qPCR-DAMS: a Database Tool to Analyze, Manage, and Store Both Relative and Absolute Quantitative Real-Time PCR data

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Supplementary Materials: Users manual

Table of Contents

Introduction

qPCR-DAMS is a database management system implemented on MS Access 2003 and Visual Basic. It is designed to analyze, manage, and store relative and absolute quantitative real-time PCR data. The system consists of 5 blocks: three blocks (Gene, Plate, and Experiment) for inputting, storing and describing raw data, and two blocks (View data and Process Data) for checking, evaluating, and processing data. Users are allowed to choose among four basic outputs: (I) Ratio relative quantification, (II) Absolute level, (III) Normalized absolute expression, and (IV) Ratio absolute quantification, and two advanced options: (V) Multiple reference relative quantification and (VI) Multiple references absolute quantification, within single software package. The coefficient of variation is monitored at each step during data processing and the accuracy is further improved by an easy data tracking and display system. In summary, qPCR-DAMS is a handy novel tool for real-time PCR users.

System requirement

Operation system: Windows 2000 or above, Windows XP, Access 2000 or above
comdlg32.ocx in the %systemroot%\System32 folder
Memory: 256 MB of RAM
Hard disk: 10 MB of hard disk space
Monitor: 1024 x 768 or greater monitor resolution
Internet: no necessary for the current version but may need for higher versions

Installing qPCR-DAMS

1. Go to web: **http://lungmicroarray.org/lbtl/info/qPCR-DAMS/index.htm**
2. Double click **qPCR-DAMS** and save to the chosen destination.
3. Double click "**qPCR-DAMS.zip**" from the destination.
4. Double "**qPCR-DAMS**", the whole package includes the software, test data, and users' manual.
5. Double click "**qPCR-DAMS.mdb**" to open the software.

***If the software is run on Window XP, a security warning will appear because of the system settings (for details please visit http://office.microsoft.com/en-us/assistance/HA011071331033.aspx). Ignore the warning and click **Open**. A panel containing the brief introduction of the software will appear. Check "Don't show this screen

again" and click **OK**. A panel **Menu** will appear. If the software is run in Window 2000 environment, there will be no security warning and the introduction panel will appear directly.

qPCR-DAMS Tasks

There are five main panels: **Gene**, **Plate**, **Experiment**, **View Data**, and **Process Data** on the database tool. Inside each panel, there are some more options to allow the users to operate.

I. Gene

The main function is to host and manage gene information in the database.

1. **Add New Gene** allows the users to input gene name, gene ID, and description.
2. **Edit Gene Information** allows the users to edit the existing gene information including gene name, gene ID, and description in the database.
3. **View Gene List** allows the user to check the following items:
    a. Gene list in the database
    b. Report file name list associated with a gene
    c. Plate report generated by the detector system in a report file name

II. Plate

It is the central part of the database system that brings genes, samples, and experiments all together. The main function is to host and manage plate information as well as data generated by the detector system in the database. There are two options but only one option is needed for the users to input plate reports into the database. The import application requires comdlg32.ocx in the %systemroot%\System32 folder. (See troubleshooting for how to extract comdlg32.ocx to the folder).

1. **Import Plate Report.**
    1.1. **Add New Plate Name** allows the users to enter the name of a new plate. Plate name here means the unique name we give to each real-time PCR plate. So when a target gene and several reference genes are run on the same plate, they should have the same plate name (**Note**: plate name is important for relative quantification because normalization is plate-name dependent.)
    1.2. **Import New Plate Report** allows the users to choose report from file, to select genes from the gene list, to select plate names from the plate name list, and to import plate reports generated by the detector system into the database. The "report file name" is the name we give to each individual plate report generated by the detector system. It is different from plate name. If a target gene is run on a plate with three reference

genes, there will be only one plate name but 4 plate file names.

1.3. **Edit Sample Information** allows the users to edit sample information including treatment and description. (**Note**: Correct treatment input is important for data processing because some calculations are dependent of treatment).

1.4. **View and Edit Plate Information** allows the users to view and edit plate names, plate file names, and report files. The most important function is for data quality control. If a sample produces abnormal data, this sample can be removed manually by the users through this option. The users can also delete plate report files that are not needed and therefore the database will not accumulate useless information. In addition, the users can edit report file names, gene names, and plate names. .

2. **Add Plate Report Manually.**

2.1. **Add New Sample.**

2.1.1. **Add sample by batch**

2.1.1.1. **Paste New Sample** allows the users to enter a batch of sample names at the same time and thus save significant time for users.

2.1.1.2. **Add Sample to Database** allows the users to input the sample name into the database. If the users input a sample name that is already in the database, the database system ignores it.

2.1.1.3. **Edit Sample Information** allows the users to edit sample information including treatment and description.

2.1.2. **Add Sample One by One**

2.1.2.1. **Add New Sample** allows the users to enter sample name, treatment, and description one by one. This is useful for a limited number of samples that need to be input.

2.1.2.2. **Edit Sample Information** allows the users to edit sample information including treatment and description.

2.2. **Add New Plate Name** allows the users to enter the name of a new plate.

2.3. **Add New Plate Report Manually** allows the users to enter report file name, to select genes from the gene list, to select plate names from the plate name list, and to paste plate reports generated by the detector system into the database.

2.4. **View and Edit Plate Information** allows the users to view and edit plate names, plate file names, and report files.

III. Experiment

Experiment panel provides a platform for the users to organize all the information related to an experiment together.

1. **Researcher Information** allows the users to enter name, title, address, city, region, Zip code, country, phone number, fax number, and email address of the researchers.
2. **Add New Experiment** allows the users set up a new experiment (**Note**: only for single reference gene normalization! See “Advanced options” in “Process Data” for multiple references gene normalization). The following functions can be carried out by the users:
    a. Enter the name of a new experiment
    b. Select researcher ID from the researcher list
    c. Select a target and a reference gene from the gene list
    d. Select a group of samples that will be used as control from the sample list
    e. Enter the amplification efficiency of the target gene and the reference which will be used in the relative quantification. If no number entered, the default value will be 2
    f. Enter the threshold value of intra-plate and inter-plate variation that will be used in the data quality control
    g. Select report files for the target gene and the reference gene that will be used in data processing
3. **Edit Experiment Setting** allows the users to change the settings of an existing experiment in the database which include experiment name, description, target gene, reference gene, target gene amplification efficiency, reference gene amplification efficiency, control, threshold value of intra-plate and inter-plate variation, report files for data processing.
4. **View Experiment Setting** allows the users to check the experiment list as well as their settings in the database. In addition, the users can check the plate list involved in each experiment by plate name, the plate report file list under each plate name, as well as the detailed report file.

