

LinRegPCR

analysis of real-time quantitative PCR data

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manual under construction

1. Update history

Important changes in version 7.0 of LinRegPCR (February 2003):

- Increased speed in reading from and writing to Excel through implementation of a COM link, readrange and writerrange procedures.
- Independence of Excel's configuration: decimal points and comma's are supported.
- Change of input range format for MJ Research systems: see Table 3.
- No fixed the number of samples and the number of cycles per sample: the program adapts to the number of samples and cycles in the input range.
- *Show or Hide* accepts discontinuous multiple selections (press Ctrl or Shift while selecting samples).
- A basic on-line *Help* function is implemented.

2. Introduction and General Information

Real-time PCR is an advanced technique for quantifying mRNA's. Innovations in PCR technology in combination with new fluorescent techniques enable monitoring of the amount of formed product with each progressing PCR cycle, which makes it possible to quantify the PCR product in the exponential or log-linear phase of the PCR reaction.

Analysis of real-time PCR data is usually based on the assumption that all samples that are analysed have similar PCR efficiencies for a specific amplicon. However, in a recent paper (Ramakers et al, Neuroscience Letters, in press) we show that small variations in PCR efficiencies already lead to erroneous interpretations of expression levels when the assumption of equal efficiencies is not fulfilled.

Therefore, we propose an assumption-free alternative for PCR data analysis, which uses the raw real-time PCR data of each individual sample. With linear regression analysis you can then calculate starting concentrations of mRNA's and individual PCR efficiencies for each sample. This linear regression approach is implemented in the LinRegPCR program.

This help file gives an overview of the functions of this program. It follows the different steps in the processing of Q-PCR data starting with the export of data from the PCR apparatus and finishing with the writing of the results to Excel.

2.1 General Procedure:

1. Read data from Excel
2. Check All Data
3. Choose fit option (default: best fitting line through 4-6 points)
4. Press Fit All
if needed: adjust Y-axis
if needed: display selection of samples
5. Check the individual samples
if needed: correct the fit to the individual samples
if needed: export graphs to clipboard (right mouse button)
6. Save results to Excel

2.2 Reference:

Ramakers C, Ruijter JM, Lekanne Deprez RH, Moorman AFM. (2003) Assumption-free analysis of quantitative real-time PCR data. Neuroscience Letters, In Press

The linear regression procedure can be found in every statistics textbook.

2.3 Contacts:

Data analysis ('dry') j.m.ruijter@amc.uva.nl
PCR procedure ('wet') c.r.ramakers@amc.uva.nl

3. Installation and requirements of LinRegPCR

The version of LinRegPCR that you received has been zipped to avoid over-jealous virus scanners to prevent the program from being send as an e-mail attachment.

In the LinRegPCR.zip file you will find:

- The executable program: LinRegPCR.exe
- This help file in Adobe Acrobat readable format: LinRegPCR_help.pdf

Unzip the files into a directory and you are ready to run. No special configuration is needed. For easy use it is handy to put a shortcut to the program and the help text in your Start menu or on your Desktop.

The program requires Windows 95 or later and Microsoft Excel 97 or later.

4. Exporting data from the PCR apparatus

The LinRegPCR program reads data from an Excel spreadsheet. Therefore, the data have to be exported from your PCR apparatus and imported into Excel. All real-time PCR apparatus enable the export of the fluorescence per cycle number data into a text file format.

Note that the exported data have to be corrected for the background (fluorescence intensity of unbound fluorochrome) and/or normalized for an internal standard. LinRegPCR does not include these correction and normalization procedures.

