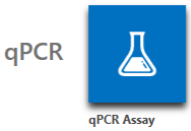
BMS Workbench Application Note 9:
Gene Expression Studies using Myra and Mic

Relative Quantification is a qPCR technique used to investigate the relative level of expression for genes of interest, with mRNA expression typically being investigated. Applications for this analysis type include the measurement of gene expression in response to a drug or comparing levels of expression of gene sof interest against an internal control or housekeeping gene(s). The *Mic* qPCR cycler has superior thermal uniformity, allowing for highly reproducible data across machines, with an added advantage of having this analysis type as a built-in feature of the *BMS Workbench* software.

This documentation will guide you through an example of setting up *Relative Quantification* analysis using an example workflow on the *Myra* and *Mic*, with analysis. The example provided examines the effects of 3 drugs on the expression of 3 genes of interest using 2 housekeeping genes as the baseline, and is intended for demonstration purposes only. You are encouraged to adapt this application note to suit your workflow.

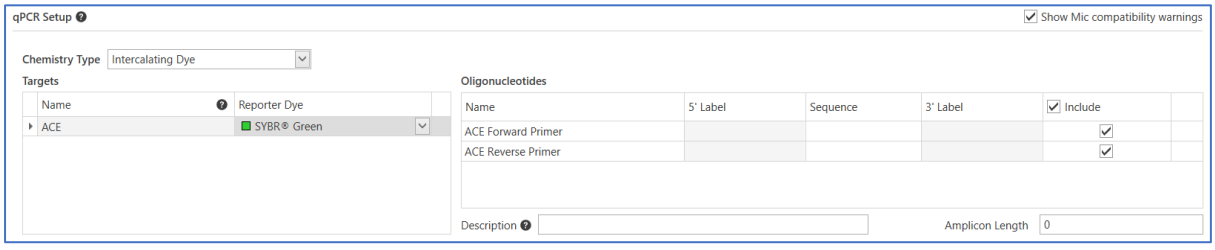
Step 1: Define the Assays

Define the *Assays* being used in **qPCR Run** and analysis. When using an *Assay* for the first time, it should be configured using the **qPCR Assay** icon on the **Start Page**.

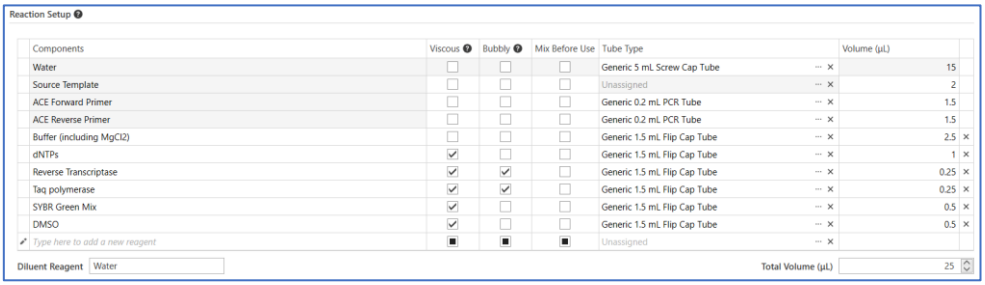


When using *Relative Quantification* analysis, your *Assays* should utilise the same *Chemistry Type*, *Run Profile* and *Reaction Setup* for compatibility on the *Mic* when loading samples. Components should also be named the same, so that when reactions are created on the *Myra*, it will utilise the same tube placed on the deck to setup all reactions.

In the provided example, 5 assays have been created for each gene being examined. It is important that each gene is defined as its own assay, as each gene needs to be assigned to a group and designated a role in *Relative Quantification* analysis. *ACE*, *ACTN3* and *Calcineurin* are the genes of interest, for which gene expression is being investigated. Houskeeping genes *B2M* and *GAPDH* represent controls. Each of the assays utilise a SYBR® Green *Intercalating Dye*. Forward and reverse primers are included in the final reaction, as reflected by the *Include* checkbox being ticked in the *Oligonucleotides* table.