IV. View data

This panel provides an easy way to track the archived data in the system. Furthermore, the users can validate their processed results by directly sorting the data to the raw data exported from the detector system. This function may be extremely important for clinical laboratories that handle thousands of samples, or laboratories using real-time PCR for microarray data validation, who work with numerous genes because all the data table in the database system are linked. No matter at which point the user starts viewing data, they can finally reach the

raw data exported from the detector system. This panel is composed of four sub-panels: **View data by gene, View data by sample, View data by plate,** and **View data by experiment.**

V. Process Data

This panel allows the users to choose the appropriate data processing module and to calculate the expression levels of the interested gene under certain quality controls for the selected experiments. Four basic data processing modules are provided by qPCR-DAMS to process data with single a reference gene normalization and an advanced option allows the users to use a multiple reference gene normalization (For detailed conceptions and mathematical procedures of each module, see **Concepts and Mathematical structures**). The main functions covered by this panel are as follows:

1. **Basic data processing**
    i. **Ratio relative quantification** allows the users to perform relative quantification and express the results as ratios.
    ii. **Absolute level** allows the users to perform absolute quantification without a reference gene.
    iii. **Normalized absolute expression** allows the users to perform absolute quantification with normalization from a reference gene.
    iv. **Ratio absolute quantification** allows the users perform absolute quantification and express the results as ratios.
2. **Advanced option** allows the users to calculate more accurate relative expression levels by using multiple reference gene normalization. This function is based on the experiment's result generated from the basic data processing modules.
    i. **Multiple references relative quantification** allows normalization with multiple reference genes based on relative quantification experiments.
    ii. **Multiple reference absolute quantification** allows normalization with multiple reference genes based on absolute quantification experiments.
3. **Quality control** helps the users to improve the validity of the processed data by three error checking steps. The errors found by step 1 and 2 can be marked and excluded manually by the users.
    i. **Fix unrecognized Ct or quantity error**: If Ct is undetermined or the quantity is 0, qPCR-DAMS updates the values of 40 to Ct or 0.01 to quantity to avoid the mathematical problem of calculating the ratio. This option will permanently change the data in the system, so please carefully check your data before selecting **Yes**.

ii. **Step 1 error check**: A threshold value for the intra-plate variation can be set to find out the abnormally amplified samples from the replicates on the same plate.

iii. **Step 2 error check**: A threshold value for the inter-plate variation can be set to find out the abnormally amplified samples from the replicates on the different plates.

4. **Data display**: qPCR-DAMS can output separate report for all the main calculation steps so as to meet the requirement of different users. This function also helps the users to improve the data validity through step-by-step monitoring or re-checking of the processed data. Because several factors such as bad sample, bad reaction, cross contamination, and pipetting error, may all cause misleading results. Many errors cannot be recognized by the built-in error check procedures, which are largely based on standard deviation calculation. This step helps the users to find these "escaped" errors. We found it is even more helpful when using this function together with **View Data by Sample**. To know the meaning of each data-to-display, see **Concepts and Mathematical Structures**.

   i. **Ratio relative quantification** displays Ct, normalized expression, intra-plate sample ratio, inter-plate sample ratio and final ratio.

   ii. **Absolute level** displays target intra-plate quantity, target inter-plate quantity, and absolute level.

   iii. **Normalized absolute expression** displays target intra-plate quantity, reference intra-plate quantity, target inter-plate quantity, reference inter-plate quantity, normalized expression, and final normalized expression.

   iv. **Ratio absolute quantification** displays target intra-plate quantity, reference intra-plate quantity, target inter-plate quantity, reference inter-plate quantity, normalized expression, and final ratio.

   v. **Multiple references relative quantification** displays inter-reference sample ratio and final ratio.

   vi. **Multiple reference normalization for absolute quantification** displays inter-reference sample ratio and final ratio.

D. How to use qPCR-DAMS

Here we present an example that is suitable for all the data processing modules of qPCR-DAMS. New users can try this example and the test data we provided. Test data can be downloaded at:

http://lungmicroarray.org/lbtl/info/qPCR-DAMS/index.htm

Suppose we want to know the effect of a drug on the mRNA expression of GABA receptor rho2 subunit (rho2) in rat brain. Therefore, we applied the drug to rats and collect whole brains from day 1 to day 6 (D1~D6) after the treatment. Rat brains collected on the day before drug application were used as controls (D0). Three independent experiments (a, b, and c) were carried out and the samples were named like D0a and D1c. Total RNA was isolated from those samples and 1 μg of the RNA was reverse transcribed into cDNA. The mRNA expression levels of rho2 were quantified with real-time PCR by both relative and absolute quantitative methods at the same time. Briefly, rho2 was run together with three house-keeping genes: β-actin (ACTB), Ubiquitin C (UBC), and Glyceraldehyde-3-phosphate dehydrogenase (GAPD) on the same 384-well-plate. The standard curves of each gene were also run on that plate. Duplicate wells were used for all the samples and the plate-run was repeated once (on 03/25/2005 and 03/30/2005, respectively). After exporting the experiment results (report file) from the detector system, the data were ready to process by qPCR-DAMS.

I. Enter information

I.1 Enter New Gene

1. Select **Gene** on the **Menu** or press **G** on the keyboard. A **Gene Control Panel** appears.
2. Select **Add New Gene**. An **Add New Gene Information** panel will pop up. Enter gene information as follows:

Gene ID	***Gene Name***	***Description***
D38494	Rho2	GABA receptor rho2 subunit, enriched in retina
NM_031144	ACTB	beta-actin, a cytoskeletal structure protein
NM_017314	UBC	Ubiquitin C, protein degradation
X02231	GAPD	Glyceraldehyde-3-phosphate-dehydrogenase, oxidoreductase in glycolysis and gluconeogenesis

3. Close **Add New Gene Information** panel and Select  from the Gene Control panel.

I.2 Enter New Plate

The users can either import plate report or add plate report manually. (Note: a comdlg32.ocx is required to run the import application. For details, see **Trouble Shooting**).

