



Powerful and interactive
RTqPCR data analysis
with Linear Mixed Models

User's Guide




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Quick Start

- Load data
 - Choose data file, xlsx, csv or txt (Comma, Tab or Semicolon separated).
 - Define column/variable classes (**Notice:** All but CT variable columns should be “factor”. CT as “numeric” – “num”). Do not forget to click [*Submit Class Change*] if you make changes.
- Specify Model
 - Drag CT values into *Response* bucket.
 - Drag Gene and Treatment(s) into *Fixed Effects* bucket.
 - (Optionally) drag variables such as SampleID, PlateID, BlockID, etc., into *Random Effects* bucket.
 - Click [*Run Model!*].
- Assign Comparisons
 - Select Reference Gene(s) from list.
 - Select the Column that contains the experimental condition that will be considered as baseline (control) and then select the Control Treatment itself.
 - Click [*Confirm Contrasts*].
- Get Result Table and Plot
 - Inspect, explore Results and click [*Download Result Table*] to save as a csv file.
 - Select axes for Plot and click [*Create Plot*].
 - Download Plot by clicking the camera [] button on the top right-hand side of the plot.

Before Loading Data

1, xlsx

2

1, tab delimited txt

1, comma delimited txt

3

1, semicolon delimited txt

Sample_ID	Treatment	Gene	CT	Timepoint	Plate_ID	Calibrator
S10	Water	UBI3	17.53604096	0h	PL4	
S11	Water	UBI3	17.30711124	0h	PL4	
S12	Water	UBI3	17.25564171	0h	PL4	
S13	Water	UBI3	17.40090061	0h	PL4	
S14	Water	UBI3	17.23961803	0h	PL4	
S15	Water	UBI3	16.75159613	0h	PL4	
S16	BTH	UBI3	17.04715584	0h	PL4	
S17	BTH	UBI3	17.18971344	0h	PL4	
S18	BTH	UBI3	19.0479966	0h	PL4	
S19	MBI600	UBI3	17.18658658	0h	PL4	
S20	MBI600	UBI3	17.47423254	0h	PL4	
S21	MBI600	UBI3	17.03833513	0h	PL4	
S10	Water	UBI3	17.53604247	0h	PL4	
S11	Water	UBI3	17.37675946	0h	PL4	
S12	Water	UBI3	17.07535196	0h	PL4	
S13	Water	UBI3	17.22157669	0h	PL4	
S14	Water	UBI3	17.02068313	0h	PL4	
S15	Water	UBI3	16.66497351	0h	PL4	
S16	BTH	UBI3	16.91268856	0h	PL5	
S17	BTH	UBI3	17.21732554	0h	PL5	
S18	BTH	UBI3	18.99186394	0h	PL5	
S19	MBI600	UBI3	17.20443476	0h	PL5	
S20	MBI600	UBI3	17.53211577	0h	PL5	
S21	MBI600	UBI3	17.17129934	0h	PL5	
S10	Water	RdR1	16.50917003	0h	PL5	
S11	Water	RdR1	16.39591033	0h	PL5	
S12	Water	RdR1	16.3754339	0h	PL5	
S13	Water	RdR1	15.87348341	0h	PL5	
S14	Water	RdR1	16.83802513	0h	PL5	
S15	Water	RdR1	15.9480237	0h	PL5	
S16	BTH	RdR1	15.05574644	0h	PL5	
S17	BTH	RdR1	15.2334278	0h	PL5	
S18	BTH	RdR1	16.33166124	0h	PL5	
S19	MBI600	RdR1	16.32138619	0h	PL5	
S20	MBI600	RdR1	16.15611946	0h	PL5	
S21	MBI600	RdR1	16.36128136	0h	PL5	
S10	Water	RdR1	16.87775983	0h	PL4	
S11	Water	RdR1	16.3424288	0h	PL4	
S12	Water	RdR1	16.35859477	0h	PL4	
S13	Water	RdR1	16.15556337	0h	PL4	
S14	Water	RdR1	16.7657637	0h	PL4	
S15	Water	RdR1	16.34346327	0h	PL4	
S16	BTH	RdR1	15.02162814	0h	PL4	
S17	BTH	RdR1	15.87323312	0h	PL4	
S18	BTH	RdR1	15.9622267	0h	PL4	

• Data file preparation

1. rQPCR accepts .xlsx, .csv or .txt files with column data that are separated by either "tab", "comma" or "semicolon" characters. Their general structure is depicted here.
2. The first row should be the descriptive name of each variable.
3. Data structure must be in the, so-called, long format.

Example: Assume an experimental assay that involves testing three different concentrations of an antibiotic. All of them should be in the same column, under the general header, *Antibiotic*, or *Treatment A*, or "[the name of the Antibiotic]", etc. The control of this treatment (i.e., "water" or "no Antibiotic" or "control", etc., should be included under the same header, as well.

If this experiment was held at, say, three different conditions (i.e., temperatures, time-points), then another column should be populated with all the levels of this condition, under the general header (*Treatment B*, *Temperature*, *Condition*, etc.).

Before Loading Data

1. **Important!** Ensure that the column that contains the gene names is titled as “Gene(s)” or “gene(s)” (or even “GENE(S)”). If not, QPCRinR will ask the user to indicate the “gene” column and will rename it accordingly.
2. For Inter-Run Calibration, “calibrator” samples are required (see Model Specification). These samples must be “tagged” in a separate column with a specific name (i.e., “Calib”). Everything else in this column can be empty; any header name is allowed.