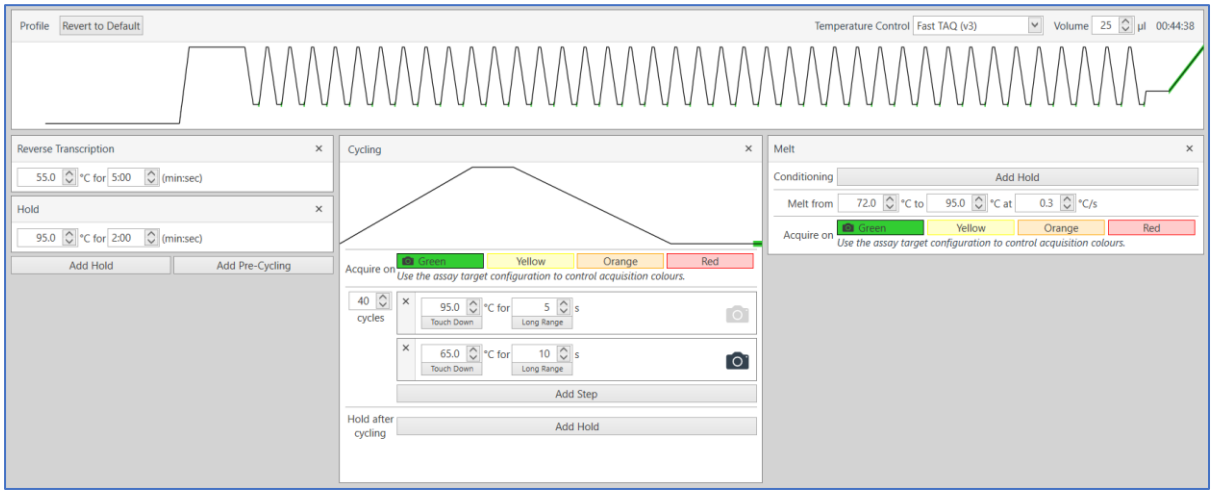


The same *Components*, including names, *Tube Type* and volumes, have been input into the *Reaction Setup* table.



No *Controls* are utilised in this assay setup; hence the *Controls* table has not been filled in.

An *Assay Profile* has been configured for each assay, taking care to ensure all cycling conditions are the same, including holds.



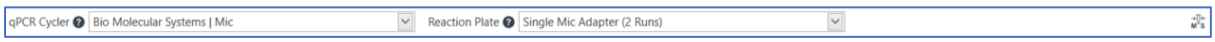
Importantly, no *Analysis Settings* have been configured for any *assay*. Usually, as in this example, *Relative Quantification* analysis requires the creation of a *Mic* **qPCR Project** file, to allow for comparison of each

Step 2: Setup Myra

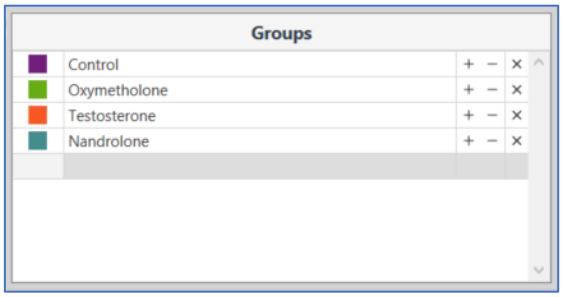
Create a new **Reaction Driven qPCR Setup** run file using the appropriate icon on the **Start Page**. If you are using a sample preparation method, you can choose to utilise the **Sample Prep and qPCR Setup** option on the **Start Page**, instead.

Add the number of *Assays* using the + symbol next to the *Assays* heading.