A. Import Plate Report

1. Click the **Plate** button on the main menu or press **P** on the keyboard. A **Plate Control Panel** will appear.
2. Select **Add New Plate Name** from the **Import New Plate Report** panel, and then input "03252005" in the **Plate Name**. **Plate ID** "1" will appear automatically.
3. Click **Add Next Record** and input "03302005" into the **Plate Name**. **Plate ID** "2" will appear automatically.
4. Click  to back to the **Plate Control Panel** and select **Import New Plate Report**.
5. Select **Choose Data File**. Highlight "rho2_03252005" from the destination and select **Open**. The path of the file and the file name will show up automatically in the **Report File** and **Report File Name** blanks.
6. Select "Rho2 " from the **Gene ID** list.
7. Select "03252005" from the **Plate Name** list.
8. Select **Import Data** and input the data into the database (Note: the format of the report should be cleared before importing. To clear the format, open the plate report Excel file, click on the top left square and change the whole table black. Then select **Edit** > **Clear** > **Formats**.)
9. Select Choose Data File and enter more plate information according to the following table:

Report File Name	***Gene Name***	***Plate Name***
ACTB_03252005	ACTB	03252005
UBC_03252005	UBC	03252005
GAPD_03252005	GAPD	03252005
Rho2_03302005	Rho2	03302005
ACTB_03302005	ACTB	03302005
UBC_03302005	UBC	03302005
GAPD_03302005	GAPD	03302005

[**Note**: If experiment report was generated by other system such as ABI 7500, arrange column of data to the same order as that system(Well, Type, Sample Name, Replicate, Ct, Quantity, Std Dev, Mean).

10. Click **Edit Sample Info** from the **Import Plate Report** panel. An **Edit Sample Info** panel will appear.
11. Sample information can be input according to the following table:

Sample Name	***Treatment***	***Description***
D0a, D0b, and D0c	D0	Brain tissue before drug treatment
D1a, D1b, and D1c	D1	Brain tissue after drug treatment for 1 day
D2a, D2b, and D2c	D2	Brain tissue after drug treatment for 2 days
D3a, D3b, and D3c	D3	Brain tissue after drug treatment for 3 days
D4a, D4b, and D4c	D4	Brain tissue after drug treatment for 4 days
D5a, D5b, and D5c	D5	Brain tissue after drug treatment for 5 days
D6a, D6b, and D6c	D6	Brain tissue after drug treatment for 6 days

12. Click  to back to the main menu.
13. It is easy to make mistake when entering plate information. Go to **View and Edit Plate Information** from the **Plate Control Panel** to correct wrong information or delete unwanted plate information.
14. Close all other panels and back to the main Menu.

B. Add Plate Report Manually

1. Click the **Plate** button on the main menu or press **P** on the keyboard. A **Plate Control Panel** will appear.
2. Select **Add New Sample** from the **Add Plate Report Manually** panel and a **Sample Control Panel** will pop up.
3. You can enter new sample one by one when sample number is small or you can also enter sample by batch when sample number is large.

   3.1 Enter sample by batch

   1) Click **Paste New Samples** from the left top of the panel (block **Add Sample by Batch**). A **Paste New Samples by Batch** panel will pop up. Click the left square to the blank row to change to whole row under **Sample Name** black.

2) Double click to open the “qPCR-DAMS Test Data”, which contained 8 report files exported from the ABI 7700 detector system. Open the file “rho2_03252005” and copy all the sample names in column and then paste to the black row.
3) A dialog will pop up “You are about to paste 18 record(s), are you sure you want to paste those record(s)? Click **Yes** and then close this panel.
4) Click **Input Sample to Database**. A dialog “You are about to append query that will modify database, are you sure……?” Click **Yes**.
5) Because there are repeated sample names, a new dialog “You are about to append 18 rows……Are you sure you want to append the selected rows?” Click **Yes**. A dialog “Microsoft Office Access can’t append all the records in the append query” will pop up, click **Yes** and close this panel.
6) Click **Edit Sample Info** from block **Add Sample by Batch**. An **Edit Sample Info** panel will appear.
7) Input related **Sample Name**, **Treatment**, and **Description.**
8) Click  to back to the main menu.

3.2 Enter sample one by one (if you have not done add sample by batch, you can use this step)

1) Click **Add New Sample** button on the right top of the panel (block **Add Sample One by One**). An **Add New Sample** panel will pop up.
2) Input related **Sample Name**, **Treatment**, and **Description.**
3) Click “next” symbol and input relative information for other samples
4) Click on  to exit.

I.3 Enter New Experiment

1. Click **Experiment** button on the main menu or press **E** on the keyboard. An **Experiment Control Panel** will pop up.
2. Select **Researcher Information** and a researcher table will open. Input “Jin” and “Nili” to the blank of **Last Name** and **First Name**, respectively. Other information is optional. Close this table.
3. Select **Add New Experiment**, and then input “Rho2 subunit expression in brain 1” under the **Experiment Name**. An **Experiment ID** “1” will be generated automatically.

4. Input “ACTB normalization” in the **Description**.
5. Select “ACTB” and “rho2” from the **Reference gene** and **Target gene** list, respectively.
6. Enter 1.99 and 2 for the **Refer E** (reference gene amplification efficiency) and **Target E** (target gene amplification efficiency), respectively. If amplification efficiency is not selected, the default number will be 2.
7. Select “Nili” from the **Researcher ID** list and “D0” from the **Control** list. Input “6%” and “30%” for the **Intra-Plate Threshold** and **Inter-Plate Threshold** (Note: The users can enter their own threshold value).
8. Select “ACTB_03252005” and “ACTB_03302005” from list for the **Reference Gene Plate Report**; “rho2_03252005” and “rho2_03302005” for the **Target Gene Plate Report.**
9. Select “**Add Next Experiment**” and enter two more experiments according to the following settings:

Experiment 2

Experiment Name: Rho2 subunit expression in brain 2; **Description**: UBC normalization; **Target Gene**: rho2; **Reference Gene**: UBC; **Target E**: 2; **Reference E**: 1.98; **Control**: D0; **Researcher ID**: Nili Jin; **Intra-plate threshold**: 6%; **Inter-plate threshold**: 30%; **Reference Gene Plate Report**: UBC_03252005, UBC_03302005; **Target Gene Plate Report**: Rho2_03252005, Rho2_03302005.