- **Technical and Biological Replication**

Technical replication is important in RT-qPCR experiments. QPCRinR requires that technical replicates in the data set are essentially the same except the C_T value. In other words, two technical replicates' rows contain identical inputs except their C_T values.

Accordingly, biological replicates share one more difference: All but C_T values and sample_IDs (or the equivalent column name) are equal among biological replicate rows in the data set.

Welcome screen


1

1. Navigation tabs

2. Open User's Guide


3. Begin the analysis

Welcome Load Data Model Specification Contrast Assignment Results



QPCRinR

Powerful and interactive
RT-qPCR data analysis
with Linear Mixed Models



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v.0.9.7.3

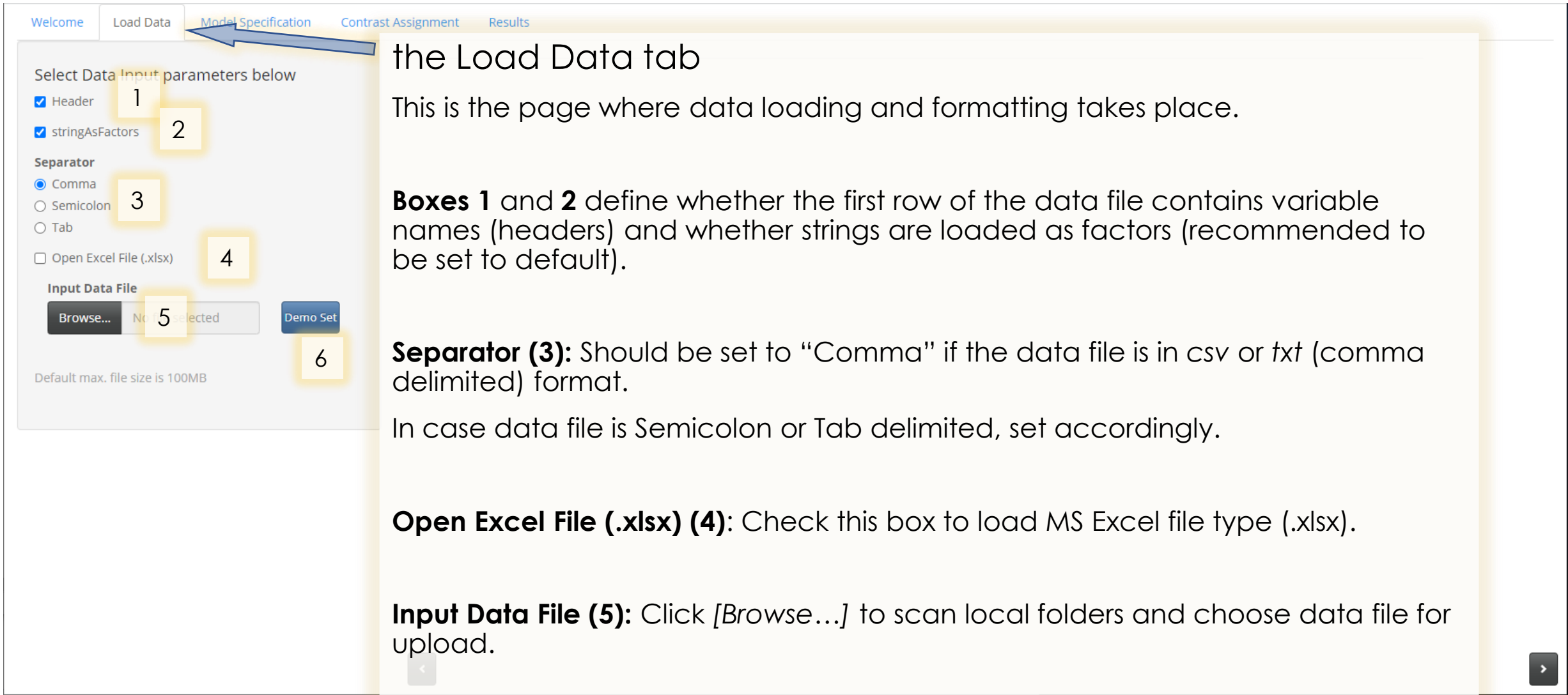
2

QPCRinR User's Guide

3

Launch!!

Load Data



The screenshot shows the 'Load Data' tab selected in a software interface. The interface has a top navigation bar with tabs: 'Welcome', 'Load Data', 'Model Specification', 'Contrast Assignment', and 'Results'. The 'Load Data' tab is active. Below the navigation bar, there is a section titled 'Select Data Input parameters below'. This section contains several options: a checked checkbox for 'Header' (callout 1), a checked checkbox for 'stringAsFactors' (callout 2), a 'Separator' section with radio buttons for 'Comma' (selected, callout 3), 'Semicolon', and 'Tab', an unchecked checkbox for 'Open Excel File (.xlsx)' (callout 4), an 'Input Data File' section with a 'Browse...' button (callout 5) and a 'Demo Set' button (callout 6). At the bottom of the 'Input Data File' section, it says 'No file selected' and 'Default max. file size is 100MB'. A blue arrow points from the 'Load Data' tab to the 'Model Specification' tab.

the Load Data tab

This is the page where data loading and formatting takes place.