| | A | B | C | D | E | F | G | H | I | J | " | BK | BL |
|----|---|---------|----------------|---------|----------------|---------|---------------|---------|---------------|---------|---|---------------|---------|
| 1 | LightCycler Baseline Adjustment Data Export | | | | | | | | | | | | |
| 2 | User: LightCycler01 | | | | | | | | | | | | |
| 3 | Run Date: LightCycler01 | | | | | | | | | | | | |
| 4 | File Modification Date: | | | | | | | | | | | | |
| 5 | Run Version: 3.5.28 | | | | | | | | | | | | |
| 6 | Program: amplification | | | | | | | | | | | | |
| 7 | Fluorescence Compensation: Off | | | | | | | | | | | | |
| 8 | | | | | | | | | | | | | |
| 9 | GAPDH 1/10 +rt | | GAPDH 1/10 +rt | | GAPDH 1/10 +rt | | GAPDH 1/5 +rt | | GAPDH 1/5 +rt | | " | GAPDH 1/5 +rt | |
| 10 | Cycles | F1 | Cycles | F1 | Cycles | F1 | Cycles | F1 | Cycles | F1 | " | Cycles | F1 |
| 11 | 10 | 0.0250 | 11 | 0.0298 | 10 | 0.0150 | 10 | 0.0298 | 11 | 0.0340 | " | 9 | 0.0192 |
| 12 | 11 | 0.0250 | 12 | 0.0298 | 11 | 0.0150 | 11 | 0.0298 | 12 | 0.0590 | " | 10 | 0.0192 |
| 13 | 12 | 0.0250 | 13 | 0.0538 | 12 | 0.0390 | 12 | 0.0538 | 13 | 0.0830 | " | 11 | 0.0432 |
| 14 | 13 | 0.0250 | 14 | 0.1028 | 13 | 0.0390 | 13 | 0.0788 | 14 | 0.1560 | " | 12 | 0.0682 |
| 15 | 14 | 0.0740 | 15 | 0.1518 | 14 | 0.0880 | 14 | 0.1268 | 15 | 0.2540 | " | 13 | 0.0922 |
| 16 | 15 | 0.0980 | 16 | 0.2498 | 15 | 0.1120 | 15 | 0.2498 | 16 | 0.4740 | " | 14 | 0.1412 |
| 17 | 16 | 0.1710 | 17 | 0.4208 | 16 | 0.2100 | 16 | 0.4448 | 17 | 0.8640 | " | 15 | 0.2632 |
| 18 | 17 | 0.3180 | 18 | 0.7378 | 17 | 0.3320 | 17 | 0.7868 | 18 | 1.5730 | " | 16 | 0.4582 |
| 19 | 18 | 0.5620 | 19 | 1.3488 | 18 | 0.5760 | 18 | 1.4458 | 19 | 2.8670 | " | 17 | 0.8252 |
| 20 | 19 | 1.0500 | 20 | 2.4718 | 19 | 1.0400 | 19 | 2.6428 | 20 | 5.0890 | " | 18 | 1.5092 |
| 21 | 20 | 1.9300 | 21 | 4.3518 | 20 | 1.8950 | 20 | 4.7428 | 21 | 8.5810 | " | 19 | 2.7302 |
| 22 | 21 | 3.4440 | 22 | 7.4538 | 21 | 3.3600 | 21 | 8.0398 | 22 | 13.4410 | " | 20 | 4.9272 |
| 23 | 22 | 5.9830 | 23 | 12.2638 | 22 | 5.8020 | 22 | 12.8008 | 23 | 19.2530 | " | 21 | 8.3222 |
| 24 | 23 | 10.0860 | 24 | 18.0028 | 23 | 9.7340 | 23 | 18.6378 | 24 | 24.2830 | " | 22 | 13.0592 |
| " | " | " | " | " | " | " | " | " | " | " | " | " | " |
| 40 | 39 | 34.2860 | 40 | 35.6828 | 39 | 32.7130 | 39 | 35.1208 | 40 | 34.1980 | " | 38 | 35.0622 |
| 41 | 40 | 34.1640 | | | 40 | 32.5670 | 40 | 35.0238 | | | " | 39 | 35.3792 |
| 42 | | | | | | | | | | | " | 40 | 35.2572 |

Table 1: Tab-delimited output file of the Roche Lightcycler, imported in Microsoft Excel.

The range enclosed in the red line is imported into LinRegPCR.

The columns A to BL and rows 9 to 42 are supplied in the Read-from-Excel dialog

In the following the export procedure of the Roche Lightcycler (LC) system is described in detail. Users of other systems may find this section useful in finding the equivalent functions in their apparatus.

4.1 LightCycler (Roche)

To export the data set from a LC run, start the LC software program.