Experiment 3

Experiment Name: Rho2 subunit expression in brain 3; **Description**: GAPD normalization; **Target Gene**: rho2; **Reference Gene**: GAPD; **Target E**: 2; **Reference E**: 2.0; **Control**: D0; **Researcher ID**: Nili Jin; **Intra-plate threshold**: 6%; **Inter-plate threshold**: 30%; **Reference Gene Plate Report**: GAPD_03252005, GAPD_03302005; **Target Gene Plate Report**: Rho2_03252005, Rho2_03302005.

10. Close all other panels and back to the main Menu.

Check Error and Process Data

Basic modules

I. Ratio Relative Quantification

1. Click **Process Data** and a **Choose Process Module** panel will appear. Select **Ratio Relative Quantification** from the new panel.
2. Under **Choose Experiment** select “Rho2 expression in brain 1” from the list.

3. Click "**Fix Unrecognized Ct Error**" and a dialog "You are about to run a query that will modify data in your table" will appear. Select **Yes** and a dialog "You are about to up-date 0 rows" will appear. Click **Yes**. If the dialog show "You are about to up-date N rows", it means that plate reports in this experiment include wells with "Undetermined" Ct. Click **Yes** and the "Undetermined" Ct will be changed to 40 by the system.
4. Click **Step 1 Error Check**. The pop-up table shows that "D2c" on report file Rho2_03302005 produced an intra-plate variation higher than 6%, the intra-plate threshold value. Close this table.
5. From the main **menu**, choose **View Data** > **View Data by Sample**. Click on the "+" in front of the "D2c" and the list of report data about "D2c" will show up. The report shows well "B18" is quite different from the other replicates. Close the **View Data** panel and select **Plate** > **Plate Control Panel** > **View and Edit Plate Information.** Select "03302005" > "Rho2_03302005". Check the well "B18" and return to the **Experiment Report** panel. Perform **Step 1 Error Check** again and the pop-up table shows no sample has intra-plate variation higher than the threshold. Close this table.
6. Click **Step 2 Error Check**. The pop-up table shows D2a produces an inter-plate variation higher than 30%. Go **View Data**> **View Data by Sample**>**D2a**. No abnormal is found. Ignore this problem and close the **Check Error by Inter CV** table.
7. Select the result type (**Ct**, **Normalized Expression**, **Intra-plate Sample Ratio**, **Inter-plate Sample Ratio**, and **Final Ratio**) to process.
8. Select **Preview** to show results. The final result of experiment 1 are as follows:

Treatment	Final Ratio	SD Final Ratio
D0	1	0.209548
D1	1.743938	0.292553
D2	1.474616	0.059399
D3	2.773921	0.229582
D4	2.838308	0.738051
D5	1.934002	0.832779
D6	0.730862	0.002113

9. To export experiment result for further data processing, right click mouse on the report and select **Export** (We suggest choosing "Microsoft Excel 97-2003" as the file type). Click **Save** to save the exported report in the proper destination.
10. To print the processed data directly, click **Print** from the **Experiment Reports** panel.

II. Absolute levels

Procedures are basically the same as Ratio Relative Quantification. See Ratio Relative Quantification for details.

1. Choose **Process Data**>**Choose Process Module**>**Absolute levels**.
2. **Choose Experiment** "Rho2 expression in brain 1" from the list.
3. **Fix Unrecognized Quantity Error**. A dialog "You are about to run a query that will modify data in your table" will appear. Select **Yes** and a dialog "You are about to update 4 rows" will appear. From **View Data** (see detail later) it shows the unrecognized quantity errors are from NTC but not from samples to quantify. Click **Yes** and the unrecognized quantity will be updated to 0.01 by the system.
4. **Step 1 Error Check** to find out and filter abnormal samples.
5. **Step 2 Error Check** to find out and filter more abnormal samples.
6. Select the result type (**Target Intra-plate Quantity**, **Target Inter-plate Quantity**, and **Absolute Level**) to process.
7. Select **Preview** to show results. The final result of experiment 1 are as follows:

Treatment	Mean Log Qty	StDev Log Qty
D0	3.776928	0.279465
D1	3.382855	0.170282
D2	3.213073	0.183467
D3	4.117435	0.228602
D4	4.149184	0.070496
D5	3.783094	0.148153
D6	3.818442	0.191278

8. Right click on the experiment report to export.
9. Click Print from the **Experiment Reports** panel to print.

III. Normalized Absolute Expression

1. Select **Process Data**>**Choose Process Module**>**Normalized Absolute Expression**.
2. **Choose Experiment** from the experiment list.
3. **Fix Unrecognized Quantity Error** to update the unrecognized quantity.
4. **Step 1 Error Check** to find out and filter abnormal samples.
5. **Step 2 Error Check** to find out and filter more abnormal samples.

