

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 receive more citations, as detailed along this manual.

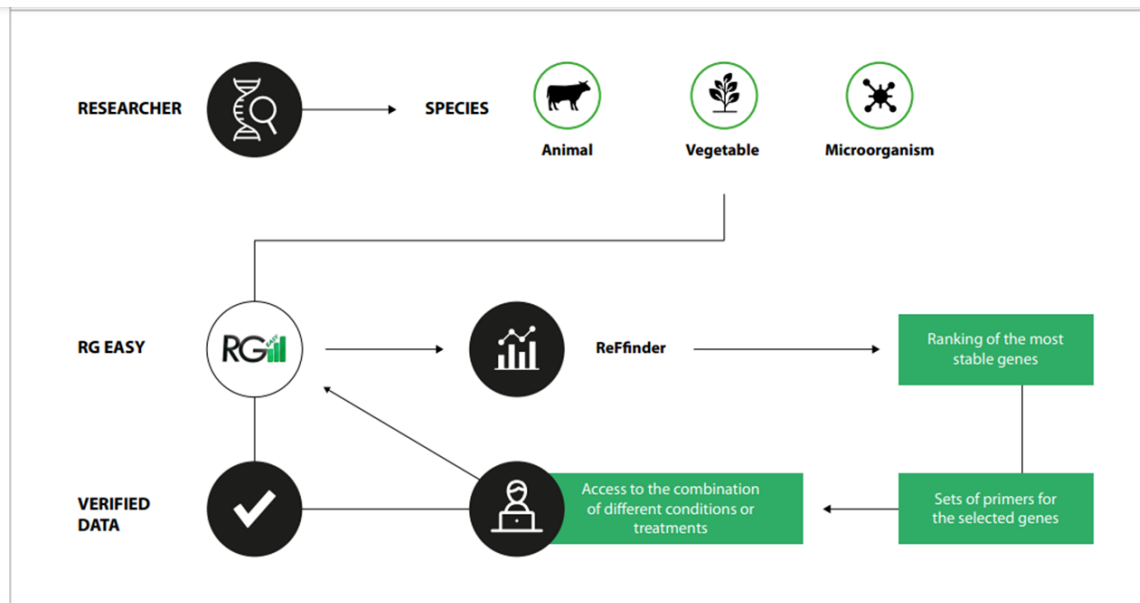


Figure 1- Maintenance and workflow of the RGeasy tool. From the registration of animal, plant or microorganism species, researchers deposit on RGeasy's database their data (Cq's values), which are immediately verified. Then, users can run all possible combinations of conditions/treatments for each study. The new combinations of treatments are 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, the 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).

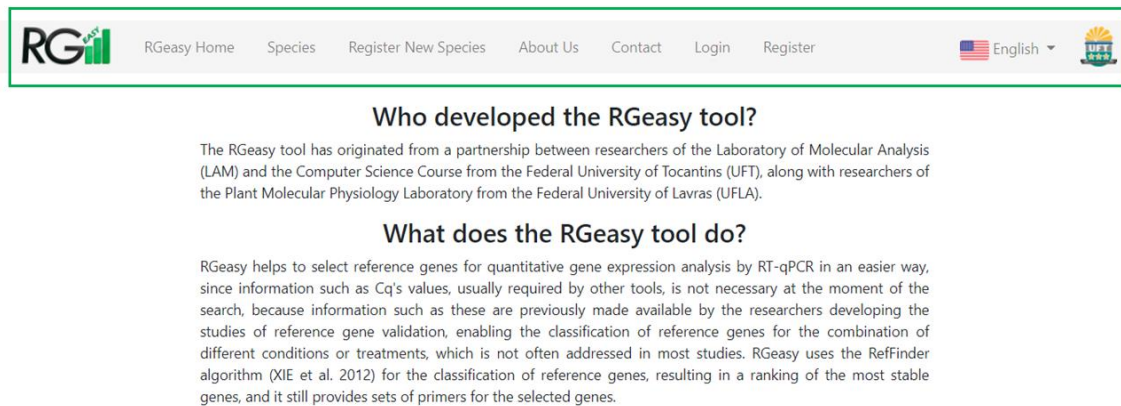


Figure 2- RGeasy's initial graphic interface

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.

