f_0% Analyzer

User Manual

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Contents

1. Getting started ……...… 3

1.1. Overview ……. 3

1.2. Enabling Macros ..……...… 3

1.3. Loading the Solver Add-in …….. 4

2. f_0% Analyzer user interface …….. 5

2.1. The Main sheet …… 5

2.2. The Tabular_data_entry sheet ……. 5

3. f_0% Analyzer workflow …… 6

3.1. Importing fluorescence data …… 6

3.2. Plate setup ..…….……...…. 6

3.3. Settings I ……..…… 7

3.4. Settings II …… 9

3.5. Results ……... 10

3.6. Standard curve parameters ……..… 10

4. Troubleshooting and support …….. 11

4.1. Error messages ……..…… 11

4.2. General considerations …….…… 11

4.3. Support …….. 11

1. Getting started

1.1. Overview

f_0% Analyzer is a free Macro-enabled Microsoft Excel-based program that is supported by Excel 2010 and later versions. It is developed to help researchers in analyzing quantitative PCR runs that depend on intercalating dyes with high accuracy. The program is based on the procedures described in "**Gamal, M., Ibrahim, M.A. Introducing the f_0% method: a reliable and accurate approach for qPCR analysis.** *BMC Bioinformatics* 25, 17 (2024). https://doi.org/10.1186/s12859-024-05630-y". f_0% Analyzer can analyze a 384-well plate at once with a maximum of 50 cycles per reaction. It supports up to 96 genes and 96 groups. The program predicts the percentage of the initial fluorescence (f_0%) of each reaction by solving the following equation:

$$f_x = f_m - \frac{f_m}{\left(1+DE^{x-C_i}\right)^{1/D}} + ax + b \qquad \text{eq. (1)}$$

x: cycle number x. f_x: fluorescence at cycle x. f_m: maximum fluorescence. D: rate of efficiency decay. E: starting efficiency. C_i: inflection cycle. a: baseline slope. b: baseline intercept

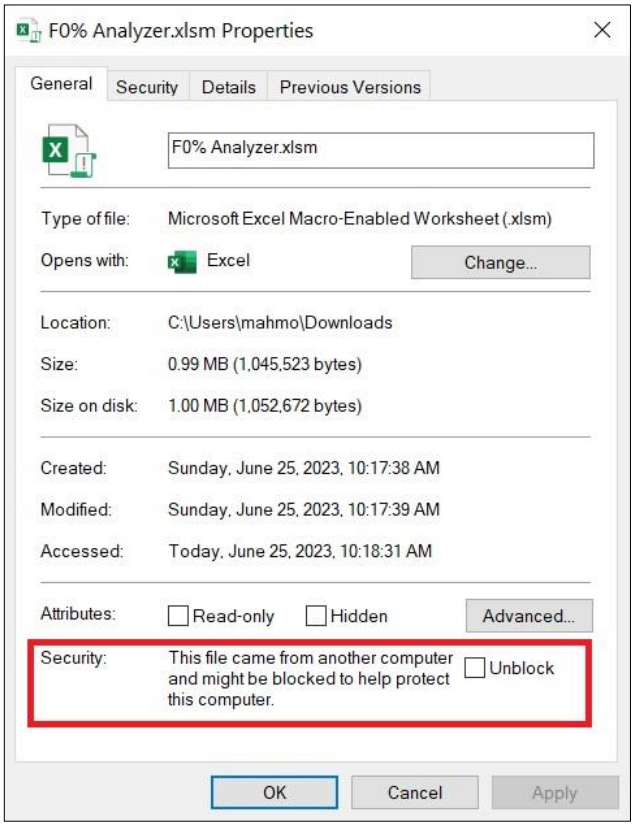
To calculate f_0%, the program should be provided with the fluorescence data of all cycles. f_0% proportionates directly with the amount of the target gene so it's used for further relative or absolute quantification.