6. Select the result type (**Target Intra-plate Quantity**, **Reference Intra-plate Quantity, Target Inter-plate Quantity**, **Reference Inter-plate Quantity**, **Normalized Expression**, and **Final Normalized Expression**) to process.
7. Select **Preview** to show results. The final result of experiment 1 is as follows

Treatment	AvgOfNE	StDevOfNE
D0	-2.58834	0.125609
D1	-2.32123	0.081542
D2	-2.42219	0.05125
D3	-2.17002	0.014316
D4	-2.20441	0.074602
D5	-2.26875	0.106302
D6	-2.68052	0.025788

8. Right click on the experiment report to export results.
9. Select **Print** to print results.

IV. Ratio Absolute Quantification

1. Choose **Process Data**>**Choose Process Module**>**Ratio Absolute Quantification**.
2. **Choose Experiment** from the experiment list.
3. **Fix Unrecognized Quantity Error** to update the unrecognized quantity.
4. **Step 1 Error Check** to find out and filter abnormal samples.
5. **Step 2 Error Check** to find out and filter more abnormal samples.
6. Select the result type (**Target Intra-plate Quantity**, **Reference Intra-plate Quantity, Target Inter-plate Quantity**, **Reference Inter-plate Quantity**, **Normalized Expression**, and **Final Ratio**) to process.
7. Select **Preview** to show results. The final result of experiment 1 is as follows:

Treatment	AvgOfRatio	StDevOfRatio
D0	1	0.302815
D1	1.80651	0.333273
D2	1.41819	0.169115
D3	2.519575	0.083532
D4	2.357008	0.379941
D5	2.061153	0.49238
D6	0.77861	0.04683

8. Right click on the experiment report to export results.
9. Select **Print** to print results.

Advanced modules

V. Multiple References Relative Quantification

1. Click **Process Data** and then select **Multiple References Relative Quantification** under the **Advanced Options**.
2. **Choose Experiment** from the experiment list and then click **Add**. If dialog "You are about to run an append……" pop up, click **Yes**. A dialog will remind you to add experiment "You are about to append N rows". Click **Yes**. This experiment will display under the **Experiment Selected**. (**Note**: Please choose and add experiments one by one)
3. To delete a selected experiment, choose that experiment from **Experiment Selected** and click **Delete**. The same dialogs will appear. Click **Yes**.
4. Select the result type (**inter-ref sample ratio** and **final ratio**) and click **process**. If you choose experiment 1 and 2, the final result is as follows:

Treatment	Final Ratio	StDev of Final Ratio
D0	0.998119	0.132892
D1	1.06107	0.145566
D2	0.689422	0.144023
D3	1.737226	0.031687
D4	1.577832	0.139648
D5	1.279272	0.337033
D6	0.925746	0.02834

If you choose experiment 1, 2, and 3, the final result is as follows:

Treatment	Final Ratio	StDev of Final Ratio
D0	0.996138	0.097144
D1	0.915643	0.165894
D2	0.623733	0.095590
D3	1.539501	0.062812
D4	1.350501	0.085950
D5	1.117348	0.336757
D6	0.720779	0.028339

VI. Multiple References Absolute Quantification

1. Click **Process Data** and then select **Multiple References Absolute Quantification** under the **Advanced Options**.
2. **Choose Experiment** from the experiment list and then click **Add**. If a dialog "You are about to run an append……" pop up, click **Yes**. A dialog will remind you the addition of

experiment “You are about to append N rows”. Click **Yes**. This experiment will show under the **Experiment Selected**. (**Note**: Please choose and add experiments one by one)

3. To delete a selected experiment, choose that experiment from **Experiment Selected** and click **Delete**. The same dialogs will appear. Click **Yes**.
4. Select the result type (**inter-ref sample ratio** and **final ratio**) and click **process**. If you choose experiment 1 and 2, the final result is as follows:

Treatment	Final Ratio	StDev of Final Ratio
D0	0.999155	0.302663
D1	1.446325	0.322752
D2	0.897775	0.256326
D3	2.299829	0.070734
D4	1.97706	0.141585
D5	1.811127	0.295321
D6	0.902072	0.048961

If you choose experiment 1, 2, and 3, the final result will be as follows:

Treatment	Final Ratio	StDev of Final Ratio
D0	0.990916	0.193836
D1	1.108892	0.276209
D2	0.739287	0.18263
D3	1.751864	0.02755
D4	1.414202	0.103725
D5	1.405532	0.330699
D6	0.624024	0.025441

III. Edit Information

Edit Gene Information

1. Click **Gene** button on the main menu. The **Gene Control Panel** will appear.
2. Click **Edit Gene Info** and a new panel containing the input information (**Gene ID**, **Gene Name** and **Description**) of Rho2, ACTB, UBC, and GAPD will appear. Edit gene information if necessary. (**Note**: you cannot enter a new gene here!)
3. To delete any gene from the list, change that row black by clicking on the left square of that row, and then press **Delete** from the keyboard.

4. To rearrange the order of **Gene ID**, **Gene Name**, or **Description**, click on the column to change the whole column black. Right click and choose **Sort Ascending** or **Sort Descending**.
5. To export gene information, go to **File** on the Access menu, click **Export**. Choose the destination to export, input the file name, and save the file in proper formats.
6. To copy gene information, click the square on the top left of the **Edit Gene Information** table to change the whole table black. Go to **Edit** on the Access menu and click **Copy**.
7. Click  to back to the main menu.

Edit Sample Information

1. Click **Plate** button on the main menu. The **Plate Control Panel** will appear.
2. Select **Import Plate Report** > **Edit Sample Info.**, or **Add Plate Report Manually** > **Add New Sample** > **Edit Sample Info.** either from the block of **Add Sample by Batch** or **Add Sample One by One**. A panel containing the input information (**Sample Name**, **Treatment**, and **Description**) will appear. Edit sample information if necessary (Note: you cannot enter a new sample name here!).
3. To delete any sample from the list, change that row black by clicking on the left square of that row, and then press **Delete** from the keyboard.
4. To rearrange the order of the listed **Sample Name**, **Treatment**, and **Description**, click on the relative column to change the whole column black. Right click and choose **Sort Ascending** or **Sort Descending**.
5. To export sample information, go to **File** on the Access menu, click **Export**. Choose the destination to export, input the file name, and save the file in desired format.
6. To copy sample information, click the square on the left top of the **Edit Sample Information** table to change the whole table black. Go to **Edit** on the Access menu and click **Copy**.
7. Click  to back to the main menu.