Boxes 1 and 2 define whether the first row of the data file contains variable names (headers) and whether strings are loaded as factors (recommended to be set to default).

Separator (3): Should be set to “Comma” if the data file is in csv or *txt* (comma delimited) format.

In case data file is Semicolon or Tab delimited, set accordingly.

Open Excel File (.xlsx) (4): Check this box to load MS Excel file type (.xlsx).

Input Data File (5): Click [*Browse...*] to scan local folders and choose data file for upload.

Load Demo Dataset (6): Click this box to load a dataset for practice.

Load Data

As soon as data uploading is completed, additional option menus and panels appear:

Drop-down menus of
6. Variable Names
(headers of the data
file.

7. and data class types.

8. Data table panel
and

9. data structure panel

Web... Results

show 10 entries Search:

experiment	Calibrators	samples	replicates	gene	times	treatment	Ct
1		19	19_1	UBI3	02h	Control	18.25
1		20	20_1	UBI3	02h	Control	17.32
1		25	25_1	UBI3	06h	Control	19.17
1		26	26_1	UBI3	06h	Control	18.11
1		31	31_1	UBI3	24h	Control	18.6
		32	32_1	UBI3	24h	Control	18.72
		13	13_1	UBI3	24h	Control	18.29
		13	13_3	UBI3	24h	Control	18.29
		19	19_1	TD20	02h	Control	25.7
		20	20_1	TD20	02h	Control	21.21

Showing 1 to 10 of 174 entries Previous 1 2 3 4 5 ... 18 Next

Variable Definition

Choose class of each variable

Important: Set all variables to "factor", EXCEPT CT which should be set as "numeric"

Variable Name

experiment 6

Variable Class Type

factor 7

Submit Class Change

```
'data.frame': 174 obs. of 8 variables:
 $ experiment : int 1 1 1 1 1 1 1 1 1 1 ...
 $ Calibrators: Factor w/ 2 levels "", "Calib": 1 1 1 1 1 1 2 2 1 1 ...
 $ samples : int 19 20 25 26 31 32 13 13 19 20 ...
 $ replicates: Factor w/ 48 levels "13_1","13_2",...: 13 15 25 27 37 39 1 3 13 15 ...
 $ gene : Factor w/ 4 levels "AC01","PPO0",...: 4 4 4 4 4 4 4 4 3 3 ...
 $ times : Factor w/ 3 levels "02h","06h","24h": 1 1 2 2 3 3 3 3 1 1 ...
 $ treatment: Factor w/ 3 levels "Control","FLG22",...: 1 1 1 1 1 1 1 1 1 1 ...
 $ Ct : num 18.2 17.3 19.2 18.1 18.6 ...
```


Load Data

Welcome Load Data Model Specification Contrast Assignment Results

Select Data Input parameters below

☒ Header

☒ stringAsFactors

Separator

☒ Comma

☐ Semicolon

☐ Tab

☐ Open Excel File (.xlsx)

Input Data File

Browse... DemoSet.txt Demo Set

Upload complete

Default max. file size is 100MB

Variable Definition

Choose class of each variable

Important: Set all variables to "factor", EXCEPT CT which should be set as "numeric"

Variable Name

experiment.Calibrators.samples..replicates.gene.times.treatment.Ct

Variable Class Type

factor

Submit Class Change

Show 10 entries

	experiment.Calibrators.samples..replicates.gene.times.treatment.Ct
1	9 19 19_1 UBI3 02h Control 18.25
2	9 20 20_1 UBI3 02h Control 17.32
3	9 25 25_1 UBI3 06h Control 19.17
4	9 26 26_1 UBI3 06h Control 18.11
5	9 31 31_1 UBI3 24h Control 18.6
6	9 32 32_1 UBI3 24h Control 18.72
7	9 Calib 13 13_1 UBI3 24h Control 18.29
8	9 Calib 13 13_3 UBI3 24h Control 18.29
9	9 19 19_1 TD20 02h Control 25.7
10	9 20 20_1 TD20 02h Control 21.21

Showing 1 to 10 of 174 entries

Previous 1 2 3 4 5 ... 18 Next

3

```
'data.frame': 174 obs. of 1 variable:
 $ experiment.Calibrators.samples..replicates.gene.times.treatment.Ct: Factor w/ 174 levels "10\t\t19\t19_2\tAC01\t02h\tControl\t24.09",...: 91 95 115 119 139 141
```

In case of inappropriate selection of the **Separator** of the data file,

1. data will appear stacked in the Data Table
 2. variable names will be concatenated in the Variable Name menu
- and
3. There will be no structure in the Structure Panel

Variable Definition
Choose class of each variable

Important: Set all variables to "factor", EXCEPT CT which should be set as "numeric"

Variable Name

experiment

1

experiment
Calibrators
samples
replicates
gene

10 9
Showing 1 to 10 of 174 entries

```
'data.frame': 174 obs. of 8 variables:
 $ experiment: int  9 9 9 9 9 9 9 9 9 9
 $ Calibrators: Factor w/ 2 levels "9", "10"
 $ samples    : int  19 20 25 26 31 32
 $ replicates : Factor w/ 48 levels "1", "2", "3", "4", "5", "6", "7", "8", "9", "10", "11", "12", "13", "14", "15", "16", "17", "18", "19", "20", "21", "22", "23", "24", "25", "26", "27", "28", "29", "30", "31", "32", "33", "34", "35", "36", "37", "38", "39", "40", "41", "42", "43", "44", "45", "46", "47", "48"
 $ gene       : Factor w/ 4 levels "ACC", "BAC", "CAC", "DCC"
 $ times      : Factor w/ 3 levels "02h", "04h", "06h"
 $ treatment  : Factor w/ 3 levels "Control", "Low", "High"
 $ Ct         : num  18.2 17.3 19.2 18.1 17.4 17.5 18.3 17.6 17.7 17.8
```