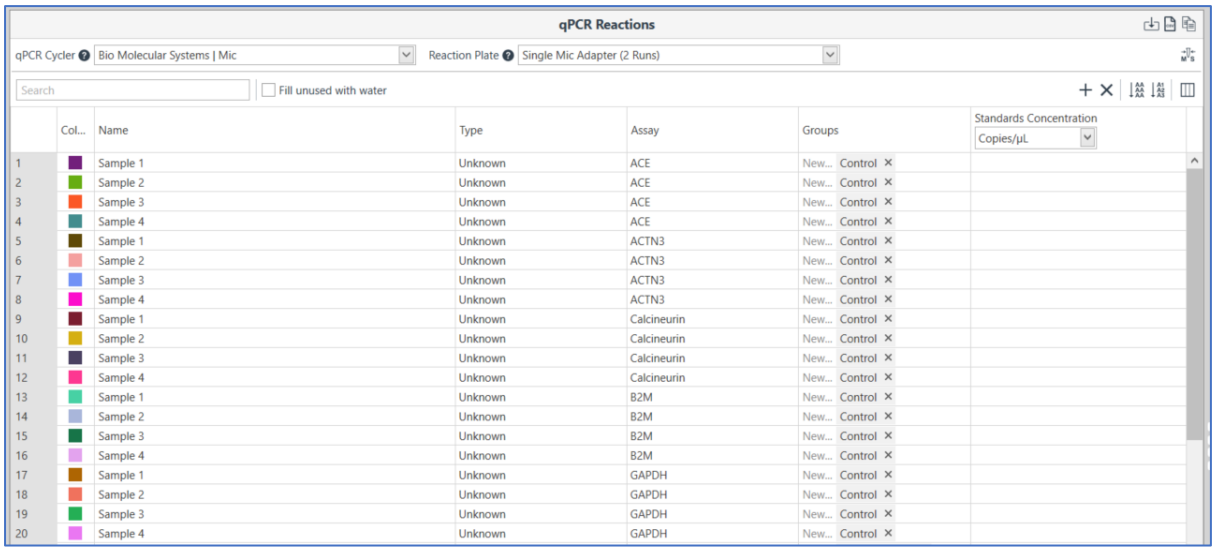
Next, use the *Reactions* editor under the *Run Setup* heading to configure reactions. Select the *qPCR Cycler* being used, followed by the *Reaction Plate.* You can also configure whether *Myra* should create mix and/or distribute samples. In this example, a *Mic* is being used, with *Myra* creating the mix and distributing samples into a *Single Mic Adapter (2 Runs)*.



In this example, 16 samples in total are being used, with 4 groups: a control and 3 groups of treatment with anabolic steroids oxymetholone, testosterone and nandrolone. Since biological replicates are being used, these groups are defined first using the *Groups* editor on the left-hand side panel. Biological replicates are essential for *Relative Quantification* analysis; technical replicates cannot be used because each replicate represents a manipulation of the original sample. It is also important to ensure there is a statistically significant number of samples required for *Relative Quantification* analysis; in this instance, this has been determined as a minimum of 85 samples.



Input each group of *Reactions*, with a group of 4 samples being assigned a different *Assay*. As *Reactions* are configured, you should assign a *Group* to prevent confusion. *BMS Workbench* will define these as biological replicates, and not technical replicates, as different *Assays* have been selected.

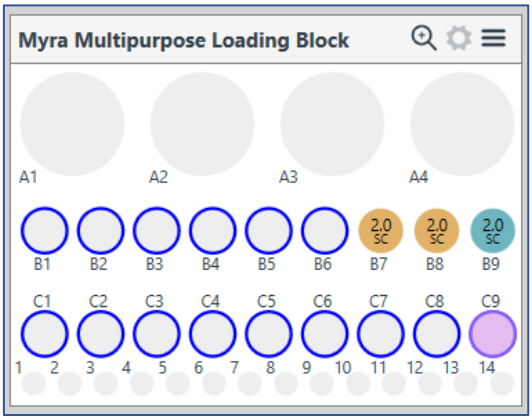


Additional NTCs have also been defined in this example, as a safeguard against contamination.

