

**RGeasy**

**Instruction Manual**

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## 1 Introduction

This manual is a guide for researchers seeking validated reference genes for gene expression analysis via RT-qPCR, as well as for those aiming to register species (animals, plants or microorganisms) used in their reference gene validations studies (Figure 1). RGeasy makes research access and development simpler, as it provides greater data dissemination, reduces cost, and decreases the time required to conduct each study. In addition, due to the increased visibility of the registered studies on RGeasy database, these studies can potentially be cited more than usual, as detailed along this manual.

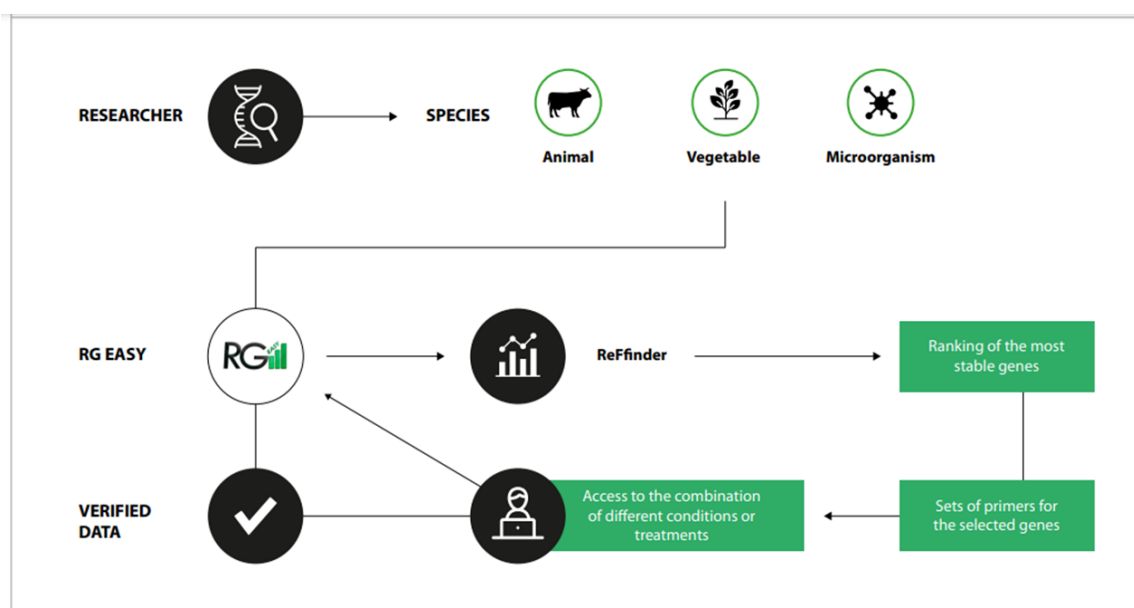


Figure 1. Maintenance and workflow of the RGeasy tool. From the registration of animal, plant or microorganism species, researchers deposit their data (Cq's values) on RGeasy database, which are immediately verified. Then, users can run all possible combinations of conditions/treatments for each study. The new combinations of treatments is ranked by RefFinder (XIE et al., 2012), and RGeasy provides, in addition to the ranking with reference genes, a set of validated primers for each reference gene.

## 2 Access to RGeasy database

When using RGeasy, user has access to the species registered on the tool by clicking on “Species” in the navigation bar located in the upper part of the initial interface (Figure 2).

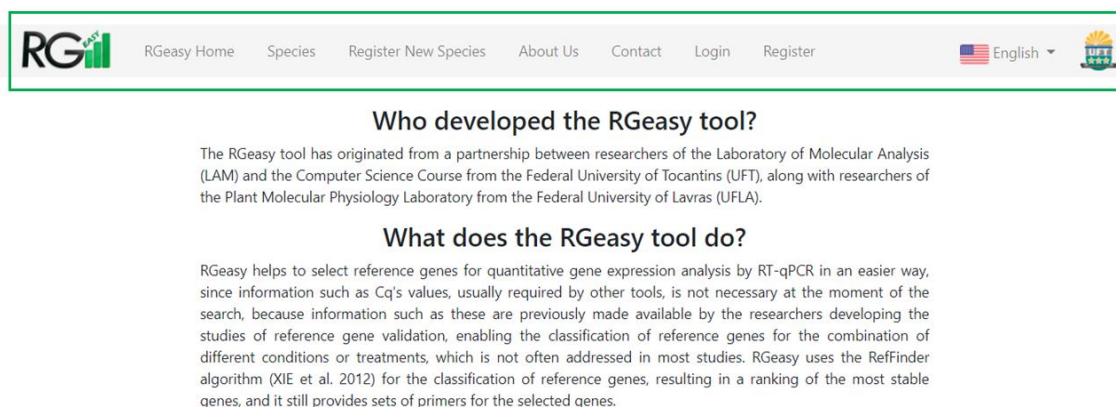


Figure 2. RGeasy's initial graphic interface

Species on RGeasy are separated into three categories: Animals, Plants, and Microorganisms (Figure 3). By clicking on the species of interest, it is automatically shown all the reference gene validation studies registered on RGeasy for that species. In this interface, the user has access to each study by clicking on its title, and under the title from each study, it is displayed the types of samples analyzed on them.