Edit Plate Information

1. Click **Plate** button on the main menu. The **Plate Control Panel** will appear.

2. Click **View and Edit Plate Information** and a new panel containing the input information (Plate ID, Plate Name, and Date) of “03252005” and “03302005” will appear.
3. To change a plate name, work on the plate table directly.
4. To change a report file name, eg. “Rho2_03252005” to “Rho2A” on plate “03252005”, click “**+**” before “03252005” to show the report file list in that plate, change “Rho2_03252005” to “Rho2A”.
5. To change the gene of a report file (if the user selects a gene for the report file), click “**+**” before the plate to show the report file list in that plate, select the right gene from the gene list.
6. To exclude abnormal wells from data processing, for example, well “B18” on plate report file “Rho2_03302005” is quite different from the other replicates, open plate list by clicking “**+**” before the plate “03302005”, and then open the report file by clicking “**+**” before “Rho2_03302005”. Mark well B18 with a “√” at the “error” column and the checked wells will not be used in further calculations. Use **Next** or **Previous** symbol to edit other plate reports. Close this panel to return to the **Plate Control Panel**. (Note: Don’t change raw data such as **Well**, **Ct** and **Quantity**!!!)
7. To delete any unwanted reported file, go to **View and Edit Plate Information**, click the “**+**” on the left of a selected **Plate Name** to show the plate report file list, click on the left square of the selected report file to change the whole row black and then select **Delete** on the keyboard.
8. To rearrange the order of any plate information or plate report information, such as **Plate name** or **Sample Name**, click on the top of the corresponding column to change the whole column black. Right click and choose **Sort Ascending** or **Sort Descending**.
9. To export plate information or plate reports, open the proper panel, go to **File** on the Access menu, and click **Export**. Choose the destination to export, input the file name, and save the file in the desired format.
10. To copy plate information, click the square on the left top of the **Edit Plate Information** table to change the whole table black. Go to **Edit** on the Access menu and click **Copy**.
11. Close all the other panels and go back to the main **Menu**.

Edit Experiment Information

1. Click **Experiment** button on the main menu. The **Experiment Control Panel** will appear.
2. Click **Edit Experiment setting**. A panel **Experiment** will appear. Use **Previous** or **Next** symbol to find the proper experiment. Edit information if necessary.
3. To remove any plate report file from the experiment, change that row black by clicking on the left square of that row, and then press **Delete** from the keyboard. To add more plate report files, click on the last row to select from the list. Close this panel to return to the **Experiment Control Panel**.
4. To delete a whole experiment, click **View Experiment Setting** to open the Experiment table. Click the left square of any unwanted experiment to change the whole row black, press **Delete** from the keyboard to remove that experiment. Close this panel to return to the **Experiment Control Panel**.
5. Click  to go back to the main menu.

View Data

View Data by Gene

1. Click **View Data** button on the main menu or press **V** on the keyboard. A **Data Control Panel** will appear.
2. Click **View Data by Gene** button and the table **Gene** will appear.
3. Click "**+**" in the front of a gene ID, the "+" will change to a "-" and a list of plates containing this gene will append to the "-".
4. Click "+" in the front of a report file name, the "+" will change to a "-" and the details of that plate report will append to the "-".
5. Close the **Gene** table and go back to the **Data Control Panel**.

View Data by Sample

1. Click **View Data by Sample** button from the **Data Control Panel** or press **S** on the keyboard. A table **Sample** will appear.
2. Click "+" in the front of a sample name, the "+" will change to a "-" and a list of the details of this sample including a plate report containing the sample, wells containing the sample on that plate, and raw data (Ct, quantity, mean, etc) generated by the detector system about the sample will append to the "-".

3. Close the table **Sample** and go back to the **Data Control Panel**.

View Data by Plate

1. Click **View Data by Plate** button from the **Data Control Panel** or press **P** on the keyboard. A table **Plate** will appear.
2. Click "+" in the front of a plate ID, the "+" will change to a "-" and a list of plates with that plate ID will append to the "-".
3. Click "+" in the front of a report file name, the "+" will change to a "-" and the according report generated by the detector system will append to the "-".
4. Close the table **Plate** and go back to the **Data Control Panel**.

View Data by Experiment

1. Click the **View Data by Experiment** button from the **Data Control Panel** or press **E** on the keyboard. A table **Experiment** will appear.
2. Click "+" in the front of an experiment ID, the "+" will change to a "-" and a list of plate reports related with that experiment append to the "-".
3. Click "+" in the front of a report file name, the "+" will change to a "-" and the input report file generated by the detector system will append to the "-".
4. Close all other panels and go back to the main **Menu**.

Conceptions

General conceptions

I. **Relative quantification**: Is the method used to reveal the relative mRNA levels of a gene in different samples or the mRNA level changes of this gene in a certain sample under different treatments. The mRNA levels of the interested gene (target gene) are normalized to an internal control gene (reference gene). The final result is normally reported as ratios.

II. **Absolute quantification**: is the method used to determine the accurate mRNA quantity of an interested gene in a certain unit of sample by using a highly accurate and reproducible calibration curve (standard curve). The final result can be expressed as absolute levels, normalized absolute expression, or ratios.

III. **Control**: On the aspect of real-time PCR, control means the group of samples (eg. untreated group or 0 time) whose expression will be used as the calibrator and the expression of other group of samples will be expressed as a value relative to it.
IV. **Normalized expression**: The expression levels of the target gene are expressed as a relative value to a reference gene in order to correct the difference of samples (eg. different starting materials and mRNA reverse transcription efficiency).

qPCR-DAMS related conceptions

I. **Ratio relative quantification**: A sample, e.g. blank, untreated or 0 time, is selected as a control. The normalized expression of the control is defined as 1. The expression levels of other samples are ratio relative to the control. To get a more accurate quantification, multiple reference genes are suggested to use in the normalization and the final ratio is the geometric means of the ratios derived from each reference gene.
II. **Absolute level**: The quantity (e.g. copy number) of the target gene is determined by a standard curve. The final result can be reported as copies/ng RNA, copies/cell, copies/ml blood, or copies/genome, etc.
III. **Normalized absolute expression**: The quantities of the target gene and the reference gene (normally a house-keeping gene) in a sample are both determined with standard curves. Because many house-keeping genes are steadily expressed in multiple samples, the final expression level of the target gene is then expressed as a normalized value in comparison with the reference gene (copies of the target gene per copy of the reference gene).
IV. **Ratio absolute quantification**: A sample, e.g. untreated or 0 time is chosen as a control. The normalized expression of all the sample groups is determined with the absolute standard quantification method, and then ratios relative to the control are calculated. Multiple reference gene normalization can also be used in this data processing module to get a more accurate quantification.