1.2. Enabling Macros

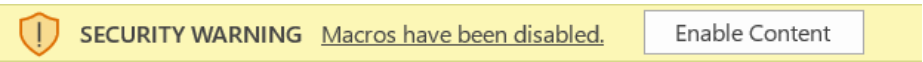
f_0% Analyzer is a Macro-enabled Microsoft Excel file. However, Microsoft's default is to block all macro-enabled files downloaded from the internet. So, before being able to use the f_0% Analyzer, you should **unblock** and **enable** the macro content.

First, to **unblock** the macro content:

1. Right-click the f_0% Analyzer file and choose **Properties** from the context menu.
2. At the bottom of the **General** tab, select the **Unblock** checkbox and select **OK**.



Then, the following security warning should appear just below the Ribbon when you open the unblocked f_0% Analyzer file for the first time:



Press **Enable Content** to enable the macro content.

N.B. If you can't enable the macros in the file, please refer to Microsoft Support.

1.3. Loading the Solver Add-in

f_0% Analyzer depends on the Solver Add-in in Excel to solve eq. (1). However, the Solver Add-in is not loaded in Excel by default. Therefore, before using the f_0% Analyzer for the first time, you should load the Solver Add-in by following these steps:

1. Go to **File** > **Options**.
2. Click **Add-Ins**, and then in the **Manage** box, select **Excel Add-ins**.
3. Click **Go**.
4. In the **Add-Ins available** box, select the **Solver Add-in** check box, and then click **OK**.

Notes:

- If the **Solver Add-in** is not listed in the **Add-Ins available** box, click **Browse** to locate the add-in.
- If you get prompted that the Solver Add-in is not currently installed on your computer, click **Yes** to install it.
- The **Solver Add-in** in Excel 2010 and later is supported, however, older versions are not.

After you load the **Solver Add-in**, please close, and restart the f_0% Analyzer to allow the Solver Add-in location to be added to the workbook references.

Note: If you encounter an error message "**Compile error: can't find project or library**" after completing the previous steps. Please, make sure that the **Solver Add-in** is referenced to the right location on your PC by following these steps:

1. Go to **File** > **Options**.
2. Click **Add-Ins**, and then in the **Manage** box, select **Excel Add-ins**.
3. Click on the **Solver Add-in** from the **Add-ins** box and record its location from the details.
4. Close **Excel Options** and press **Alt + F11** to launch the VBA editor window.
5. Open **Tools** from the top menu and select **References** from the drop-down menu.

6. In the **References** dialog box, select **Solver** then press **Browse…**.
7. Use the **Browse dialog box** to allocate **SOLVER.XLAM** in the recorded location from step 3.
8. Press **Ok**.

2. f_0% Analyzer user interface

The Excel file contains two sheets: Main and Tabular_data_entry. The Main sheet represents most of the f_0% Analyzer user interface. While the Tabular_data_entry sheet is a secondary way to enter the fluorescence data.

2.1. The Main sheet

This sheet is divided into two major sections: INPUT and OUTPUT.

2.1.1. The INPUT section contains four subsections:

- Fluorescence data: this is the primary way to enter the fluorescence data (long format). In this format, each row represents the reading of a single cycle of a specific reaction. It contains three columns: Well, Cycle, and Rn.
- Plate setup: this subsection displays the wells containing fluorescence and permits assigning a target gene, a task, a concentration (if present), a group, and an ID for each well.
- Settings I: this subsection contains a group of options for baseline correction, amplification threshold, quantification method, and efficiency calculation method.
- Settings II: it reveals the calculated efficiency for each gene and offers to edit these values before analysis.

2.1.2. The OUTPUT section contains three subsections:

- Original plate setup: this subsection redemonstrates the plate setup entered in the input section to be compared with the results.
- Results: it displays the calculated f_0% with its R^2 in addition to quantification results if present.
- Standard curve parameters: this subsection will contain the parameters of the standard curves (R^2, corrected efficiency, slope, and intercept) if present.