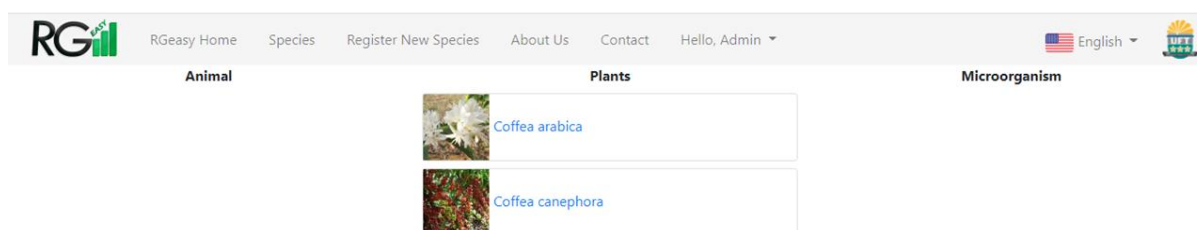


Figure 3. Species categories on RGeasy.

In order to define the desired combination of treatments or conditions, users must select the samples of interest by clicking on the icon beside them (Figure 4). The result is instantly shown by clicking on “Run RefFinder”.

Validation of reference genes for qPCR analysis of *Coffea arabica* L. somatic embryogenesis-related tissues

☐ Non-embryogenic calli
 ☐ Embryogenic calli
 ☐ Embryogenic cell suspensions
 ☐ Plantlets

☐ Somatic embryos (globular, cordiform/torpedo and cotyledonary)

Run Reffinder

Figure 4. Samples analyzed in the study entitled “Validation of reference genes for qPCR analysis of *Coffea arabica* L. somatic embryogenesis-related tissues” by Freitas et al. (2017).

Since RGeasy uses the RefFinder tool to analyze the stability of the reference genes, a table is generated on the results page with the ranking of genes according to the following algorithms: RefFinder, Delta CT, Bestkeeper, Normfinder and Genorm (Figure 5).

Result Obtained by the New Samples Combination:

Method	1	2	3	4	5	6	7	8	9	10	11	12
Delta CT	PP2A	24S	TUB	EF1a	ACT	APRT	CYCL	RPL39	UBQ	14-3-3	AP47	GAPDH
BestKeeper	24S	APRT	RPL39	PP2A	EF1a	CYCL	TUB	ACT	14-3-3	UBQ	AP47	GAPDH
Normfinder	PP2A	24S	TUB	EF1a	ACT	APRT	CYCL	RPL39	14-3-3	UBQ	AP47	GAPDH
Genorm	24S   RPL39	PP2A	EF1a	TUB	ACT	APRT	CYCL	UBQ	14-3-3	AP47	GAPDH	
Recommended comprehensive ranking	24S	PP2A	RPL39	TUB	EF1a	APRT	ACT	CYCL	UBQ	14-3-3	AP47	GAPDH

Comprehensive Ranking	Delta CT	BestKeeper	Normfinder	Genorm
Genes Geomean of ranking values				
24S	1.41			
PP2A	1.86			
RPL39	3.72			
TUB	4.21			
EF1a	4.23			
APRT	4.74			
ACT	5.89			
CYCL	6.96			
UBQ	9.49			
14-3-3	9.49			
AP47	11.00			
GAPDH	12.00			

Figure 5 RGeasy’s result for the different samples analyzed by Freitas et al (2017).

In addition, on the results page, RGeasy provides a table with some additional information for each reference gene, according to the stability ranking from RefFinder. For each reference gene, the primer pair, the correlation coefficient ( $R^2$ ), the amplification efficiency, the accession number, and the database from which the sequence was obtained, are made available to the user (Figure 6).