1. Click on **Data Analysis** and open the file you want to export.
2. (LC version 3.0) Click on **Select-a-program** (upper left corner) and choose **'Amplification'**
3. Click the **Quantification** button.
4. in LC version > 3.0: select the option **Fit Points** instead of the default **Second Der. Max.**
5. Select the **Step 2: Noise band** tab and drag the red noise band line to the lowest value on the Y-axis.
6. From the menu select: **Quantification – Export - Noise band**, name the file you want to export (.TXT extension) and save it.
7. Exit the LC program.

| | A | B | C | D | E | F | G | H | I | J | " | AO | AP |
|----|------|--------------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|
| 1 | Well | Primer/Probe | Cycle 1 | Cycle 2 | Cycle 3 | Cycle 4 | Cycle 5 | Cycle 6 | Cycle 7 | Cycle 8 | " | Cycle 39 | Cycle 40 |
| 2 | 1 | 633 | 0.02789 | -0.02063 | 0.00081 | 0.02470 | 0.01242 | 0.00578 | 0.00066 | -0.00354 | " | 18.92255 | 19.35929 |
| 3 | 2 | 633 | 0.03827 | 0.01390 | 0.02953 | 0.03739 | 0.01639 | 0.00418 | 0.00099 | -0.00163 | " | 20.58293 | 20.92072 |
| 4 | 11 | 633 | 0.15135 | 0.11969 | 0.07477 | 0.04410 | 0.02426 | 0.00869 | 0.00094 | -0.00239 | " | 0.11078 | 0.13660 |
| 5 | 13 | 633 | 0.01070 | -0.02854 | 0.00228 | 0.02472 | 0.00927 | 0.00274 | -0.00107 | -0.00298 | " | 16.94027 | 17.46689 |
| 6 | 14 | 633 | 0.02167 | -0.01240 | 0.00539 | 0.01796 | 0.00196 | -0.00326 | -0.00170 | 0.00008 | " | 22.50565 | 23.03503 |
| 7 | 25 | 633 | -0.00863 | -0.08177 | -0.03243 | 0.01563 | 0.00097 | -0.00190 | 0.00114 | 0.00072 | " | 16.14327 | 16.88367 |
| 8 | 26 | 633 | 0.08257 | 0.02895 | 0.02775 | 0.03196 | 0.01311 | 0.00495 | 0.00285 | 0.00036 | " | 23.50941 | 24.14959 |
| 9 | 37 | 633 | 0.00180 | -0.08505 | -0.03568 | 0.01778 | 0.00530 | 0.00201 | 0.00062 | -0.00062 | " | 17.16877 | 18.08989 |
| 10 | 38 | 633 | 0.05383 | -0.02245 | 0.00249 | 0.03225 | 0.01211 | 0.00254 | 0.00073 | -0.00015 | " | 22.62773 | 23.53618 |
| 11 | 49 | 633 | -0.03675 | -0.12516 | -0.05790 | 0.00296 | -0.01067 | -0.00611 | -0.00069 | 0.00161 | " | 14.72465 | 16.13018 |
| 12 | 50 | 633 | 0.01101 | -0.05658 | -0.01843 | 0.01569 | -0.00027 | -0.00084 | -0.00046 | -0.00174 | " | 21.52746 | 22.71413 |
| 13 | 61 | 633 | -0.05318 | -0.10671 | -0.04734 | -0.00151 | -0.01491 | -0.00735 | 0.00225 | 0.00085 | " | 12.99186 | 14.44224 |
| 14 | 62 | 633 | 0.05420 | -0.00819 | 0.00795 | 0.02508 | 0.00513 | 0.00169 | 0.00203 | -0.00240 | " | 19.72627 | 21.26981 |
| 15 | 73 | 633 | -0.05101 | -0.11235 | -0.05029 | 0.00360 | -0.00733 | -0.00466 | -0.00052 | -0.00138 | " | 0.38874 | 0.41332 |
| 16 | 74 | 633 | 0.07614 | 0.01263 | 0.01977 | 0.03181 | 0.01230 | 0.00486 | 0.00063 | -0.00303 | " | 17.18584 | 19.44158 |
| 17 | 85 | 633 | -0.06422 | -0.08632 | -0.03330 | 0.00393 | -0.00285 | -0.00194 | 0.00029 | 0.00040 | " | 0.36526 | 0.4034 |
| 18 | 86 | 633 | 0.06220 | 0.01056 | 0.01717 | 0.02787 | 0.00944 | 0.00164 | -0.00121 | -0.00275 | " | 12.98381 | 15.59209 |
| 19 | 3 | 635 | 0.05476 | 0.02137 | 0.02238 | 0.01255 | 0.00440 | -0.00232 | -0.00522 | " | 20.05741 | 20.90722 | |
| 20 | 4 | 635 | 0.07933 | 0.05061 | 0.04559 | 0.04186 | 0.02223 | 0.00754 | -0.00025 | -0.00269 | " | 18.69888 | 19.38409 |
| 21 | 15 | 635 | 0.05066 | 0.02101 | 0.02063 | 0.02269 | 0.01104 | 0.00498 | 0.00105 | -0.00289 | " | 18.83375 | 19.91577 |
| 22 | 16 | 635 | 0.07695 | 0.04240 | 0.03841 | 0.03686 | 0.01711 | 0.00436 | 0.00123 | -0.00014 | " | 23.49263 | 25.08151 |
| 23 | 23 | 635 | 0.11535 | 0.08939 | 0.05684 | 0.03429 | 0.01862 | 0.00753 | 0.00072 | -0.00453 | " | 0.11544 | 0.14061 |
| 24 | 27 | 635 | 0.07938 | 0.04184 | 0.03823 | 0.03567 | 0.01513 | 0.00286 | -0.00047 | 0.00016 | " | 15.85049 | 17.13839 |
| " | " | " | " | " | " | " | " | " | " | " | " | " | " |
| 95 | 82 | 641 | 0.08360 | 0.05846 | 0.03214 | 0.01778 | 0.00939 | 0.00370 | 0.00153 | -0.00146 | " | 7.82142 | 10.26863 |
| 96 | 93 | 641 | -0.03496 | -0.01935 | -0.01946 | -0.01750 | -0.00805 | -0.00356 | -0.00105 | 0.00025 | " | 0.00819 | 0.03077 |
| 97 | 94 | 641 | 0.08067 | 0.05324 | 0.02455 | 0.01119 | 0.00520 | 0.00116 | -0.00042 | -0.00164 | " | 1.01889 | 1.38072 |