QPCRinR performs factorial analysis. Users must inspect and, if necessary, change the class of the data variables to "factor", except CT values. In the Variable Definition menu:

1. Select Variable Name
2. Select Class type
3. Click [Submit Class Change]

Important: Check the structure panel to confirm the class change

Variable Definition
Choose class of each variable

Important: Set all variables to "factor", EXCEPT CT which should be set as "numeric"

Variable Name

experiment

Variable Class Type

integer

2

factor
integer
numeric

10 9
Showing 1 to 10 of 174 entries

```
'data.frame': 174 obs. of 8 variables:
 $ experiment: int  9 9 9 9 9 9 9 9 9 9
 $ Calibrators: Factor w/ 2 levels "9", "10"
 $ samples    : int  19 20 25 26 31 32
 $ replicates : Factor w/ 48 levels "1", "2", "3", "4", "5", "6", "7", "8", "9", "10", "11", "12", "13", "14", "15", "16", "17", "18", "19", "20", "21", "22", "23", "24", "25", "26", "27", "28", "29", "30", "31", "32", "33", "34", "35", "36", "37", "38", "39", "40", "41", "42", "43", "44", "45", "46", "47", "48"
 $ gene       : Factor w/ 4 levels "ACC", "BAC", "CAC", "DCC"
 $ times      : Factor w/ 3 levels "02h", "04h", "06h"
 $ treatment  : Factor w/ 3 levels "Control", "Low", "High"
 $ Ct         : num  18.2 17.3 19.2 18.1 17.4 17.5 18.3 17.6 17.7 17.8
```

Variable Definition
Choose class of each variable

Important: Set all variables to "factor", EXCEPT CT which should be set as "numeric"

Variable Name

experiment

Variable Class Type

factor

Submit Class Change

3

10 9
Showing 1 to 10 of 174 entries

```
'data.frame': 174 obs. of 8 variables:
 $ experiment: Factor w/ 2 levels "9", "10"
 $ Calibrators: Factor w/ 2 levels "9", "10"
 $ samples    : int  19 20 25 26 31 32
 $ replicates : Factor w/ 48 levels "1", "2", "3", "4", "5", "6", "7", "8", "9", "10", "11", "12", "13", "14", "15", "16", "17", "18", "19", "20", "21", "22", "23", "24", "25", "26", "27", "28", "29", "30", "31", "32", "33", "34", "35", "36", "37", "38", "39", "40", "41", "42", "43", "44", "45", "46", "47", "48"
 $ gene       : Factor w/ 4 levels "ACC", "BAC", "CAC", "DCC"
 $ times      : Factor w/ 3 levels "02h", "04h", "06h"
 $ treatment  : Factor w/ 3 levels "Control", "Low", "High"
 $ Ct         : num  18.2 17.3 19.2 18.1 17.4 17.5 18.3 17.6 17.7 17.8
```

Load Data

4

1

2

3

4

The Data Table panel and the Structure panel can be used to evaluate the proper input of data.

1. The Data Table is expandable and searchable and allows the exploration of the layout and the values of the data set.
2. In the Structure panel, inspect the number of levels of each factor.
3. **Very Important:** The class of CT values should be "numeric" (num) for the analysis to go on.
4. After the examination of data input, proceed to Model Specification

experiment	Calibrators	samples	replicates	gene	times	treatment	Ct
1	9	19	19_1	UBI3	02h	Control	18.25
2	9	20	20_1	UBI3	02h	Control	17.32
3	9	20	20_1	UBI3	02h	Control	19.17
4	9	26	26_1	UBI3	06h	Control	18.11
5	9	32	32_1	UBI3	24h	Control	18.6
6	9	32	32_1	UBI3	24h	Control	18.72
7	9	13	13_1	UBI3	24h	Control	18.29
8	9	13	13_1	UBI3	24h	Control	18.29
19	10	19_1	19_1	TD20	02h	Control	25.7
20	10	20_1	20_1	TD20	02h	Control	21.21

```
'data.frame': 174 obs. of 8 variables:
 $ experiment : Factor w/ 2 levels "9","10": 1 1 1 1 1 1 1 1 1 ...
 $ Calibrators: Factor w/ 2 levels "", "Calib": 1 1 1 1 1 1 2 2 1 1 ...
 $ samples    : Factor w/ 21 levels "13","15","17",...: 4 5 11 16 17 1 1 4 5 ...
 $ replicates : Factor w/ 48 levels "13_1","13_2",...: 13 13 27 37 39 1 3 13 15 ...
 $ gene       : Factor w/ 4 levels "AC01","PP00",...: 4 4 4 4 4 4 4 4 3 3 ...
 $ times      : Factor w/ 3 levels "02h","06h","24h": 1 1 2 2 3 3 3 3 1 1 ...
 $ treatment  : Factor w/ 3 levels "Control","FLG22",...: 1 1 1 1 1 1 1 1 1 1 ...
 $ Ct         : num 18.2 17.3 19.2 18.1 18.6 ...
```

Model Specification

the Model Specification tab

QPCRinR implements Linear (Mixed) Models for the analysis of qPCR experiments. This framework offers possibilities for incorporating complex designs and controlling for several variance components (See main article and references therein for details).