Mathematical structures

The following mathematical structures give the main steps to calculate the expression levels of a gene of interest. In relative quantification, although both crossing point (CP) and threshold cycle (Ct) can be used in calculation in this software, only Ct is mentioned in the

following text for the convenience of description. Furthermore, in the following text, several letters will be used specifically to represent some meaning in each model. *I* represents the number of repeated wells on the same plate; *J*, the number of repeated plates; *K*, the number of reference genes chosen for normalization; *L*, the number of different groups of samples (treatment); *N*, the number of biological replicates of a sample.

I. Ratio relative quantification

I.a Single reference relative quantification

Step 1

Calculate intra-plate mean value of Ct of a sample (***Ct*$_{sample}$**). This step is to calculate the mean value of Ct for the samples or controls from the replicated wells on the same plate-run.

Equation 1: Formula to calculate the *Ct*$_{sample}$

$$Ct_{sampleL} = \frac{Ct_{sampleL-well1} + Ct_{sampleL-well2} + \ldots\ldots + Ct_{sampleL-wellI}}{I}$$

Ct$_{sampleL}$ is the mean Ct of sample *L* from all the repeated wells; *Ct*$_{sampleL\text{-}wellI}$ is the Ct of sample *L* in well *I*.

Step 2

Calculate intra-plate normalized expression (***Intra-plate NE***). In this step, the normalized expression (NE) of each sample and control on the same plate-run is calculated. **This step is plate name dependent, which is distinct from the absolute quantification method.**

Equation 2: Formula to calculate the intra-plate NE

$$NE = \frac{(E_{ref})^{Ct_{ref}}}{(E_{target})^{Ct_{target}}}$$

E$_{target}$, target gene PCR amplification efficiency; *E*$_{ref}$, reference gene PCR amplification efficiency; *Ct*$_{target}$, intra-plate mean Ct of the target gene; *Ct*$_{ref}$, intra-plate mean Ct of the reference gene.

Step 3

Calculate the intra-plate sample ratio (***Intra-plate Ratio$_{sample}$***). In this step, the relative expression of samples on the same plate-run is calculated as the ratio relative to the control. The *intra-plate Ratio$_{sample}$* is calculated by 2 sub-steps.

1) Calculate *MNE$_{control}$* by averaging all the biological replicates of the *NE$_{control}$*.
2) Calculate *intra-plate Ratio$_{sample}$* by dividing *NE$_{sample}$* with *MNE$_{control}$*.

Equation 3: the combined formula to calculate the intra-plate Ratio$_{Sample}$

$$Intra-plateRatio_{sampleL} = \frac{NE_{sampleL}}{MNE_{control}} = \frac{NE_{sampleL}}{(\frac{NE_{control1}+NE_{control2}+.....+NE_{controlN}}{N})}$$

Intra-plate Ratio$_{sampleL}$ is the intra-plate ratio of sample *L*; *NE$_{sampleL}$*, NE of sample *L*; *NE$_{controlN}$*, NE for the *N*th biological replicate of the control; *MNE$_{control}$*, mean value of *NE$_{controlN}$* from all the biological replicates.

Step 4

Calculate the inter-plate sample ratio (***Inter-plate Ratio$_{sample}$***). In this step, the mean values of the intra-plate ratio of individual samples from multiple runs are calculated.

Equation 4: formula to calculate the *inter-plate Ratio$_{sample}$*

$$Inter-plateRatio_{sampleL} = \frac{Ratio_{sampleL-plate1}+Ratio_{sampleL-plate2}+.....+Ratio_{sample-plateJ}}{J}$$

Inter-plate Ratio$_{sampleL}$ is the mean value of *intra-plate Ratio$_{sampleL}$* from all the repeated plate-runs; *Ratio$_{sample-plateJ}$* is the *intra-plate Ratio$_{sampleL}$* on the *J*th repeated plate.

Step 5

Calculate the final ratio. In this step, the final ratio of a sample to control is obtained by averaging the inter-plate ratio from all the biological replications.

Equation 5: formula to calculate the final Ratio from single reference gene normalization

$$FinalRatio = \frac{Inter-plateRatio_{sample1}+Inter-plateRatio_{sample2}+KK+Inter-plateRatio_{sampleN}}{N}$$

I.b Multiple references relative quantification

Single reference gene normalization may generate biased quantitative result because the selected reference gene itself may have differential expression in many cases. However, multiple reference gene normalization can improve the accuracy of the quantification. This function is actually a further calculation on the results obtained from single reference gene normalization. Therefore, step 1 to 4 is completely the same as described in the "Single reference relative quantification**"** and the additional steps are as follows:

Step 6

Calculate the inter-reference sample ratio (***Inter-ref Ratio$_{sample}$***): It is obtained from the geometric means of *inter-plate Ratio$_{sample}$* derived from all different reference genes.

Equation 6: formula to calculate the *inter-ref Ratio$_{sample}$*

$$Inter-refRatio_{sampleL} = \sqrt[K]{Ratio_{sampleL-ref1} \times Ratio_{sampleL-ref2} \times ..... \times Ratio_{sampleL-refK}}$$

Inter-ref Ratio$_{sampleL}$ is the geometric mean of the Ratio of the sample *L* from all the reference genes; *Ratio$_{sampleL\text{-}refK}$* is the inter-plate ratio of sample *L* calculated from the *K*th reference gene.

Step 7

Calculate the final Ratio: In this step the final ratio of a sample to control is obtained by averaging the *inter-ref Ratio$_{sample}$* from all the biological replicates.

Equation 7: formula to calculate the final Ratio from multiple reference gene normalization

$$FinalRatio = \frac{Inter-refRatio_{sample1} + Inter-refRatio_{sample2} + ... + Inter-refRatio_{sampleN}}{N}$$

II. Absolute levels

In absolute quantification, the theoretical relationship of the quantity and Ct can be expressed in the following formula which was derived from the standard curve:

$$Y = b + aX$$

Y, Log quantity of a standard or sample; X, Ct of the standard or sample; *b,* intercept point Y-value of the standard curve; and *a,* slope of the standard curve.

Because Log quantities but not quantities of unknowns have linear relationship with the Ct values, Log quantities are used in the calculations of absolute expression levels.