Gene: UBQ		
Primer Sequence (Forward)	Primer Sequence (Reverse)	
TTTCCTGGCGTGGGTATTG	CGGGTTTATCTCTCCAACGAAT	
R2	e*	Accession n
0.99276	95.0	DV686961.1
Bank		
GenBank National Center for Biotechnology Information (NCBI)		

Gene: PSAB		
Primer Sequence (Forward)	Primer Sequence (Reverse)	
TTTCCTGGCGTGGGTATTG	CGGGTTTATCTCTCCAACGAAT	
R2	e*	Accession n
0.9923	92.0	GT648763.1
Bank		
GenBank National Center for Biotechnology Information (NCBI)		

Figure 6 Representation of the general information made available by RGeasy for two reference genes, *Ubiquitin (UBQ)* and *photosystem I P700 chlorophyll a apoprotein A2 (PSAB)*, present on RGeasy's database.

When selecting reference genes through RGeasy, it is essential to include, in the Materials and Methods section of the user's article, the correct form of RGeasy's citation, which is unique, according to the research being developed (Figure 7).

#### How to Cite

The RGeasy tool (citation soon) was used for the selection of reference genes through a new combination of treatments, obtained from the study developed by Freitas, et al. (2017) and ranked by the RefFinder (XIE et al., 2012) tool.

#### References:

- [Reference Genes Easy](#)
- [RefFinder](#)
- [Validation of reference genes for qPCR analysis of Coffea arabica L. somatic embryogenesis-related tissues](#)

Figure 7 The correct way of citing RGeasy in each study is present at the end of the result's page.

## 3 Registering new species

### 3.1 Registering a single species

In order to register new species, the user should click on the "Register New Species" icon located in the navigation bar (Figure 8). The user should first insert a image file that best characterizes the species that is being registered. Then, the user should add the scientific name of the species, as well as the title of the article, the DOI (Digital Object Identifier), publication year, authors names and the Cq's values of the study (Figure 8).

Figure 8 Registration a new species on RGeasy database.

Once each required information is filled out, the user should not press the “Enter” key, since the tool will understand that all fields have been completed and automatically generate the table displaying the Cq value (Figure 11) of the samples for each reference gene being analyzed. However, since not every field is filled out, this action will result in an error message, as shown in figure 9.

Figure 9 Error message generated due to blank fields during registration of a new species on RGeasy’s database.

Author’s names should be inserted in the “Authors” field and must be separated exclusively by commas (Figure 10).

RG gen

RGeasy Home Species Register New Species About Us Contact Login

English

Species Image  
Choose an Image

Add the Species Here:  
Coffea arabica x

Article DOI Year  
Validation of reference genes for qPCR analysis of Coffea arabica L. somatic embryo 10.1007/s11240-016-1147-6 2017

Authors  
Natália Chagas Freitas,Horllys Gomes Barreto,Christiane Noronha Fernandes-Brum,Rafael Oliveira Moreira,Antonio Chalfun-Junior,Luciano Vilela Paiva

Figure 10 Filling out the author's field during registration of a new species on RGeasy's database.

After filling out the required fields (Figure 11) and pressing the “Enter” key, a table that allows the user to edit the data if necessary will be generated.

RG gen

RGeasy Home Species Register New Species About Us Contact Login

English

Species Image  
Choose an Image

Add the Species Here:  
Coffea arabica x

Article DOI Year  
Validation of reference genes for qPCR analysis of Coffea arabica L. somatic embryo 10.1007/s11240-016-1147-6 2017

Authors  
Natália Chagas Freitas,Horllys Gomes Barreto,Christiane Noronha Fernandes-Brum,Rafael Oliveira Moreira,Antonio Chalfun-Junior,Luciano Vilela Paiva

Cq Data

Samples	24S	ACT	GAPDH	CYCL	EF1aTUB	PP2A	AP47	RPL39	APRT	UBQ 14-3-3		
Non-embryogenic calli	23.81	19.41	17.2173	19.1452	18.2951	23.8537	24.3669	24.181	19.9301	23.2265	25.3652	19.682
Non-embryogenic calli	23.78	19.42	17.2173	19.1647	18.2763	23.9093	24.3962	24.2006	19.912	23.2641	25.3652	19.7414
Non-embryogenic calli	23.795	19.415	17.2173	19.1549	18.2857	23.8815	24.3815	24.1908	19.921	23.2453	25.3652	19.7117

Generate Table

Figure 11 Registration a new species on RGeasy's database.

Once the user press on the “Generate table” icon (Figura 11), the table cannot be further edited and a pop-up window will be exhibited, asking the user to select the species category (Animal, Plant or Microorganism) of the new species that is being added (Figure 12).



The screenshot shows the RGeasy web interface. A modal dialog box is centered on the screen with the text: "Which group of organisms does the new species belongs to?". Below this text are three radio button options: "Animal", "Plants", and "Microorganism". An "OK" button is at the bottom of the dialog. In the background, the "Cq Data" table is visible, showing columns for Samples, 24S, ACT, GAPDH, CYCL, EF1aTUB, PP2A, AP47, RPL39, APRT, and UBQ 14-3-3. The table contains three rows of data for "Non-embryogenic calli".