Drag items in the desired bucket

1 Model Components

- experiment
- Calibrators
- samples
- replicates
- gene
- times
- Ct
- treatment

2 Response - Drag CT value here

3 Fixed Effects

4 Random effects

1. The user can interactively define the model components according to the design of her study by dragging variables from the left-side list to the relevant buckets (2, 3, 4).

2. CT variable should always occupy the *Response* bucket, alone.
3. Gene names should be dragged in the *Fixed Effects* bucket. Several other experimental treatments (regimes, genotypes, mutants, chemical/compound drug concentrations, temperatures, time-points, etc.) should be placed in the same container.
4. Sample_IDs and other sources of random effects (batches, blocks, experiments, etc.) should be dragged into *Random Effects* bucket (optional but recommended).

The screenshot shows a web interface for model specification. At the top, there are tabs: 'Welcome', 'Load Data', 'Model Specification' (active), 'Contrast Assignment', and 'Results'. Below the tabs, there is a checkbox for 'Inter-Run Calibration'. The main area is titled 'Drag items in the desired bucket'. On the left, under 'Model Components', there are three items: 'experiment', 'Calibrators', and 'replicates'. On the right, under 'Response - Drag CT value here', there is a box containing 'Ct'. Below these, there is a checkbox labeled '1' with the text 'Or Input model formula manually'. Below this, there is a text input field labeled '2' with the text 'Please enter model formula' and the example formula 'i.e., ct ~ gene * treatment1 * treatment2 + (1 | sample)'. Below the input field is a button labeled 'Run Model!'. At the bottom left, there is a navigation arrow button.

For more complex model specification, the option for manual model input is available.

The formula should be expressed in accordance with R programming, as an *lm* or *lmer* function input.

1. Check box (1).
2. Type the desired model formula in box (2):
 - I. *Response*: the [Ct] variable followed by '~'.
 - II. *Fixed Effects*: each [variable] followed by '+' or '*' when modelling interactions with the next variable.
 - III. *Random Effects*, optional but recommended: Input variables after the string: "+(1 | [variable1])". For interactions and nesting, the general formula would be: "+(1 | [variable1]:[variable2])"

Example: $Ct \sim \text{gene} * \text{treatment} * \text{times} + (1 | \text{samples}) + (1 | \text{samples} : \text{gene})$

Diagram illustrating the components of the example formula:

- Response**: Ct
- Fixed Effects**: $\text{gene} * \text{treatment} * \text{times}$
- Random Effects**: $(1 | \text{samples}) + (1 | \text{samples} : \text{gene})$

Please, refer to R language manuals for more information about formula expressions.

Model Specification

Inter-Run Calibration (IRC)

In case multi-plate experiments are involved, QPCRinR offers the possibility to analyze them jointly, provided that Calibrator samples are included.

Calibrator samples are identical runs (i.e., cDNA of the same sample tubes) in every plate of the assay. They should be technically and biologically replicated and should encompass all the genes of the assay.

Occasionally, the latter is not always possible. QPCRinR is designed to perform IRC with at least two genes per Calibrator sample.

For IRC to be applied, the data file should include a column that “marks” the Calibrator samples with a name “tag” and a column with Plate IDs/names.

1. To perform IRC, check the *Inter-Run Calibration* box at the side menu.
2. Write the “tag” -**NOT the header!**- of Calibrator samples in the first box that appears.
3. Provide the Name (header) of the column of the data set that refers to Plate IDs/names (here: *experiment*).

The image shows the 'Model Specification' window of the QPCRinR software. It has three tabs: 'Welcome', 'Load Data', and 'Model Specification'. The 'Model Specification' tab is active. On the left, there is a sidebar with a checkbox for 'Inter-Run Calibration' (checked) and two input fields: 'Please insert Calibrators' Tag' (containing 'Calib') and 'Please insert Plate Column Name' (containing 'experiment'). On the right, there is a 'Model Components' section with a list of components: 'experiment', 'Calibrators', and 'replicates'. Below this, there is a 'Drag items in the desired bucket' section. The main area of the window shows a preview of the data structure. Below the software window, there is a screenshot of a spreadsheet. The spreadsheet has columns labeled A through I. Column A is 'samples', B is 'replicates', C is 'gene', D is 'times', E is 'treatment', F is 'experiment', G is 'Calibrators', and H is 'Ct'. The data rows show various samples and their corresponding Ct values. Red boxes with numbers 1, 2, and 3 are overlaid on the spreadsheet to indicate the locations of the 'Inter-Run Calibration' checkbox, the 'Calib' tag, and the 'experiment' column header, respectively.