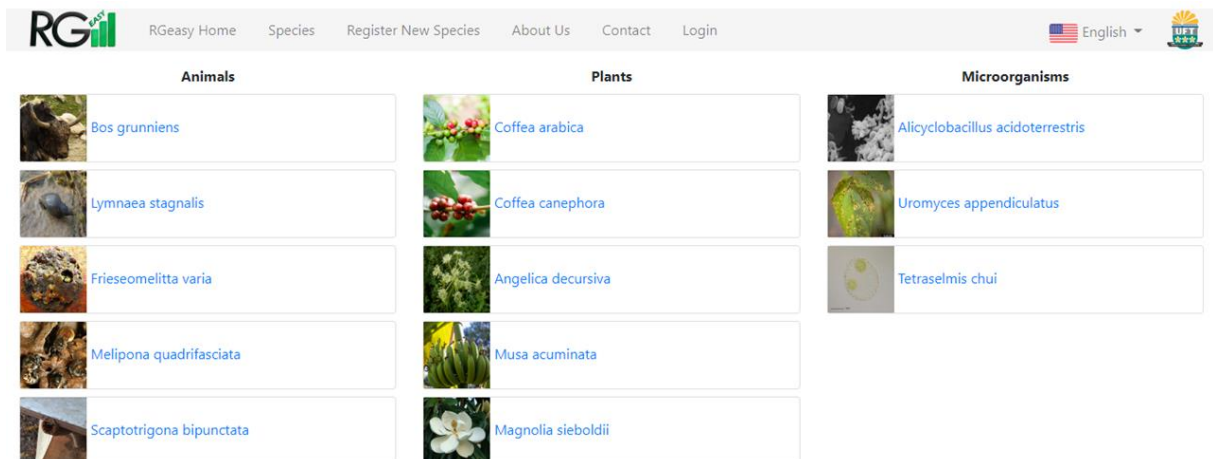


Figure 3- Species categories on RGeasy.

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”.

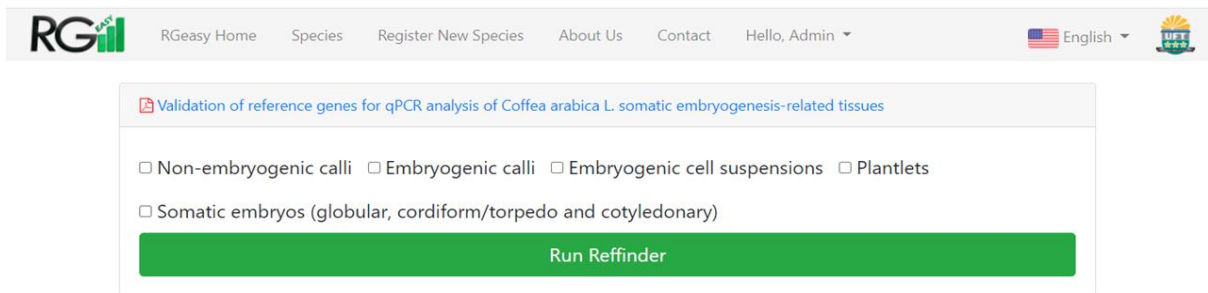


Figure 4- Samples analyzed in the study entitled “Validation of reference genes for qPCR analysis of *Coffea arabica* L. somatic embryogenesis-related tissues” by Freitas et al. (2017).

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).