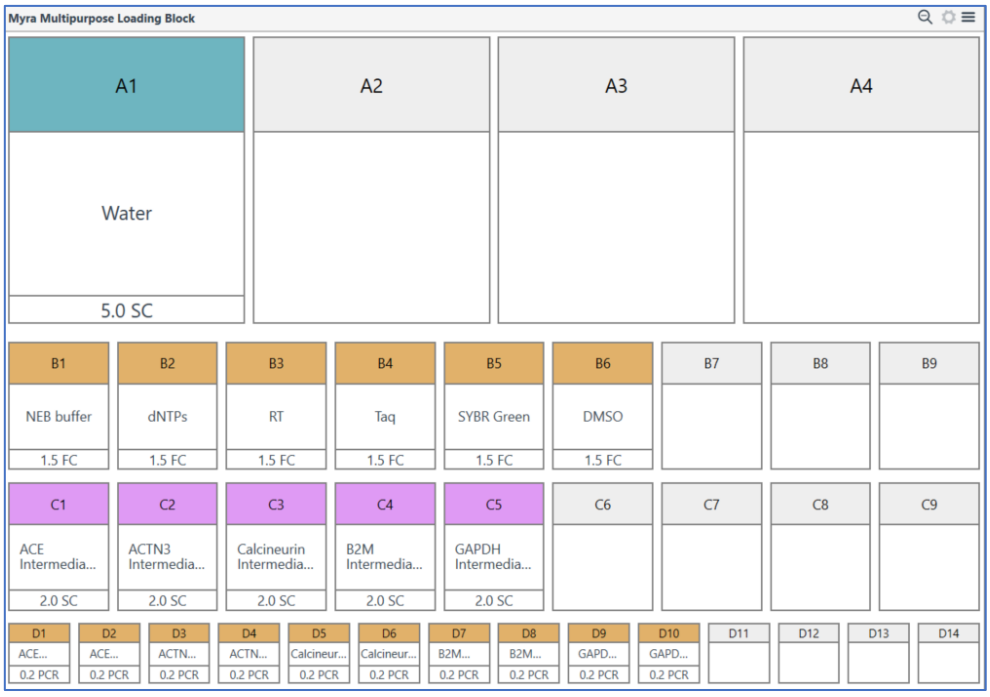
The *Deck Layout* is now ready to be configured. Any additional plates or loading blocks to hold components and templates should be selected first in the *Deck Configuration* tab and dragged into the desired position in the deck. *BMS Workbench* comes pre-loaded with a variety of plates available in *Plate Library* to facilitate loading.

Reagents and any *Intermediate Mixes* listed in the *Components* tab should then be allocated, followed by *Templates* and *Controls* listed in the *Templates* tab. Assign the *Tube Type* to components and templates that come in tube format before dragging the tubes into desired positions; *Templates* in plates do not

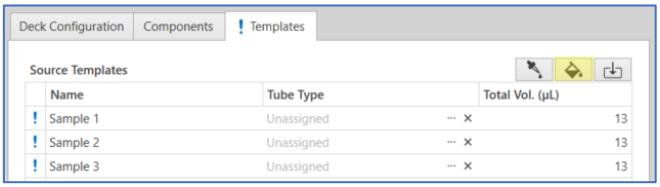
need to be assigned a *Tube Type* and can be dragged directly onto a plate. When hovering over a loading block, compatible loading positions will be highlighted blue for convenience.



In this example, each of the *Reaction Components* must be dragged into position. Since they are named the same in each *Assay*, only one tube is required for each; however, 5 different *Intermediate Mixes* are required, for each of the *Assays*, as they utilise different oligonucleotides.



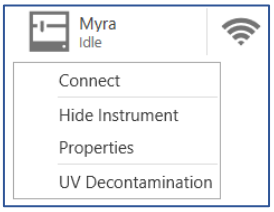
That leaves only the *Templates* to be allocated. When multiple templates are being loaded, it is best to use the *Fill consecutive wells with all unallocated items in one deck* icon to quickly load a 96-well plate in sequential order, as in the provided example.



BMS Workbench calculates the *Total Volumes* needed for each *Component* and *Template* based on the number of reactions specified, which can be found in the last column. When loading the *Deck,* this is an ideal place to ensure sufficient volumes are available for the *Component* or *Template* for *Myra* to complete the run.

Step 3: Run the Myra

When ready, connect the *Myra* to the PC using the provided 2.0m USB cable and click on the *Scan for Instruments* icon. The *Myra* instrument should automatically appear. Click on the *Myra* icon and select *Connect* from the drop-down menu.



A *Run Summary* banner will automatically appear. Press the play button when ready.