Table 2: Comma-delimited output file of the Applied Biosystems Prism system, imported in Microsoft Excel.

The range enclosed in the red line is imported into LinRegPCR.

The columns A to AP and rows 1 to 97 are supplied in the Read-from-Excel dialog

The resulting file is a Tab-delimited text file with a header of 7 lines describing the LC run, and one pair of columns per sample (Table 1).

4.2 ABI-Prism (Applied Biosystems)

The Applied Biosystems ABI-Prism systems enable the export of data to a comma-delimited text file (*.CSV) format (Table 2). Please note that the so-called “clipped” file of the ABI systems cannot be imported into LinRegPCR.

To export the data set from a ABI-Prism run, open (a detailed procedure is welcome).

LinRegPCR expects the exported fluorescence data to start from cycle 1: the cycle numbers are not read from the input.

4.3 MJ Research systems

The MJ Research export format is also comma-delimited (Table 3).

(more details are welcome)

LinRegPCR expects the exported fluorescence data to start from cycle 1: the cycle numbers are not read from the input.

| | A | B | C | D | E | F | G | H | I | J | " | CT | CU |
|----|-------|--------|-------|----------|----------|----------|----------|----------|----------|----------|---|----------|----------|
| 1 | Read# | Cycle# | Step# | A1 | A2 | A3 | A4 | A5 | A6 | A7 | " | H11 | H12 |
| 2 | 1 | 1 | 4 | 0.069888 | 0.081266 | 0.083051 | 0.065926 | 0.066484 | 0.067393 | 0.069403 | " | 0.058256 | 0.060491 |
| 3 | 2 | 2 | 4 | 0.070140 | 0.081418 | 0.084376 | 0.066149 | 0.066619 | 0.067607 | 0.069239 | " | 0.057950 | 0.061299 |
| 4 | 3 | 3 | 4 | 0.070503 | 0.081252 | 0.084172 | 0.066570 | 0.066185 | 0.067241 | 0.069153 | " | 0.057758 | 0.060699 |
| 5 | 4 | 4 | 4 | 0.070357 | 0.080904 | 0.084480 | 0.066596 | 0.066102 | 0.067266 | 0.069129 | " | 0.057617 | 0.060614 |
| 6 | 5 | 5 | 4 | 0.070357 | 0.081037 | 0.084000 | 0.066736 | 0.066172 | 0.066912 | 0.068849 | " | 0.057080 | 0.060228 |
| 7 | 6 | 6 | 4 | 0.070090 | 0.080630 | 0.084271 | 0.066003 | 0.066053 | 0.066788 | 0.068585 | " | 0.057012 | 0.060381 |
| 8 | 7 | 7 | 4 | 0.070025 | 0.080572 | 0.084046 | 0.065941 | 0.065778 | 0.066956 | 0.068753 | " | 0.056737 | 0.059858 |
| 9 | 8 | 8 | 4 | 0.069517 | 0.080351 | 0.083825 | 0.065914 | 0.065723 | 0.066659 | 0.068504 | " | 0.056312 | 0.059709 |