A	B	C	D	E	F	G	H
samples	replicates	gene	times	treatment	experiment	Calibrators	Ct
13 13_1	PP00	24h	Control	9	Calib	25.89	
19 19_1	ACO1	02h	Control	9		24.23	
20 20_1	ACO1	02h	Control	9		20.59	
25 25_1	ACO1	06h	Control	9		24.94	
26 26_1	ACO1	06h	Control	9		23.43	
31 31_1	ACO1	24h	Control	9		25.74	
32 32_1	ACO1	24h	Control	9		25.75	
13 13_1	ACO1	24h	Control	9		23.75	
21 21_1	UBI3	02h	FLG22	9	Calib	18.4	
22 22_1	UBI3	02h	FLG22	9		18.4	
27 27_1	UBI3	06h	FLG22	9		17.59	
28 28_1	UBI3	06h	FLG22	9		18.98	
33 33_1	UBI3	06h	FLG22	9		18.15	
15 15_3	UBI3	24h	FLG22	9	Calib	17.81	
21 21_1	UBI3	24h	FLG22	9		18.4	
22 22_1	TD20	02h	FLG22	9		18.54	
33 33_1	TD20	06h	FLG22	9		22.49	
34 34_1	TD20	24h	FLG22	9		21.77	
15 15_1	TD20	24h	FLG22	9		19.54	
15 15_1	TD20	24h	FLG22	9		20.5	
15 15_1	TD20	24h	FLG22	9		18.58	
15 15_1	TD20	24h	FLG22	9		19.45	

Model Specification

The screenshot shows the 'Model Specification' tab of a software interface. It includes sections for 'Inter-Run Calibration' with input fields for 'Calib' and 'experiment', a 'Run Model!' button, and a table of model fit statistics. Below this is a detailed model report showing the formula, REML criterion, scaled residuals, and random effects. Numbered callouts indicate key actions: 1 points to the 'Run Model!' button and the model report, while 2 points to the 'Run Model!' button and the 'Contrast Assignment' tab.

1. Run the specified model by clicking [Run Model!] button. If selected, IRC runs in the background and Calibration samples are removed before the main model run. When the process is over, three panels appear.

The first panel is a table of evaluation metrics for the specified model.

	logLik	AIC	BIC
	-151.94	379.88	492.74

The second is the model summary, displaying the model formula and the estimated coefficients.

By scrolling down, a third panel appears, listing the estimated marginal means of all levels of each fixed factor of the analysis.

2. After examining the model report, the user can either try an alternative model or proceed to the next panel, where the comparisons are assigned

Contrast Assignment

the Contrast Assignment tab

In this tab, the reference gene(s) and the baseline (control) treatment are defined for relative comparisons.

1. Choose the reference gene(s) from the list on the left
2. Select the variable that contains the Control treatment
3. Click [... continue...] to activate a drop-down menu of all levels of treatment selected at step 2
4. Select the level that corresponds to Control (it doesn't necessarily have to be named 'control'; it may be any level of any of the *Fixed Factors* that will be considered as the baseline).
5. Click [Confirm Selection]

2a. Alternatively, the analysis can proceed without control assignment by unchecking the [Include Control] box and clicking [Proceed without Control].

Contrast Assignment

Contrast Assignment

1

2

3

4

Welcome Load Data Model Specification Contrast Assignment Results

Choose REFERENCE Gene(s)

☐ ACO1
☐ PPO0
☐ TD20
☒ UBI3

Proceed without Control

☒ Include Control

Control in...

treatment

... continue...

Choose Control

Control

Confirm Selection

contrast	estimate	SE	df	t.ratio	p.value
ACO1_FLG22_02h	4.890	0.702	99	6.964	<.0001
PPO0_FLG22_02h	4.015	0.702	99	5.717	<.0001
TD20_FLG22_02h	1.923	0.702	99	2.738	0.0073
ACO1_S2_02h	2.312	0.702	99	3.292	0.0014
PPO0_S2_02h	1.726	0.702	99	2.457	0.0157
TD20_S2_02h	-0.145	0.702	99	-0.206	0.8371
ACO1_FLG22_06h	3.913	0.702	99	5.572	<.0001
PPO0_FLG22_06h	4.881	0.702	99	6.950	<.0001
TD20_FLG22_06h	2.926	0.702	99	4.166	0.0001
ACO1_S2_06h	2.117	0.702	99	3.015	0.0033
PPO0_S2_06h	2.207	0.702	99	3.143	0.0022
TD20_S2_06h	1.113	0.702	99	1.584	0.1163
ACO1_FLG22_24h	2.943	0.702	99	4.190	0.0001
PPO0_FLG22_24h	2.656	0.702	99	3.782	0.0003
TD20_FLG22_24h	9.729	0.702	99	13.854	<.0001
ACO1_S2_24h	3.354	0.702	99	4.776	<.0001
PPO0_S2_24h	2.192	0.702	99	3.122	0.0024
TD20_S2_24h	3.347	0.702	99	4.767	<.0001

Degrees-of-freedom method: kenward-roger

After [Confirming Selection] or [Proceeding without Control], the main panel will be populated with the results.

1. The first column is the contrast combination. As expected, the reference gene is absent. If the choice in *Contrast Assignment* tab was comparisons with control treatment (a $\Delta\Delta\text{ct}$ equivalent), then the control treatment will be absent, too.
2. The next column (*estimate*) is the estimated $\log_2\text{FoldChange}$ of each factor combination relative to the reference gene **and** the control treatment (if there was one, see 1).
3. Next, the standard error of the estimate (SE), the degrees of freedom (df), the t.ratio and the p.value of the test are shown.
4. Click on *Results* tab or the arrow at the bottom right corner to see this table in interactive and expandable format.