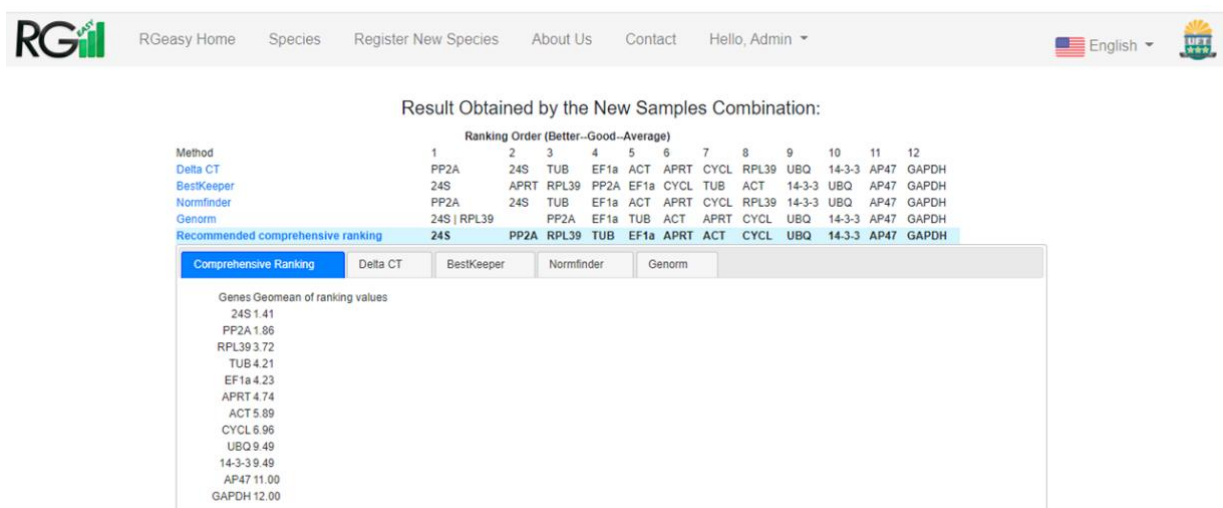


Figure 5- RGeasy’s result for the different samples analyzed by Freitas et al. (2017).

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			Gene: PSAB		
Primer Sequence (Forward)		Primer Sequence (Reverse)	Primer Sequence (Forward)		Primer Sequence (Reverse)
TTTCCTGGCGTGGGTATTG		CGGGTTTATCTCTCCAACGAAT	TTTCCTGGCGTGGGTATTG		CGGGTTTATCTCTCCAACGAAT
R2	e*	Accession n	R2	e*	Accession n
0.99276	95.0	DV686961.1	0.9923	92.0	GT648763.1
Bank			Bank		
GenBank National Center for Biotechnology Information (NCBI)			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). Then, the user is prompted to add an image that best represents the species being registered, to inform its category (Animal, Plant or Microorganism), and to provide the source of the image, when the image is obtained from the internet (Figure 8).

The screenshot shows the RGeasy website's registration interface. At the top, there is a navigation bar with the RGeasy logo and links for Home, Species, Register New Species, About Us, Contact, and Login. The language is set to English. The main form area includes a 'Species Image' section with a placeholder image and a 'Choose an Image' button. Below this is a radio button selection for the organism group: 'Animals', 'Plants' (which is selected), and 'Microorganisms'. At the bottom, there is an 'Add Image Link' text input field with a 'Not Mandatory Field' label.

Figure 8- Initial steps for registering a new species on RGeasy's database.

After providing this information, users should inform the scientific name of the species being registered, as well as the title of the article, its DOI (Digital Object Identifier), year of publication, author names, and the Cq values and the information from each reference gene (Primer sequences, correlation coefficient (R²), amplification efficiency (e), accession number, and the database where the sequence is deposited).

Add the Species Here:

Article: DOI: Year:

Authors:

Cq Values

Samples	24S	ACT	GAPDH	CYCL	EF1a	TUB	PP2A	AP47	RPL39	APRT	UBQ	14-3-3
Non-embryogenic calli	23.81	19.41	17.22	19.15	18.30	23.85	24.37	24.18	19.93	23.23	25.37	19.68
Non-embryogenic calli	23.78	19.42	17.22	19.16	18.28	23.91	24.40	24.20	19.91	23.26	25.37	19.74
Non-embryogenic calli	23.80	19.42	17.22	19.15	18.29	23.88	24.38	24.19	19.92	23.25	25.37	19.71

Reference Genes Information

Gene	Primer-Forward	Primer-Reverse	R ²	e	Accession	Bank
24S	GACCAATCGTCTTCTTCCAGAAA	TCAACTCAGCCTTGGAACATTAG	0.984	100.0	GT730897.1a	GenBank
ACT	GCCAGATGGACAAGTGATTACCA	CAGCAGCTTCCATTCCTATGATAG	0.969	100.0	GT000704.1a	GenBank
GAPDH	GGGAAGAGCTGCTTCATTAACA	CCATTGAGGGCTGGAAGAAC	0.987	95.0	GW488886.1a	GenBank

Figure 9- Required information for registering a new species on RGeasy's database.

