LinRegPCR version 2021.2

LinRegPCR is a program for the analysis of quantitative RT-PCR (qPCR) data resulting from monitoring the PCR reaction with SYBR green or similar fluorescent dyes. The program determines a baseline fluorescence and does a baseline subtraction. Then a Window-of-Linearity is set and PCR efficiencies per sample are calculated. With the mean PCR efficiency per amplicon, the Cq value per sample and the fluorescence threshold set to determnine the Cq, the starting concentration per sample, expressed in arbitrary fluorescence units, is calculated. See: Ramakers et al., NeuroSci Lett 2003; Ruijter et al., Nucleic Acids Research 2009.

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FAQs

- **When I try to read dat from Excel (Office 2016) I get an "access violoation error"**
  This may be because you are using a 64-bit version of Office. LinRegPCR cannot connect to Office 2016, 64-bit. You have to use Office 2016 32-bit.
- **I tried to import a file into LinRegPCR : the error message was "invalid variant type conversion".**
  This error message indicates that the program is trying to convert a text that is not a number into a number. This most often happens because: 1: you gave the wrong input range or 2: the decimal separator in Excel is different from the one in Windows. LinRegPCR works with the decimal separator in Windows, so you have to set Excel to use the system separator (in Excel: options menu - international Tab in the options dialog).
- **After importing data I get the error message "floating point division by zero".**
  This error occurs when there are not enough data per sample to fit a straight line. This may be because no positive values are left after baseline subtraction by your PCR apparatus. Check your input file and remove the rows or columns with these 'empty' samples. Better still: leave the baseline correction to LinRegPCR (see: Ruijter et al., 2009)
- **I did run LinRegPCR but in the output all the starting concentrations are 0.**
  This is because Excel only displays three decimal places. When you increase the number of decimal places or switch to scientific format you will see the starting concentrations are above 0. Except when no baseline can be determined or no amplification is present, then the program reports -999.
- **Why does LinRegPCR use the word 'baseline' in stead of 'background'?**
  We use the word baseline to describe the fluorescence that is observed before the amplicon specific fluorescence can be detected. Most PCR systems already use the word background for the fluorescence of a reference fluorochrome that is used to correct for experimental variations in sample volumes and well characteristics. In these systems baseline is used as we use it.
- **What is the unit of the N0 value?**
  The starting concentrations (N0) per sample are calculated in the unit of the Y-axis of the PCR amplification plot which are arbitrary fluorescence units. To convert this unit to a RNA concentration you need a calibration line of known concentration of the amplicon you are producing in the PCR.
- **LinRegPCR reports PCR efficiency values that range between 1 and 2. How must I interpret these values?**
  You are probably used to describe efficiency as a value between 0 and 1. To get these values you just subtract 1 from the efficiency that LinRegPCR reports. An efficiency of 1.85 reported by LinRegPCR can be read as an efficiency of 0.85 or 85%. We use PCR efficiencies between 1 and 2 because it makes the equations a lot easier to handle.
- **How is the starting concentration in LinRegPCR calculated?**
  LinRegPCR calculates a starting concentration (N0) per sample with the formula: N0 = Nq / (Emean^Cq) with the ^ symbol meaning 'to the power'. In this formula Nq stands for the fluorescence threshold set to determine Cq, which is the number of cycles needed to reach Nq. Emean is the mean PCR efficiency for the amplicon that is amplified in the current sample. The mean efficiency is used because the efficiency per sample is too variable to give reliable results. (see Karlen et al., 2007; Cikos et al., 2007); Ruijter et al., 2013.