Results

the Results tab

the Results tab

Results

Show 10 entries

Search:

	gene	treatment	times	estimate	SE	df	t.ratio	p.value
1	ACO1	FLG22	02h	4.89022464521627	0.70225975198053	99.0000000000003	6.96355533892515	3.68323515501156e-10
2	PPO0	FLG22	02h	4.01502859677021	0.702259751980526	98.9999999999999	5.71729845751085	1.14791792051044e-7
3	TD20	FLG22	02h	1.92269390751159	0.702259751980527	98.9999999999999	2.73786715255883	0.007333085759543
4	ACO1	S2	02h					
5	PPO0	S2	02h					
6	TD20	S2	02h					
7	ACO1	FLG22	06h					
8	PPO0	FLG22	06h					
9	TD20	FLG22	06h					
10	ACO1	S2	06h					

Showing 1 to 10 of 18 entries

Download Result Table

The output of the analysis is displayed as table on the main panel of the Results tab.

1. As in Contrast Assignment Tab, the first columns are populated by the contrast combinations (*Fixed Factors* of the analysis). Here they appear separated so that the user can sort the table as she wishes.
2. The other columns are as described in the previous page.
3. Click [*Download Result Table*] to download results as a csv file.

On the side panel the plot controls are located (see next page).

Results

Define X-axis: 1

Define Y-axis: 2

Fill by: 3

Split by: 4

Merge Factors

Create Plot 5

Select properties of plot

Height: 100, 500, 3,000

Width: 100, 1,000, 3,000

X Label Rotation: 0, 30, 90

Show 10 entries

	gene	treatment	times	estimate	SE	df	t.ratio	p.value
1	ACO1	FLG22	02h	4.89022464521627	0.70225975198053	99.0000000000003	6.96355533892515	3.68323515501156e-10
2	PPO0	FLG22	02h	4.01502859677021	0.702259751980526	98.9999999999999	5.71729845751085	1.14791792051044e-7
3	TD20	FLG22	02h	1.92222222222222	0.702259751980527	98.9999999999999	2.73816277116278	0.007333085759543
4	ACO1	S2	02h	2.32222222222222	0.702259751980527	98.9999999999999	3.316277116278	0.0015745274798874
5	PPO0	S2	02h	1.72222222222222	0.702259751980527	98.9999999999999	2.45316277116278	0.015745274798874
6	TD20	S2	02h	-0.142222222222222	0.702259751980527	98.9999999999999	-0.206123536007282	0.83711710934832
7	ACO1	FLG22	06h	3.92222222222222	0.702259751980527	98.9999999999999	5.57183494732796	2.1753941070837e-7
8	PPO0	FLG22	06h	4.82222222222222	0.702259751980527	98.9999999999999	6.87183494732796	1.14791792051044e-7
9	TD20	FLG22	06h	2.92222222222222	0.702259751980527	98.9999999999999	4.16277116278	0.0000663800497309143
10	ACO1	S2	06h	2.12222222222222	0.702259751980527	98.9999999999999	3.01499539111385	0.00326472675991491

Showing 1 to 10 of 18 entries

Download Result Table

Previous 1 2 Next

The output of the analysis can be plotted as a barplot in the Results tab. On the left panel, the user can define:

1. The variable on X-axis.
2. The Y-axis should always correspond to *estimates*; therefore, Y-axis is pre-selected by default.
3. The fill color of the bars in the plot.
4. The facets (sub-panels) of the barplot.
5. When ready, click [Create Plot]

The plot can be further formatted with the Properties panel:

1. Adjust the height and
2. the width of the plot.
3. Rotate the labels of the X-axis.
4. Change the plot theme.
5. Change the color palette.

By hovering the mouse over the top right-hand corner of the plot, the user can select several functionalities. To save as a png image, click the camera [📷] button.

