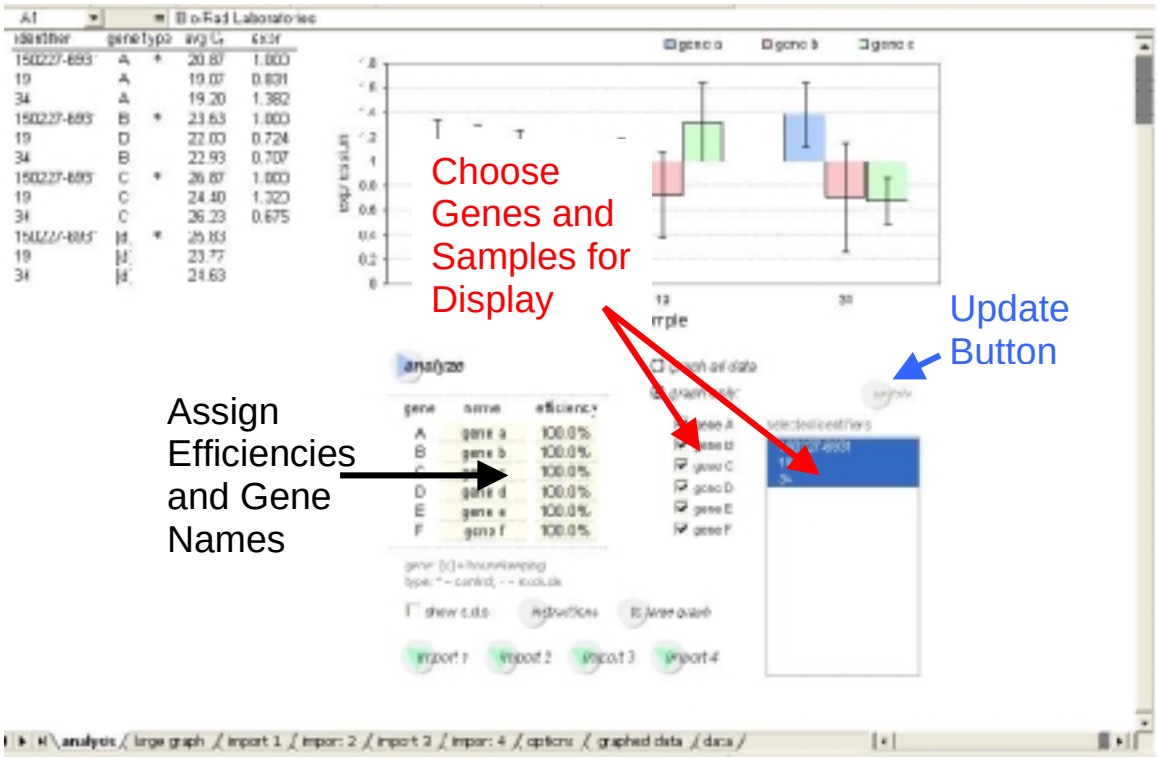
1. Assign efficiency to each gene
2. Assign a name to each gene
3. Select a subset of your data to view

For more on the functions in the analysis tab select the help button or click on the quick ref A tab.

Using the Macro as a Template

Many researchers want to use the same identifiers and gene assignments for every experiment. You can import data into a macro already populated with the appropriate sample and gene assignments. Set up import tabs as desired and turn off the check box marked "**clear existing roles before import**". This check box is located under the analyze button. (See the figure on this page)

You can now import data without clearing identifiers and gene assignments.



Additional Tabs (Not covered in workflow)

Large Graph

This is a larger presentation of the data on the analysis tab.

Options Tab

Choose among several options for the import tab and data presentation. Sort data, present y-axis in log scale and choose how to scale the data using this tab.

Graphed Data

If you want to export the calculated data for your own graphs you can copy the data from this tab.

Methodology

This is a brief outline of the calculations used in the macro.

Quick ref A

This is a guide to using the analysis tab.

Quick ref I

This is a guide to using the Import tabs.