Step 1

Calculate Log Qty of individual wells (Log $Qty_{sample\text{-}well}$). In this step, the quantities (Qty) of the target (e.g. Copy number) in individual wells are transformed to Log value.

Step 2

Calculate intra-plate mean value of Log Qty (intra-plate Log Qty). In this step, the Log Qtys of samples from the replicated wells on the same plate-run are averaged.

Equation 8: Formula to calculate the intra-plate Log Qty

$$Intra-plate LogQty_{sampleL} = \frac{LogQty_{sampleL-well1} + LogQty_{sampleL-well2} + \mathrm{KK} + LogQty_{sampleL-wellI}}{I}$$

Intra-plate $LogQty_{sampleL}$ is the mean Log quantity of sample *L* from *I* repeated wells on the same plate-run; $LogQty_{sampleL\text{-}wellI}$ is the individual Log quantity of sample *L* in the *I*th well.

Step 3

Calculate final absolute level (***Inter-plate Log Qty*** or ***ME***). In this step, the Log Qtys of a sample from all the replicated plate-runs are averaged. This value can be considered as mean expression (ME) of that sample. **This step is plate name independent, which is distinct from relative quantification method.**

Equation 9: Formula to calculate the ME

$$ME_{sampleL} = \frac{LogQty_{sampleL-plate1} + LogQty_{sampleL-plate2} + \ldots\ldots + LogQty_{sampleL-plateJ}}{J}$$

$ME_{sampleL}$, mean expression of the sample *L*; $LogQ_{sampleL\text{-}plateJ}$, the intra-plate LogQty of sample *L* on the *J*th plate.

III. Normalized absolute expression

In this method, in addition to the target gene, quantity of the reference gene is also determined by absolute quantification. Therefore step 1 to 3 is exactly the same as described in "**Absolute levels**", however, two more steps are required. **Those steps are plate name independent, which are distinct from the relative quantification method.**

Step 4

Calculate the normalized mean expression (***NME***). In this step, the *ME* of the reference gene is subtracted from the target gene and the result is the normalized mean expression (*NME*).

Equation 10: Formula to calculate NME

$$NME = ME_{target} - ME_{ref}$$

ME_{target}, mean value of the log Qty of the target gene in a sample; ME_{ref}, mean value of the log Qty of the reference gene in a sample.

Step 5

Calculate final absolute level (**MNME**). In this step, the *NME* of samples from all the biological replicates is averaged. The result is the final normalized absolute expression of the target gene in that sample, which can be considered as mean normalized mean expression (*MNME*).

Equation 11: Formula to calculate MNME

$$MNME = \frac{NME_{sample1} + NME_{sample2} + \ldots\ldots + NME_{sampleN}}{N}$$

IV. Ratio absolute quantification

IV.a Single reference gene normalization for ratio absolute quantification

All the other steps in the calculation are the same as those in "**Normalized absolute expression**". However, an additional step, which is constituted of 3 sub-steps, is required to obtain the relative expression (Ratio) of a sample to the control.

1) Calculate the Log Ratio of individual samples ($\text{Log Ratio}_{sample}$) by subtracting $\text{MNME}_{control}$ from NME_{sample}.
2) Calculate the Ratio of individual samples (Ratio_{sample}) by converting the Log Ratio_{sample} to Ratio_{sample}.

3) Calculate the final Ratio, which is obtained by averaging all the biological replicates of $Ratio_{sample}$.

Equation 12: Combined formula to calculate the final Ratio from single reference gene normalization

$$Ratio = \frac{10^{LogRatio_{Sample1}} + 10^{LogRatio_{Sample2}} + ... + 10^{LogRatio_{SampleN}}}{N}$$

$$= \frac{10^{(NE_{sample1} - MNE_{control})} + 10^{(NE_{sample2} - MNE_{control})} + ... + 10^{(NE_{sampleN} - MNE_{control})}}{N}$$

$$= \frac{10^{[NE_{sample1} - \frac{(NE_{control1} + NE_{control2} + ... + NE_{controlN})}{N}]} + 10^{[NE_{sample1} - \frac{(NE_{control1} + NE_{control2} + ... + NE_{controlN})}{N}]} + ... + 10^{[NE_{sampleN} - \frac{(NE_{control1} + NE_{control2} + ... + NE_{controlN})}{N}]}}{N}$$

IV.b Multiple reference gene normalization for ratio absolute quantification

The theory behind this function is the same as the multiple reference relative quantification. After calculation of the $Ratio_{sample}$ from each individual reference gene, the geometric means of $Ratio_{sample}$ derived from all different reference genes is calculated, and then final ratio of the sample is calculated from all the biological replicates. The formula in calculation is completely the same as Equation 6 and 7 and will not be repeated here.

Formulas used for all the models

I. Standard deviation (SD)

Equation 13: Formula to calculate standard deviation

$$SD = \sqrt{\frac{\sum (X - M)^2}{N - 1}}$$

SD, standard deviation; $\sum$, sum; X, individual value; M, mean of all the individual value; N, number of replications.

II. Coefficient of variation (CV)

Equation 14: Formula to calculate CV:

CV % = (SD/M) x 100%

F. Bug Report and Troubleshooting

Please report any bug to

liulin@okstate.edu

Troubleshooting:

If plate report cannot be imported, one possible reason is that the formats of the report have not been cleared. The other reason is because the Comdlg32.ocx is outdate or missing. Follow the listed steps to extract the Comdlg32.ocx.

1. Download the comdlg32.ocx (~60 Kb Zip)
2. Use WinZip or any other utility to unzip the file
3. Extract comdlg32.ocx to **%systemroot%\System32** folder (C:\WINNT\system32)
4. Click on Start > Run, and then type the following command:
   REGSVR32 %Systemroot%\System32\comdlg32.ocx

A dialog will show up like this:

RegSvr32

DllRegisterServer in C:\WINDOWS\System32\comdlg32.ocx succeeded.

OK

Restart Windows, and then launch your application.

G. Licenses

This software is free for academic use and downloadable at

http://www.cvm.okstate.edu/research/Facilities/LungBiologyLab/

For commercial use, please contact

liulin@okstate.edu