- **I have always used the comparative Ct method to calculate the expression of a target relative to a reference gene. How do I do that with the results of LinRegPCR?**
  LinRegPCR gives you the starting concentrations (N0) of the target and the reference genes. When you have replicates per biological sample you first take the average of the N0 values in the target reaction and in the reference reactions. Then the relative expression is the ratio of these two averages for the same sample. When you use 2 or more reference genes, as you should do, the denominator of the ratio is the geometric mean of the N0 values of the reference genes. When you have only one measurement per amplicon, you calculate the ratio directly from the N0 values. You do not need to use the efficiency values and the Cq values to do this. These are only displayed in the output to give you a chance to do a comparative Ct calculation. The result will be the same. See the Equations in Box 1 in Ruijter et al., 2009.
- **LinRegPCR gives a warning about 'noisy samples'. How do I recognise a noisy sample?**
  A noisy sample is defined as a sample in which the data points do not show a continuous increase in the Window-of-Linearity. You recognise a noisy sample because in the W-o-L they have a point c that is above -or at the same level as- point c+1. Noisy samples are excluded from the calculation of the mean efficiency.
- **LinRegPCR reports a lot of baseline errors but the amplification curves show a straight log-linear phase.**
  LinRegPCR reports a baseline error when it is not possible to find a baseline value that leads to a straight continuous set of data points in the log-linear phase. However, the program also reports a baseline error when the remaining log-linear phase is to short. This may be because measurement noise causes the lower data points to be discontinuous: the fluorescence in cycle c is larger than that in cycle c+1. The baseline estimation only uses data in which Fc < Fc+1. In version 11.3 you can 'relax' this continuity criterion and allow jumps, as long as all data in the log-linear phase are around a straight line. This leads to less baseline-error samples but also leads to more variation between individual efficiency values.
- **I am wondering whether you can apply your window of linearity methodology to qPCR data obtained using a Taqman probe assay rather than the SYBR green assay? I can't think of any, as both result in fluorescence values but perhaps I have missed something?**
  The kinetics of the fluorescence of the Taqman probe is different from SYBR green. SYBR green is binding to dsDNA and is freed again at heating. So the fluorescence you see is proportional to the amount of DNA present at the end of each cycle. The Taqman probe binds to ssDNA, is digested by the polymerase and then becomes fluorescent. And stays fluorescent. So the fluorescence you see is an accumulation of the probe that has ever bound to the ssDNA. Therefore, this fluorescence increases more rapidly than the SYBR green fluorescence. However, on a log-fluorescence scale this amplifiaction curve becomes parallel to the curve that would have been observed when the reporter fluorescence was not cumulative. Therefore, the derived PCR efficiency is correct. Because the values in the Taqman curve are higher, its Cq value is too low. However, this bias is only dependent on the PCR efficiency and can thus be corrected. (see Tuomi et al., 2010); Ruijter et al., 2014).
- **I try to read data into LinRegPCR and always get the error message "Variant or safe index out of bounds".**
  This is the error message that occurs when you try to read only 1 sample. LinRegPCR expects data for at least 2 samples.
- **The qPCR experiment I perform required more than one plate. How can I correct for differences between plates?**
  See: Ruijter JM, Ruiz Villalba A, Hellemans J, Untergasser A, van den Hoff MJB. Removal of between-run variation in a multi-plate qPCR experiment. Biomolecular Detection and Quantification 5: 10-14, 2015.

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References

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Version History

LinRegPCR version 2021.2 released Dec 2021

In this version an import format for the Agilent ARIA MX real-time PCR machine was added. This import format is based on the Amplification data export to Excel. This output lists the fluorescence data per well and cycle in an Excel sheet of three columns. LinRegPCR expects a range from cell A1 (including the sheet header) to the last fluorescence value of the last well (row 4033 for a 96 well plate).

LinRegPCR version 2021.1 released June 2021

In this version a problem with importing fluorescence data from the QuantStudio was fixed. LinRegPCR erroneously removed wells with negative data. Although negative fluorescence values should not be present, they can occur as result of the background correction by the machine (background defined as the fluorescence that is not dependent on the fluorochrome, e.g. autofluorescence of the plastics). Note that empty wells, and wells with only negative fluorescence values, will still be removed. In that case, a warning is shown and the user is urged to check the data. When a lot of negative values are present in the raw data (Rn) it may be better to import the delta Rn values which are baseline-corrected by the machine (baseline defined as the fluorescence that is fluorochrome, but not amplification, dependent). This choice is made in the Read-from-Excel dialog. After reading delta Rn data, the baseline correction of LinRegPCR has to be performed by clicking the red button or skipped by setting the 'baseline corrected' menu option after data import.