Split by
gene

Merge Factors Create Plot

Select properties of plot

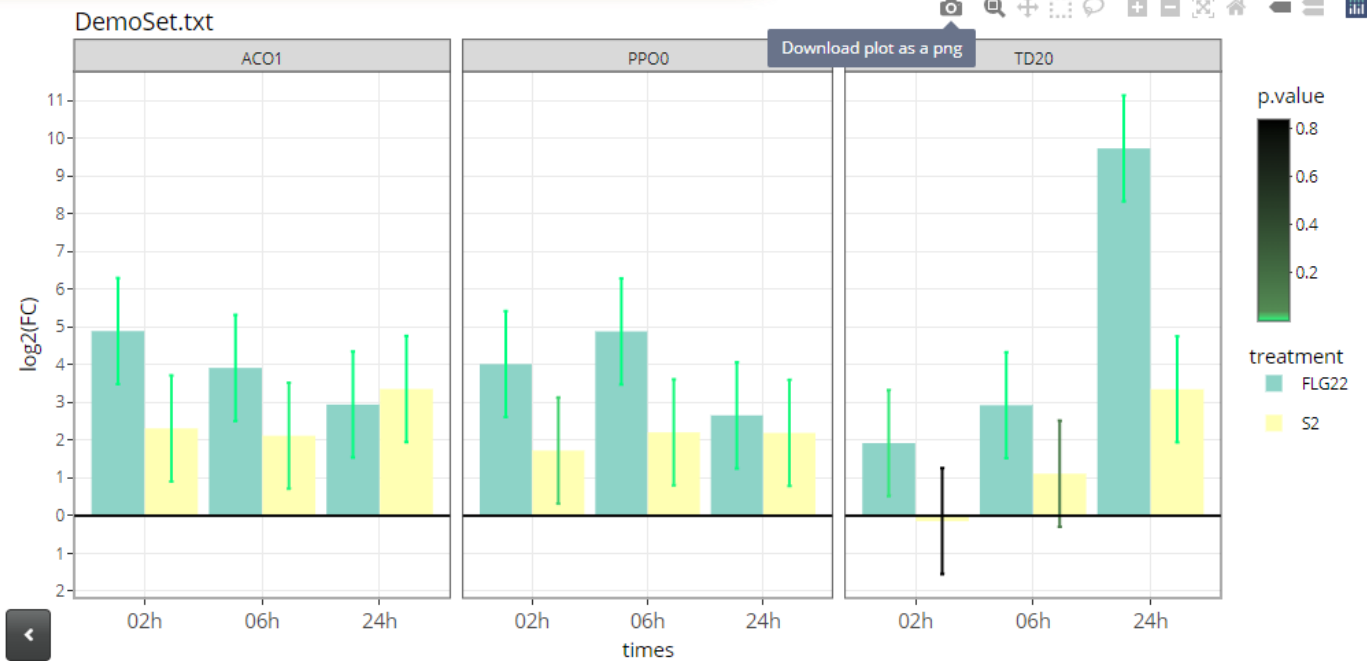
Height
100 500 3,000

Width
100 1,000 3,000

X Label Rotation
0 90

Plot theme
theme_bw

Color Palette
Set3



* In cases where the number of factors exceeds the maximum number that the plot can show separately (>3), it is possible to merge some of them by clicking [Merge Factors].

- A pop-up window will appear listing all the *Fixed Factors* of the specified model.
- Select two or more and Click [Submit] to verify the merging selection.

Merge Factors

Choose Factors to Merge

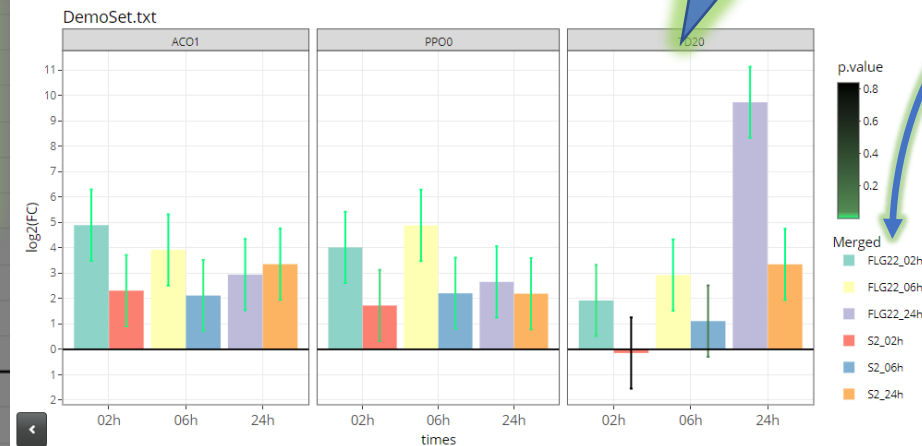
- ☐ gene
- ☒ treatment
- ☒ times

Submit

Cancel

- The merged factors will constitute a new category in the lists of the left panel, under the title "Merged". The original state of factors is also retained.
- The plot is then created as described in the previous page.
- The combined levels of the factors will be separated by an underscore character ("_").

* Here, factors "treatment" and "times" have been merged to a new factor "Merged"



- As shown here, merging of factors can also be the option if the user desires to use more color combinations.

Data

Demo data file is a partial subset extracted from:

Dimopoulou, A., Theologidis, I., Liebmann, B., Kalantidis, K., Vassilakos, N., & Skandalis, N. (2019). *Bacillus amyloliquefaciens* MBI600 differentially induces tomato defense signaling pathways depending on plant part and dose of application. *Scientific Reports*, 9(1). <https://doi.org/10.1038/s41598-019-55645-2>

Source code and Support

- <https://github.com/theojohn/QPCRinR>

Basic Literature

- Bates, D., Mächler, M., Bolker, B. M., & Walker, S. C. (2015). Fitting Linear Mixed-Effects Models Using lme4. *Journal of Statistical Software*. *Journal of Statistical Software*, 67(1), 1–48. <https://doi.org/10.18637/JSS.V067.I01>
- Lenth, R. V., Buerkner, P., Herve, M., Love, J., Miguez, F., Riebl, H., & Singmann, H. (2022). CRAN - Package emmeans. <https://cran.r-project.org/web/packages/emmeans/index.html>
- Pabinger, S., Rödiger, S., Kriegner, A., Vierlinger, K., & Weinhäusel, A. (2014). A survey of tools for the analysis of quantitative PCR (qPCR) data. In *Biomolecular Detection and Quantification* (Vol. 1, Issue 1, pp. 23–33). Elsevier GmbH. <https://doi.org/10.1016/j.bdq.2014.08.002>
- Steibel, J. P., Poletto, R., Coussens, P. M., & Rosa, G. J. M. (2009). A powerful and flexible linear mixed model framework for the analysis of relative quantification RT-PCR data. *Genomics*, 94(2), 146–152. <https://doi.org/10.1016/j.ygeno.2009.04.008>