2.2. The Tabular_data_entry sheet

This sheet provides another way to enter the fluorescence data (wide format). In this format, each row represents a reaction or a well while each column represents a cycle.

3. f_0% Analyzer workflow

3.1. Importing fluorescence data

Fluorescence data could be exported from the software associated with your qPCR instrument. Consult the manual of your specific software to perform this step. Usually, the exported file is a tab-delimited (.txt) or a comma-delimited (.csv) file that could be opened with Microsoft Excel. Two types of fluorescence data could be exported: normalized reporter (Rn) data and ΔRn data. Rn represents the fluorescence signal of the reporter (e.g., SYBR Green) divided by the fluorescence signal of the passive reference dye (e.g., ROX). On the other hand, ΔRn represents Rn after baseline subtraction. f_0% Analyzer contains belt-in parameters to subtract the baseline, so it performs better on the Rn data.

The exported data may be in the long or the wide format where each line represents a cycle or a well, respectively. Both formats are suitable for analysis with the f_0% Analyzer. In the case of using the long format, the data should consist of three columns representing the well ID, the cycle number, and Rn. While the wide format devotes the first column for the well ID and the following columns are named after cycles (i.e., Cycle 1, Cycle 2, etc.,) with Rn filling the body of the table. The user should adapt the fluorescence data to one of those formats in an external Excel sheet. Then, it will be easy to copy the data from this sheet and paste it into the fluorescence data subsection in the Main sheet (long format) or the Tabular_data_entry sheet (wide format). After pasting the data in the Tabular_data_entry sheet, the user should press >> **Next** >>. Then, the data will be transferred to the fluorescence data subsection in the Main sheet. After pasting or transferring the data to this subsection, pressing >> **To Plate Setup** >> will initiate the plate setup subsection by extracting the wells that contain data from the fluorescence data subsection.

Important considerations:

- f_0% Analyzer can analyze a 384-well plate at once. Larger data must be divided.
- f_0% Analyzer can't analyze reactions that have more than 50 cycles. If so, remove the cycles after the 50^{th} cycle before placing the data into the f_0% Analyzer file.
- Some qPCR instruments export the melt curve data as cycles after the amplification cycles. If the user forgets to remove the melt curve data, the analysis results will be severely deteriorated and generally invalid.

3.2. Plate setup

When further relative or absolute quantification is needed, plate setup becomes an extremely important step. Plate setup displays the used wells and allows assigning a target gene, a task, a concentration, a group, and an ID for each well. These values should be given with high accuracy; f_0% Analyzer will treat (Gapdh and gapdh) as different targets and (group1 and group 1) as different groups.

3.2.1. Wells, this column is filled automatically when pressing >> **To Plate Setup** >>, and it can't be edited manually.

3.2.2. Target gene, filling the target gene column is necessary, even if only one target is used to allow efficiency calculation and further quantification.

3.2.3. Task, there are three options for the task:

- Std for standard reactions, these reactions should have an absolute or relative concentration and they are indispensable for building standard curves.
- Sample for reactions with unknown results and they represent most of the analysis.
- NTC for no amplification controls, no template controls, and no reverse transcriptase controls. Negative control reactions are essential for checking the validity of the experiment.

3.2.4. Std. Conc., it should be filled with the concentration of the standard reactions to be used in further quantification steps. Both absolute and relative concentrations are accepted. Adding concentrations to reactions other than the Std. will have no effect and it will be ignored by the f_0% Analyzer.

3.2.5. Group, filling this column will be important if normalized fold change is needed.

3.2.6. Sample ID, this field is required when relative quantification (fold change or normalized fold change) is needed.

After completing the plate setup subsection, press on >> **To Settings** >> button to transfer the data to the Settings I subsection.

3.3. Settings I

This subsection contains a group of options required for the analysis process.