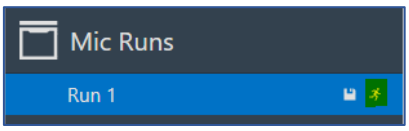
If any new component is being run, *Myra* will require calibration. Refer to the *BMS Workbench* manual, pages 24 to 25 for more information on how to perform calibration.

Step 4: Transfer Mic Tubes to the Rotor

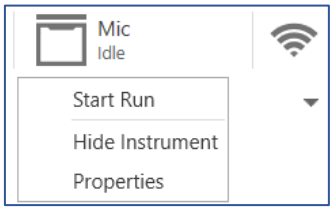
Once complete, carefully take the *Mic* rack off the *Myra* deck and use the provided capping tool to firmly cap the tubes using the provided caps before placing them into the *Mic* rotor, taking care to insert *Mic* tubes starting from position 1. To ensure thermal uniformity, fill tubes with water at the reaction volume and place them into all unused positions. There is an option to *Fill unused with water* in the *Samples* page to direct *Myra* to setup all unfilled tubes with water.

Step 5: Start the Mic Run

Mic runs can be started from the **Reaction Driven qPCR Setup** run file using the *Running man* icon.



A new window will automatically open. Click on the *Scan for instruments* icon and select the desired *Mic*. Select *Start Run* in the drop-down menu.



Step 6: Create a qPCR Project

Typically, *Relative Quantification* analysis requires multiple runs. In this example, 2 runs were necessary as a total of 80 reactions using biological replicates and 10 reactions representing NTCs were configured. It is hence necessary to use the **qPCR Project** file type, which allows for the comparison of multiple run files in a single analysis. **qPCR Project** eliminates bias from comparison of multiple run files by using a single optimal threshold for all data, including an inter-run fluorescence correction factor.

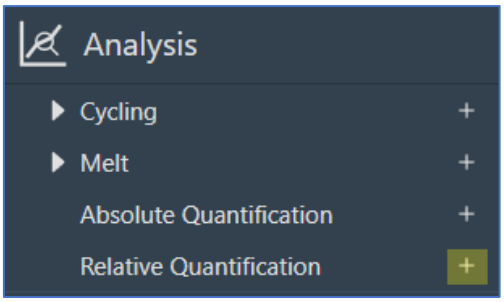
To create a new **qPCR Project**, open a **New Document** and use the **qPCR Project** icon on the **Start Page**.

Add *Runs* using the + symbol next to the *Runs* heading in the Navigator Bar. This will open a Windows dialogue box; navigate to the location of your runs and add them using the prompts.

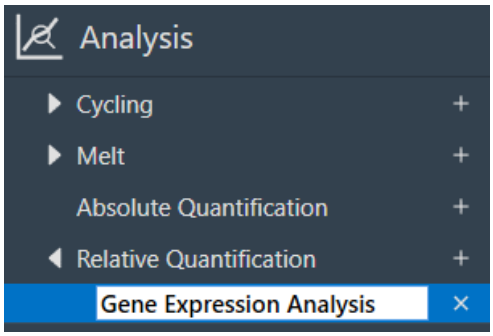
Step 7: Perform Relative Quantification Analysis.

First, double-check that *Samples* have been annotated correctly in the *Samples* editor. Click on the *Samples* heading in the Navigator Bar. The *Groups* listed here are used by *BMS Workbench* for analysis and must be assigned correctly for the analysis.

Next, add a *Relative Quantification* analysis type by clicking on the + symbol next to *Relative Quantification* in the Navigator Bar.

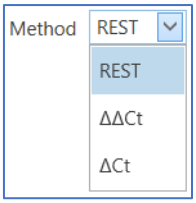


Rename the analysis by clicking on *New*.

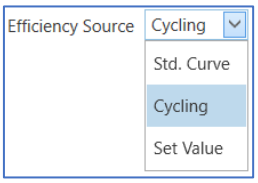


The *Parameters* bin needs to be filled in for *Relative Quantification* analysis. The default calculation method is *REST* (Relative Expression Software Tool). The *REST* method is recommended because it uses non-parametric error techniques that are suitable for gene expression analysis of qPCR data. Non-parametric error techniques do not rely on assumptions about the data set to be a valid statistic. The

ΔΔCt and *ΔCt* methods are also available from the drop-down menu to the right of *Method*, but is not recommended as it relies on the data set being normally distributed and of equal variance for validity, which is not expected to be the case for qPCR data in relative expression data sets.