Samples	24S	ACT	GAPDH	CYCL	EF1aTUB	PP2A	AP47	RPL39	APRT	UBQ 14-3-3
Non-embryogenic calli	23.81	19.41	17.2173	19.1452	18.2951	23.8537	24.3669	24.181	19.9301	23.2265
Non-embryogenic calli	23.78	19.42	17.2173	19.1647	18.2763	23.9093	24.3962	24.2006	19.912	23.2641
Non-embryogenic calli	23.795	19.415	17.2173	19.1549	18.2857	23.8815	24.3815	24.1908	19.921	23.2453

Figure 12 RGeasy’s users must inform the species group of the new species being added after clicking on “Generate table”.

Below the table containing the Cq values, a description from each candidate reference gene being analyzed should be given by the user (Figure 13). This information must be carefully completed, since it will be available for other researchers using RGeasy.

The figure shows two identical forms side-by-side. Each form has a header section with "Gene: 14-3-3" (left) and "Gene: RPL7" (right). Below the gene name is a dropdown menu set to "Vegetable" and another dropdown menu set to "Coffea canephora". The main body of each form is enclosed in a green border and contains two columns of input fields. The left column is for "Primer Sequence (Forward)" and the right column is for "Primer Sequence (Reverse)". Below these are four input fields labeled "R2", "e\*", "Accession", and "Bank".

Figure 13 Gene information users must complete for each candidate reference gene of the new species being registered on RGeasy’s database.

The process of registering a new species is completed when users press “Generate”, once every field related to the candidate reference genes is filled out.

## 4.2 Registering two or more species

Researchers carrying out studies that analyze the stability of candidate reference genes for two or more species can make the registration of the different species through a single registration process (Figure 14). However, this is true only if the same group of candidate reference genes is being analyzed for each species. Otherwise, species should be separately registered and thus the article will have to be registered more than once.

**A**

RGeasy Home Species Register New Species About Us Contact Login English

Species Image  
Choose an Image

Add the Species Here:

Coffea arabica

Article DOI Year  
10.1007/s11240-016-1147-6 2015

Authors

Cq Data

Generate Table

**B**

RGeasy Home Species Register New Species About Us Contact Login English

Species Image  
Choose an Image

Add the Species Here:

Coffea arabica, Coffea canephora

Article DOI Year  
10.1007/s11240-016-1147-6 2015

Authors

Cq Data

Generate Table

Figure 14 Registration process of two or more species. The name of the second species should be added right after the name of the first species, with these names being separated by a comma. Registration of one species (A). Registration of two or more species (B).

During the registration of the Cq values, it is essential to identify each sample with the name of the species to which it refers (Figure 15), so thus RGeasy can correctly differentiate each tissue. Otherwise, the tool may recognize two or more samples as only one sample. The further steps of registering two or more species are similar the those of registering a single species.

Cq Data

Tissue	14-3-3	RPL7	PSAB	ACTINA	DMXT	GAPDH	ADH2	UBQ
C. arabica Root	18.83401129	22.34721738	18.9538811	22.27552175	22.09	21.15589526	22.48976486	19.22130877
C. arabica Root	18.79161145	22.25786822	19.06681386	22.19844382	21.99	21.17575063	22.34972896	19.24057826
C. arabica Root	18.80009142	22.36707275	18.99152535	22.28515649	22.23	21.29488284	22.56445067	19.05751817

Cq Data

Tissue	14-3-3	RPL7	PSAB	ACTINA	DMXT	GAPDH	ADH2	UBQ
C. arabica Ro	18.83401129	22.34721738	18.9538811	22.27552175	22.09	21.15589526	22.48976486	19.22130877
C. arabica Ro	18.79161145	22.25786822	19.06681386	22.19844382	21.99	21.17575063	22.34972896	19.24057826
C. arabica Ro	18.80009142	22.36707275	18.99152535	22.28515649	22.23	21.29488284	22.56445067	19.05751817
C. canephora	27.05958129	31.08357957	26.96269581	28.89458898	28.29	26.04031594	29.09945913	27.44937779
C. canephora	26.9747816	32.27490168	27.25443877	28.80787631	28.43	26.28850805	28.77270871	27.39156934
C. canephora	26.76278237	30.58719535	26.87799624	28.96203217	28.56	26.10980973	29.0527805	27.3337609

Figure 15 Description of how to correctly identify each sample on RGeasy. In this case, root tissue was used for two different species (*C. arabica* and *C. canephora*) (Fernandes-Brum et.al, 2017) and as highlighted in green, the identification of each sample in this case was made by writing name of the species followed by the name of the tissue.