Once each required information is filled out, the user should press the “Enter” key. However, if not every field is filled out, this action will result in an error message, as shown in figure 10.

RG^{est} RGeasy Home Species Register New Species About Us Contact Hello, Admin ▾ English ▾ UFE

The accession field is required.

The article field is required.

The bank field is required.

The doi field is required.

The e field is required.

The genes field is required.

Figure 10- 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 11).

The screenshot shows the RGeasy database registration interface. At the top, there are navigation links: RGeasy Home, Species, Register New Species, About Us, Contact, and Login. A language selector is set to English. The main form area includes a 'Species Image' section with a 'Choose an Image' button and a text input for 'Add Image Link' containing 'Canva https://www.canva.com/'. Below this is the 'Add the Species Here:' section with a dropdown menu showing 'Coffea arabica'. The 'Article' section has a text input for the article title, a 'DOI' input with 'https://doi.org/10.1007/s11240-016-1', and a 'Year' input with '2017'. The 'Authors' section at the bottom has a text input containing the names: 'Natália Chagas Freitas, Horllys Gomes Barreto, Christiane Noronha Fernandes-Brum, Rafael Oliveira Moreira, Antonio Chalfun-Junior, Luciano Vilela Paiva'. The 'Which group of organisms does the new species belong to?' section has radio buttons for 'Animals', 'Plants' (selected), and 'Microorganisms'.

Figure 11- Filling out the author's field during registration of a new species on RGeasy's database.

In order to provide the Cq's values and the information related to each reference gene (Figure 9), users should previously organize their data in an spreadsheet, one for each field, as shown in Figures 12 and 13, and then paste the spreadsheets in the correct field (Figure 9).

	A	B	C	D	E	F	G	H	I	J	K	L	M
1	Samples	24S	ACT	GAPDH	CYCL	EF1a	TUB	PP2A	AP47	RPL39	APRT	UBQ	14-3-3
2	Non-embryogenic calli	23.81	19.41	17.22	19.15	18.30	23.85	24.37	24.18	19.93	23.23	25.37	19.68
3	Non-embryogenic calli	23.78	19.42	17.22	19.16	18.28	23.91	24.40	24.20	19.91	23.26	25.37	19.74
4	Non-embryogenic calli	23.80	19.42	17.22	19.15	18.29	23.88	24.38	24.19	19.92	23.25	25.37	19.71
5	Non-embryogenic calli	23.54	19.41	17.32	19.20	18.29	23.59	24.33	24.05	19.75	22.86	25.37	19.56
6	Non-embryogenic calli	23.50	19.50	17.28	19.18	18.29	23.73	24.43	24.10	19.79	22.79	25.33	19.50
7	Non-embryogenic calli	23.52	19.46	17.30	19.19	18.29	23.66	24.38	24.08	19.77	22.83	25.35	19.53
8	Non-embryogenic calli	23.65	19.47	17.34	19.08	18.12	23.91	24.54	24.38	19.88	22.98	25.39	19.90
9	Non-embryogenic calli	23.67	19.47	17.36	19.11	18.12	23.94	24.56	24.40	19.88	22.91	25.39	19.91
10	Non-embryogenic calli	23.66	19.47	17.35	19.09	18.12	23.92	24.55	24.39	19.88	22.94	25.39	19.91
11	Embryogenic calli	23.40	20.63	18.42	18.24	17.93	22.76	23.81	24.44	19.76	23.57	23.54	19.57
12	Embryogenic calli	23.39	20.63	18.44	18.28	17.91	22.69	23.81	24.47	19.74	23.80	23.48	19.67
13	Embryogenic calli	23.40	20.63	18.43	18.26	17.92	22.72	23.81	24.45	19.75	23.69	23.51	19.62
14	Embryogenic calli	23.22	20.65	19.25	18.90	18.80	22.91	23.79	24.43	19.61	23.70	23.29	19.05
15	Embryogenic calli	23.22	20.63	19.25	18.76	18.73	22.91	23.87	24.41	19.67	23.70	23.27	19.06
16	Embryogenic calli	23.22	20.64	19.25	18.83	18.84	22.91	23.83	24.42	19.64	23.70	23.28	19.06
17	Embryogenic calli	23.50	20.96	18.76	18.70	17.67	23.16	24.69	25.08	19.75	24.56	23.78	19.78
18	Embryogenic calli	23.54	20.96	18.74	18.65	17.69	23.17	24.68	25.21	19.70	24.60	23.80	19.78
19	Embryogenic calli	23.52	20.96	18.75	18.67	17.68	23.16	24.68	25.29	19.66	24.58	23.79	19.78