You can also select your *Efficiency Source* here. The default recommended method is *Cycling*, which uses *Cycling* data to avoid the potential bias of technical error in the construction of the standards or their measurement, but you can also use *Std. Curve* or *Set Value*. When the efficiency source is set to cycling, the analysis will use the efficiency calculated in the *Cycling* analysis, which is the most accurate reflection of the qPCR data. It is also possible to set the *Efficiency Source* to a *Set Value* that has been determined from a *Standard Curve*.



In the provided example, the *REST* method is being used with an *Efficiency Source: Cycling*.

Next, define whether your genes are a *Gene of Interest*, a *Reference Gene* or *(None)* to indicate they have no role. Here, *ACE*, *ACTN3* and *Calcineurin* are designated *Gene of Interest*, whereas *B2M* and *GAPDH* are assigned as *Reference Gene*.

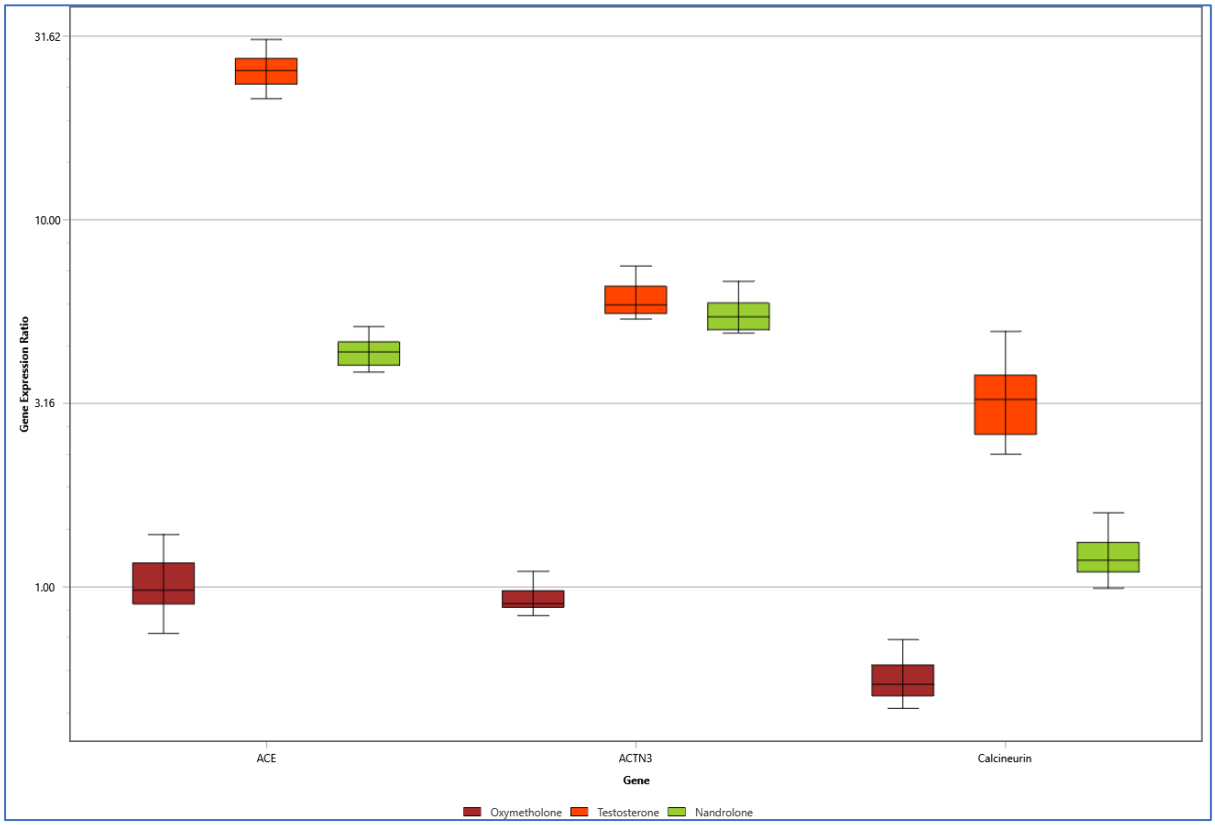
	Gene	Role	Efficiency
	ACE	Gene Of Interest	
	ACTN3	Gene Of Interest	
	Calcineurin	Gene Of Interest	
	B2M	Reference Gene	
	GAPDH	Reference Gene	