3.3.1. Baseline correction

The preferred fluorescence data for the f_0% Analyzer is the normalized reporter (Rn) data which has not been corrected for the baseline. So, the default option is "Yes" for baseline correction. If the user chooses baseline correction, he will be asked to choose a starting cycle for the analysis. This step is intended to remove the first cycles when they deviate obviously from the direction of the baseline and this in turn will increase the accuracy of the analysis. If the fluorescence data is baseline-corrected and the user is sure from the correction process, he can turn off baseline correction by choosing "No".

N.B. If baseline correction is turned on for baseline-corrected data, it will perform fine adjustments to the baseline without affecting the validity of the result.

3.3.2. Amplification Threshold

The amplification threshold discriminates true from false amplifications. Its default value is the difference between the highest and the lowest fluorescence values throughout the run divided by 100. Then, the amplification threshold is compared with the fluorescence change at the C_i of all reactions before analysis. If the amplification threshold is greater than the fluorescence change at the C_i of a specific reaction, this reaction will be excluded from the analysis process with a "Below Amplification Threshold!" message in the Results subsection.

N.B. The amplification threshold could be manually modified.

3.3.3. Final Cycle

The default value for this option will be the last cycle in the run if it is not greater than 50. As the maximum number of cycles that the f_0% Analyzer can handle is 50. This option is helpful when the user wants to analyze the results while excluding one or more cycles from the end of the run. Also, if cycles from the melt curve were included by mistake in the provided fluorescence data, adjusting the final cycle option to the final amplification cycle would eliminate the problem.

3.3.4. Quantification method

f_0% Analyzer supports two modes of relative quantification in addition to absolute quantification. Generally, there are four options available for quantification:

- None: no quantification will be performed.
- Fold change: a type of relative quantification where the target gene expression is expressed as a fold change to the reference gene(s) of the same sample. Fold change requires assigning an ID for each sample and choosing one or more reference genes.
- Normalized fold change: another type of relative quantification where the fold change of different samples is normalized to the geometric mean of the samples of the control group of the same target. Normalized fold change requires assigning an ID for each sample, assigning a group for each sample, choosing one or more reference genes, and choosing a reference group.
- Absolute quantification: where a standard curve is built, and a concentration is calculated for each sample on the same scale as the standard concentrations. Absolute quantification requires at least three different levels of standards with assigned concentrations.

3.3.5. Efficiency calculation

Efficiency could be obtained by one of two methods: free *E* mode and standard curve.

- Free *E* Mode: This method relies on a special use of eq. (1), where *E* is left as a variable to be predicted with the other equation parameters. The predicted *E* or efficiencies from single reactions aren't used directly but averaged per target to enhance the accuracy and reproducibility of the analysis. Furthermore, we found that the prediction of *E* by the free *E* mode becomes very accurate when the reaction cycles are truncated to one cycle just after the inflection cycle (C_i). However, another study claims that the best results are obtained when calculations are limited to just one cycle below the C_i (Tellinghuisen, 2021). Therefore, in f_0% Analyzer, we left the user to choose the final cycle to be used in the free *E* mode.
- Standard curve: In this method, f_0% of the standard reactions are predicted using the fixed *E* mode of eq. (1) where *E* is a constant that is assumed to equal 2. Then, the standard curve is built by regressing $log_{10}(f_0\%)$ on $log_{10}(conc.)$. And *E* is calculated from the slope of the regression line.

f_0% Analyzer suggests using the standard curve method for efficiency calculation when the data for a standard curve is present. However, the user can change the efficiency calculation method to Free *E* mode if needed. Furthermore, when the f_0% Analyzer couldn't find the data for a standard curve, the only available option for efficiency calculation becomes the Free *E* mode. In all cases, the user can omit efficiency calculation by choosing None in the efficiency calculation method. In this case, the f_0% Analyzer assigns an efficiency of 2 that could be manually edited in the next step.