Figure 12- Cq's values should be organized in columns, one for each reference gene, and the sample type should be described in the lines of the spreadsheet, which is then pasted in the 'Cq values' field when registering a new species.

	A	B	C	D	E	F	G
1	Gene	Primer-Forward	Primer-Reverse	R2	e	Accession	Bank
2	24S	GACCAATCGTCTTCTTCCAGAAA	TCAACTCAGCCTTGAAAACATTAG	0.984	100.0	GT730897.1a	GenBank
3	ACT	GCCAGATGGACAAGTGATTACCA	CAGCAGCTTCATTCTATGATAG	0.969	100.0	GT000704.1a	GenBank
4	GAPDH	GGGAGAGCTGCTTCATTTAACA	CCATTGAGGGCTGGAAGAAC	0.987	95.0	GW488886.1a	GenBank
5	CYCL	TGGTCCAGGGATTTGTCCAT	CGGTCTTGTCCGGTCAGAT	0.997	96.0	GT007167.1a	GenBank
6	EF1a	GGTGGTTTTGAAGCTGGTATTCT	TGTTGCAGCAGCAGATCATT	0.997	92.0	GR996930.1a	GenBank
7	TUB	TCGGGCTGCCTCATGGAT	TTGTCGGGCTGAAGATCTG	0.995	90.0	GT707405.1a	GenBank
8	PP2A	ACCTATGGTGAAATGAAGATGGA	AGGCGGCGAGATGAATCTTT	0.973	97.0	GT005097.1a	GenBank
9	AP47	GGGTACGCTCACCATTTTCATC	AGCCAACAGCACCAGTAACTTG	0.947	97.0	DV690764.1a	GenBank
10	RPL39	GCGAAGAAGCAGAGGCAGAA	TTGCCATTGTAGCGGATGGT	0.991	87.0	GT720707.1a	GenBank
11	APRT	TGGAGAACGGGCTCTGGTAGT	ACGCGCTCAAGTAGCCTGAT	0.992	92.0	GR996015.1a	GenBank
12	UBQ	AATCCGTCCCCGCATGTT	CCAGTGCATCCTGTTGTCTCA	0.999	99.0	Cc05_g12790b	Sol Genomics Network (SGN) database
13	14.3.3	AGCTCAGCAAGATATGTGATGAA	TGGTAGTCACCCTTCAATTTTCA	0.955	80.0	SGNU356404b	Sol Genomics Network (SGN) database
14							

Figure 13- Information related to each reference gene, including primer sequences, correlation coefficient (R2), amplification efficiency (e), accession number, and the database where the sequence is deposited, should be organized in columns, and each reference gene in lines of the spreadsheet, which is then pasted in the 'Reference Genes Information' field when registering a new species when registering a new species.

Once all required information for registering a new species is filled out, the user should click on the 'Create' icon to complete this process.