You also need to define whether each *Group* is *Control*, *Treatment* or *(None)* to indicate they have no role. Here, *(Control)* is marked as *Control*, whereas the other groups are *Treatment*.

	Group	Role
	Control	Control
	Oxymetholone	Treatment
	Testosterone	Treatment
	Nandrolone	Treatment

The *Relative Quantification* chart and *Results* table will update automatically. The chart will display the gene expression rate as a box-and-whisker plot. In the provided example, the graph has been toggled to group the chart by the treatment group, with a linear *y*-axis. Each box-and-whisker plot is coloured using the *Group* settings.

A *Gene Expression Ratio* of 1:1 compared to the control group, for example the oxymetholone groups for *ACE* and *ACTN3*, indicate no difference in the relative expression levels of these genes and is reported as *No difference* in the *Results* table. A *Gene Expression Ratio* higher than 1:1 indicate a higher expression level in that treatment group, which is reported as an *Up* result in the *Results* table. Here, testosterone and nandrolone have increased the expression of all three genes. A *Gene Expression Ratio* lower than 1:1 indicate a lower gene expression level, as with the oxymetholone decreasing expression of the *Calcineurin* gene.



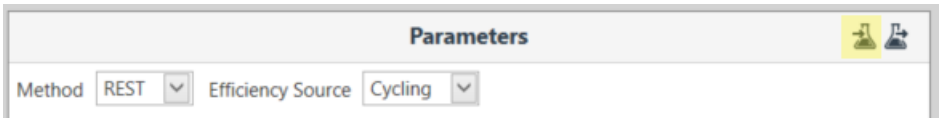
Statistics from the chart are also specified in the *Results* table, including *Std. Error*, *P Value* and *Result*.

Results

Gene	Treatment Group	Expression Ratio	Std. Error	95% C.I.	P Value	Result
ACE	Oxymetholone	1.004	0.850 - 1.195	0.745 - 1.386	0.9935	No difference
ACTN3	Oxymetholone	0.929	0.859 - 1.020	0.834 - 1.100	0.1795	No difference
Calcineurin	Oxymetholone	0.556	0.485 - 0.674	0.466 - 0.717	0.0195	Down
ACE	Testosterone	25.590	23.033 - 28.430	21.382 - 31.009	0.0270	Up
ACTN3	Testosterone	6.091	5.487 - 6.698	5.362 - 7.481	0.0270	Up
Calcineurin	Testosterone	3.216	2.463 - 4.173	2.296 - 4.960	0.0165	Up
ACE	Nandrolone	4.347	3.959 - 4.797	3.843 - 5.114	0.0235	Up
ACTN3	Nandrolone	5.545	4.983 - 6.009	4.909 - 6.790	0.0235	Up
Calcineurin	Nandrolone	1.212	1.057 - 1.442	0.989 - 1.589	0.0410	Up

These results indicate that testosterone and nandrolone increase the expression of *ACE*, *ACTN3* and *Calcineurin* in comparison to housekeeping genes *B2M* and *GAPDH*. Testosterone increases the expression of *ACE* most, at over 25-fold in comparison to housekeeping genes. Oxymetholone has no impact on the expression of *ACE* or *ACTN3*; however, it does decrease the expression of *Calcineurin* by half. Importantly, these results are based on a *P Value* calculated, where values less than 0.05 are considered significant; if your experiment assigns significance based on a different *P Value*, you should take that into account when using this analysis type.

If you would like to retain your analysis for the future, you can *Save analysis settings to the assay target* using the highlighted icon below.



Revision Number	Revised On	Revision Description
A	26/06/2023	First Release