Scaling and Controls:

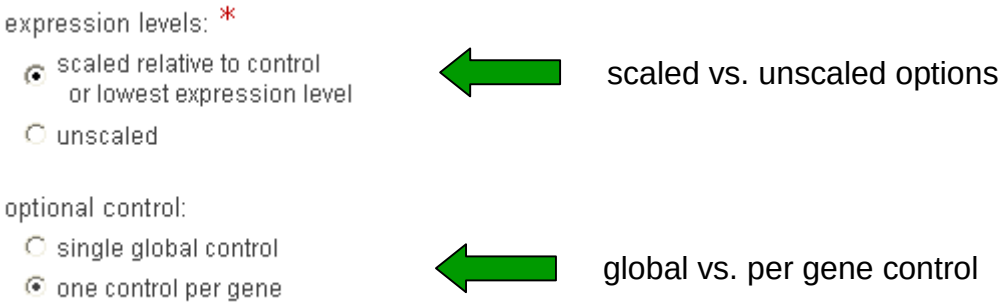
Scaling:

Scaling is nothing more than assigning a sample an expression value of one and rescaling all other samples relative to that sample's expression value. The macro as received from Bio-Rad has the scaling option on.

For each gene evaluated the lowest expressing sample will be assigned a value of one. This results in what the geNorm home page refers to as rescaled normalized expression.

If you choose the option labeled "unscaled" the sample with the lowest mean C_T (often the highest expressing sample) for each gene will have a value close to one. However no logic is applied to your results to ensure that any sample has a value of exactly one.

The following options can be accessed on the options tab.



scaled vs. unscaled options

global vs. per gene control

Controls:

You may choose a control to scale your results to the sample of your choice rather than one chosen arbitrarily by the macro. This is optional and done by highlighting a sample and using the control button located on the import tab (see page 11).

If no controls are assigned in the import tabs the results will be displayed as described above under scaling.

The macro will assign the control an expression value of one and scale all other expression values accordingly. This is useful if you want to easily evaluate fold expression relative to a particular biological condition. (e.g. no compound applied or wild type). This does not change the results of your experiment. The relative levels of expression will be the same from one sample to the next.

Interpreting Results:

Keep in mind that comparing the expression value of one gene to the value of another gene is not necessarily valid.

If a sample has a relative expression value of 2 for gene A and 2.1 for gene B this does not mean that gene A message levels are very similar to gene B message levels. Remember that all expression values are scaled relative to another sample in the experiment. Comparisons of expression within a gene are always safe. Comparisons within a sample from one gene to the next are not always valid.

Documented Behaviors

These are a few behaviors that may be different from what the user expects.

Explicit Sorting of Controls

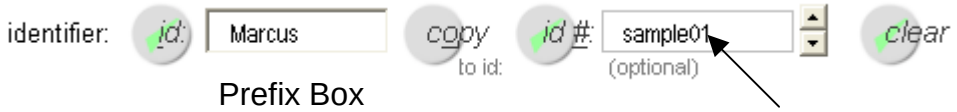
If you use the graph series order > explicit option on the options tab and you have a control defined, the control will be sorted to the end of the x-axis on the graph. Remove the asterisk located next to the control in the samples list and the control will sort in the order you specify.

Run time Errors

If the software recognizes a problem with the data structure it will usually indicate to you what it thinks the problem is. If you edit a gene in the plate setup without using the gene buttons under your plate you may get a runtime error. Check that all samples are assigned a gene A-F and have not been inadvertently edited.

Copy identifier from plate into auto increment field

This useful function works but requires that you click on the sample increment box indicated in the figure below. When you do this, the prefix will update to match the one in the prefix box.



Very High Expression Values

Very large expression values may not be viewable on the analysis tab but will appear as ######. To view these values go to the “graphed data” tab and resize the column width.

Acknowledgements

We thank Dr. Jo Vandesompele for his patience and guidance. We also thank him for granting us permission to incorporate the mathematical models used in this macro.