After fulfilling the options provided in the Settings I subsection, the user shall press >> **Calculate *E*** >> to calculate efficiencies and switch to the Settings II subsection.

N.B. After pressing >> **Calculate E** >>, Excel will take some time (seconds to minutes, according to the number of reactions) to calculate efficiencies using the Solver tool.

3.4. Settings II

This subsection displays the used target genes along with the calculated efficiencies. In this step, the user can edit the efficiency of any target manually. Changing efficiencies manually should be considered when analyzing too few reactions while a larger set of reactions were used earlier to calculate a more robust efficiency value with the same reaction conditions.

After reviewing the efficiency values for each target gene, the user should press >> **Analyze** >> to calculate f_0% for each reaction.

N.B. After pressing >> **Analyze** >>, Excel will take some time (seconds to minutes, according to the number of reactions) to calculate f_0% using the Solver tool.

3.5. Results

After pressing >> **Analyze** >>, the user will be transferred to the OUTPUT section. This section displays the results subsection besides the original plate setup subsection. So that the results can be arranged according to any criteria used in the original plate setup.

The results subsection contains three columns:

- F0%: The raw result produced by the analysis process. f_0% can be used directly instead of copy number if absolute quantification is not needed.
- R^2: This column gives an idea about the accuracy of the predicted f_0%. Generally, an $R^2 < 0.99$ suggests an invalid f_0%.
- Quantification column: The name of this column varies according to the chosen quantification method in the Settings I subsection. By default, this column is empty and displays values only after pressing << **Quantify** >>.

To understand the mathematical background of each quantification method, please refer to our published paper "Introducing the f_0% Method: A Reliable and Accurate Approach for qPCR Analysis".

3.6. Standard curve parameters

This is a special subsection that displays the parameters of the standard curve(s) used in absolute quantification. It is valuable only when absolute quantification is used.

In addition to the target column, this subsection displays another four columns representing various parameters of the standard curve:

- R^2: This parameter gives an idea about the accuracy of the standard curve. Generally, $R^2 > 0.99$ is considered accurate.
- Corrected E: This parameter stands for corrected efficiency which is the apparent efficiency after correcting for efficiency differences between targets. Corrected E tends to be always close to two.
- Slope: This is the slope of the regression line used to build the standard curve. It is used to calculate the corrected E and tends to be close to 1.
- Intercept: It is the point at which the line of the standard curve passes through the Y-axis ($\log_{10}(f_0\%)$). The intercept shall refer to ($\log_{10}(f_0\%)$) equivalent to one copy of the target if the concentration of the standard reactions is expressed in copy numbers.

4. Troubleshooting and support

4.1. Error messages

Message	Solution(s)
Cannot run the macro "F0% Analyzer'!name of the macro' The macro may not be available in this workbook or all macros may be disabled.	Enable macros. Section (1.2)
Compile error: can't find project or library	Load the Solver Add-in. Section (1.3)
The cell or chart you're trying to change is on a protected sheet. To make a change, unprotect the sheet. You might be requested to enter a password.	Avoid making changes outside the allowed space.

4.2. General considerations

- f_0% Analyzer can analyze a 384-well plate at once with a maximum of 50 cycles per reaction, supporting up to 96 genes and 96 groups.
- Reset buttons are present in the Main and the Tabular_data_entry sheets. Reset buttons remove all data and restore the default state of the sheet.
- Low R^2 (< 0.99) in the results subsection indicates an invalid result. This mostly occurs when forgetting to remove the melt curve cycles from the data.
- We implemented self-explanatory error messages for many errors, however, we might not face all errors.
- The formats of the sheets are protected to avoid unintended changes in the formatting, and it is not recommended to unprotect the sheets.

4.3. Support

If you face any problems while using the f_0% Analyzer, try to strictly follow the instructions in the user manual. If you fail to solve your problem, feel free to contact us at mahmoud.gamal@cu.edu.eg.