| 10 | 9 | 9 | 4 | 0.069512 | 0.080482 | 0.083669 | 0.065827 | 0.065502 | 0.066596 | 0.068226 | " | 0.056344 | 0.059821 |
| 11 | 10 | 10 | 4 | 0.069135 | 0.079704 | 0.083588 | 0.065418 | 0.065653 | 0.066623 | 0.068166 | " | 0.056213 | 0.059299 |
| 12 | 11 | 11 | 4 | 0.069150 | 0.079809 | 0.083238 | 0.065739 | 0.065267 | 0.066323 | 0.068189 | " | 0.055994 | 0.059099 |
| 13 | 12 | 12 | 4 | 0.068585 | 0.079684 | 0.083305 | 0.065380 | 0.065455 | 0.066279 | 0.068175 | " | 0.055943 | 0.059077 |
| 14 | 13 | 13 | 4 | 0.068759 | 0.079397 | 0.083161 | 0.065125 | 0.065075 | 0.065631 | 0.068046 | " | 0.055713 | 0.058966 |
| 15 | 14 | 14 | 4 | 0.068776 | 0.078834 | 0.083012 | 0.064833 | 0.065210 | 0.065672 | 0.067853 | " | 0.055822 | 0.058639 |
| 16 | 15 | 15 | 4 | 0.068316 | 0.078562 | 0.082902 | 0.064742 | 0.065037 | 0.065459 | 0.067286 | " | 0.056076 | 0.059039 |
| 17 | 16 | 16 | 4 | 0.068213 | 0.078323 | 0.082511 | 0.064222 | 0.064822 | 0.065734 | 0.067411 | " | 0.056327 | 0.059449 |
| 18 | 17 | 17 | 4 | 0.067886 | 0.078148 | 0.082475 | 0.064247 | 0.064298 | 0.065378 | 0.067914 | " | 0.057245 | 0.060218 |
| 19 | 18 | 18 | 4 | 0.067751 | 0.077916 | 0.082521 | 0.064453 | 0.064996 | 0.065612 | 0.068610 | " | 0.059115 | 0.062289 |
| 20 | 19 | 19 | 4 | 0.067764 | 0.077613 | 0.082597 | 0.064559 | 0.064541 | 0.065349 | 0.069383 | " | 0.062494 | 0.065299 |
| 21 | 20 | 20 | 4 | 0.067392 | 0.077982 | 0.082886 | 0.064949 | 0.064563 | 0.065390 | 0.071314 | " | 0.068261 | 0.071031 |
| 22 | 21 | 21 | 4 | 0.067313 | 0.077586 | 0.083685 | 0.065874 | 0.064941 | 0.065497 | 0.074826 | " | 0.077373 | 0.079811 |
| 23 | 22 | 22 | 4 | 0.067616 | 0.077946 | 0.085749 | 0.067375 | 0.065616 | 0.066126 | 0.080648 | " | 0.089574 | 0.091559 |
| 24 | 23 | 23 | 4 | 0.067779 | 0.078071 | 0.089703 | 0.070023 | 0.066735 | 0.067478 | 0.088909 | " | 0.104866 | 0.105866 |
| " | " | " | " | " | " | " | " | " | " | " | " | " | " |
| 39 | 38 | 38 | 4 | 0.230077 | 0.253777 | 0.277841 | 0.203453 | 0.212400 | 0.229807 | 0.230217 | " | 0.259250 | 0.242149 |
| 40 | 39 | 39 | 4 | 0.239172 | 0.261948 | 0.282682 | 0.206777 | 0.218138 | 0.235939 | 0.232810 | " | 0.261328 | 0.244350 |
| 41 | 40 | 40 | 4 | 0.246893 | 0.270118 | 0.286027 | 0.208711 | 0.222383 | 0.241427 | 0.234369 | " | 0.263283 | 0.245627 |