LinRegPCR version 2020.2 released October 2020

In this version a bug in the handling of empty wells in Step-One-Plus input was corrected. Empty wells, those without fluorescence values in the Excel output of Step-One-Plus are removed during data import. NOTE: when you read amplicon and tissue annotation information from Excel you will have to remove these wells manually from the annotation sheets. You can export the imported data, without empty wells to Excel (choose tab Data - Input Data - grid to Excel) to help you find out which wells are removed. The first column in this grid is a concatenation of the well and the target name columns in the original input.

LinRegPCR version 2020.1 released March 2020

This version support RDML version 1.3. The main change are the pre-defined strings that distinguish the different monitoring chemistries in the RDML output. In the LinRegPCR interface some chemistry names have been extended and "saturating DNA binding dye" is added to list of choices in the Read-from-Excel dialog.

It was noted that in the compact output the column "Individual PCR Eff" was not present when no amplicon groups or tissue annotation were imported. This error has been corrected.

LinRegPCR version 2020.0.0.0.3 released January 2020

In this version two errors in the program's behavior were corrected:
1. After setting amplicon groups, re-entering the Amplicon Groups tab triggered the program to run through all reactions and to set a common W-o-L. This unwanted action was undone
2. When no amplicon groups or tissues were defines, the program did not report individual PCR efficiencies. Although this was already the case since 2012, the quality check based on the individual PCR efficiency was deemed important enough to always report these values.

LinRegPCR version 2020.0.0.0.1 released January 2020

This version of the windows version of LinRegPCR was programmed in parallel with a web-version that will be released in 2020. The intention was to make sure that both versions give the same results. Most changes in the code are in the background and do not affect the reported results. However, some differences with results of earlier versions may occur:

- The assignment of the NoPlateau flag. The implementation of the rules was such that mainly NoAmplification reactions were reported as NoPlateau. Some NoPlateau reactions were not reported. The current version will not report a N0 value when NoPlateau or BaselineError are true.
- The default threshold and the Cq value reported. This change was implemented to accommodate the users who judge the results of their qPCR experiment from the reported Cq values. To make this a valid judgement the Cq values should be determined at a common threshold. Earlier versions of LinRegPCR determined an optimal threshold per assay. The Cq value for the common threshold is determined with the mean PCR efficiency per assay, whereas earlier versions used the PCR efficiency for the individual reactions. The common threshold does not change the reported target quantity (N0). The use of the mean efficiency to determine the Cq value is more in line with using the mean efficiency per assay to calculate N0. However, this change results in slightly different N0 values.
  NOTE 1: Users, who use Cq values from LinRegPCR in Pffafl-like efficiency corrected relative expression calculations should have set a common threshold for target/reference ratios (only 1 delta Cq); for fold difference calculations (two deltas) the threshold cancels out.
  NOTE 2: Users who use the individual efficiencies will not see any change. However, remember that this approach will lead to extra variability in your results (see our 2009 and 2013 papers).
  NOTE 3: The reported and applied common threshold is the geometric mean of the thresholds that the program determined for each assay. In earlier versions this was the threshold set when the user did not define groups of reactions per assay. Because of the new calculation of Cq values, this change does not affect the reported results.
- Some of the lesser used user choices were removed from the main interface and placed on the User Settings tab. Some only become visible when the are chosen in the Options Menu.
- An error in the import of LC96 data was corrected. Because in earlier versions these data were not imported, this correction does not affect the reported results.
- OK and cancel buttons on the ReadFromExcel dialog were moved to make sure they are always visible.
- In this version also a bug in the import of QuantStudio data was corrected. This import was impaired by the fact that the column headers of the QuantStudio Excel export have been changed. LinRegPCR now expects at least the words 'Position' in the header of the well description and 'Target' in the column that specifies the assay. Empty wells are removed from the dataset during import.
  Note that QuantStudio, and other 'chip'-based qPCR systems work with very small volumes and thus relatively low specific fluorescence. This leads to very low number of datapoints in the observed exponential phase and makes that the baseline correction of LinRegPCR can fail. To circumvent this issue, the user can decide to import the baseline-corrected data that the system has exported (rename the R column to X and the delta R to R and, in the Read-from-Excel dialog tell LinRegPCR that he data are baseline-corrected).

LinRegPCR earlier versions

- A full update history (versions released since March 2008) can be found in the file UpdateHistory.pdf that is distributed with the program

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