3.2 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

RG Home Species Register New Species About Us Contact Hello, Admin English

Species Image Choose an Image

Which group of organisms does the new species belong to?
 Animals Plants Microorganisms

Add Image Link:
 Canva https://www.canva.com/

Add the Species Here:
 Coffea arabica x

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

Authors

B

RG Home Species Register New Species About Us Contact Hello, Admin English

Species Image Choose an Image

Which group of organisms does the new species belong to?
 Animals Plants Microorganisms

Add Image Link:
 Canva https://www.canva.com/

Add the Species Here:
 Coffea arabica x Coffea canephora x

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

Authors

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) and the sample type. Thus, RGeasy can correctly differentiate each sample. Otherwise, the tool may recognize two or more samples as only one sample. The further steps of registering two or more species are similar to those previously described for registering a single species.

	A	B	C	D	E	F	G	H	I
1	Samples	14-3-3	RPL7	PSAB	ACTINA	DMXT	GAPDH	ADH2	UBQ
2	C. canephora Root	18.83	22.35	18.95	22.28	22.09	21.16	22.49	19.22
3	C. canephora Root	18.79	22.26	19.07	22.20	21.99	21.18	22.35	19.24
4	C. canephora Root	18.80	22.37	18.99	22.29	22.23	21.29	22.56	19.06
5	C. canephora Root	27.06	31.08	26.96	28.89	28.29	26.04	29.10	27.45
6	C. canephora Root	26.97	32.27	27.25	28.81	28.43	26.29	28.77	27.39
7	C. canephora Root	26.76	30.59	26.88	28.96	28.56	26.11	29.05	27.33
8	C. canephora Root	18.35	22.30	20.11	22.19	23.44	20.30	22.41	20.21
9	C. canephora Root	18.28	22.30	20.18	22.16	23.56	20.35	22.51	20.36
10	C. canephora Root	18.30	22.26	20.37	22.21	23.49	20.28	22.49	20.18
11	C. arabica Root	21.80	19.87	20.52	18.77	20.42	25.71	23.70	23.93
12	C. arabica Root	21.78	19.85	20.42	18.79	20.50	25.81	23.84	23.75
13	C. arabica Root	21.78	20.15	20.48	18.93	20.28	25.89	23.75	23.79
14	C. arabica Root	23.48	23.76	23.73	21.68	22.13	30.54	26.20	26.08
15	C. arabica Root	23.42	23.94	23.78	21.65	22.22	30.62	26.15	26.08
16	C. arabica Root	23.38	23.38	23.96	21.82	22.40	30.71	26.34	26.04
17	C. arabica Root	21.67	20.18	20.57	18.66	20.02	27.67	23.59	24.14
18	C. arabica Root	21.68	20.06	20.63	18.59	19.98	27.36	23.66	24.06
19	C. arabica Root	21.77	20.29	20.63	18.62	19.97	27.43	23.66	24.11

Figure 15- As highlighted in green, when two or more species are present, each sample of the spreadsheet should be correctly named, name of the species followed by the sample type. This allows RGeasy to correctly differentiate each sample. In this example, samples analyzed belong to the study entitled “A panel of the most suitable reference genes for RT-qPCR expression studies of coffee: screening their stability under different conditions” by Fernandes-Brum et al. (2017).

References

Fernandes-Brum, C.N., de Oliveira Garcia, B., Moreira, R.O., Sággio, S.A., Barreto, H.G., Lima, A.A., Freitas, N.C., de Lima, R.R., de Carvalho, C.H.S. and Chalfun-Júnior, A. (2017) A panel of the most suitable reference genes for RT-qPCR expression studies of coffee: screening their stability under different conditions. *Tree Genet Genomes*, **13**, 1–13.

Freitas, N.C., Barreto, H.G., Fernandes-Brum, C.N., Moreira, R.O., Chalfun-Junior, A. and Paiva, L.V. (2017) Validation of reference genes for qPCR analysis of *Coffea arabica* L. somatic embryogenesis-related tissues. *Plant Cell, Tissue and Organ Culture (PCTOC)*, **128**, 663–678.

Xie, F., Xiao, P., Chen, D., Xu, L. and Zhang, B. (2012) miRDeepFinder: a miRNA analysis tool for deep sequencing of plant small RNAs. *Plant Mol Biol*, **80**, 75–84.