Table 3: Comma-delimited output file of the MJ Research PCR system, imported in Microsoft Excel.

The range enclosed in the red line is imported into LinRegPCR.

The columns A to CU and rows 1 to 41 are supplied in the Read-from-Excel dialog

Trouble shooting:

- Please let us know when you the export format of your PCR apparatus is different from the illustrated formats. This format will then be implemented in the next update of LinRegPCR. In the mean time you will have to rearrange your exported data in Excel to make them fit into one of these formats. Sorry for the inconvenience.

5. Importing data into Excel

A tab-delimited (*.TXT or *.DEL) or a comma-delimited (*.CSV) file can be opened in Excel:

1. Start Excel.
2. Drag the file from the Windows Explorer onto the running instance of Excel

Or:

3. Drag the file onto the Excel icon in the Windows status bar and wait for Excel to maximize.

Then:

4. Drop the file on the open worksheet.

In most cases Excel will directly recognise the structure of the file and split the file over the appropriate number of columns. If not, each row of data will be imported as a long line of text in the cells in column A. To split these lines into columns:

1. Click on the grey A to select the whole column A
2. Select **Data – Text to columns** from the menu
3. Check **'Delimited'** and press **Next**
4. Check **'Tab'** or **'Comma'** and press **Finish**

You can also open a text file in Excel:

1. Select **File – Open** from the menu
2. In the Open-dialog set file type to **'Text files'**
3. Choose the file you want to open
4. In the dialog check **'Delimited'** and press **Next**
5. Check **'Tab'** or **'Comma'** and press **Finish**

Trouble shooting:

- The file in Excel has to look like one of the examples in Tables 1 – 3. If this is not the case, you will have to re-arrange the data into one of these formats. Please let us know when the export format of your PCR apparatus is different from the illustrated formats. When you send us an exported file with a description of the export procedure of your apparatus, we will try to implement this format in the next update of LinRegPCR.
- Make sure Excel is in **'Ready'** mode (left bottom corner of Excel). When you are editing a cell that has to be read by LinRegPCR, importing the data in Excel will fail. LinRegPCR will display the message “Call was rejected by callee “.

6. Reading data into LinRegPCR

Do not close Excel!

1. Start LinRegPCR.

The program will immediately open the **Read from Excel** dialog (Figure 1). The active Excel book and sheet will be shown in the books and sheets listboxes. If not:

2. Choose the book and sheet from the dropdown lists that will appear when the arrow head next to the boxes is pressed.

If the book or the sheet do not show up in the listboxes:

- a. Press **cancel** and go to Excel
 - b. Save your data file as an Excel workbook (*.XLS)
 - c. Got back to LinRegPCR and choose **File – Read from Excel**.
3. Set the column letters and row numbers to define the range of cells that has to be read.

Note that this range includes the columns and/or rows that define the sample names and cycle numbers. Refer to the legends of Table 1 thru 3 for the ranges that have to be given for each format. Make sure you give the right number of extra rows and columns.

4. Select the data file format by clicking the appropriate radio button.
5. Press OK.

LinRegPCR will now read your data from Excel and do some pre-processing. Note that

- Negative values will be ignored
- When two cycles have the same fluorescence intensity the second cycle of these two will be ignored

The data that are read can be inspected in the table on the All Data page. These data still include negative and double fluorescence values. On the Sample Data page the data that are actually used for calculation of the best fitting line in the current sample are shown.

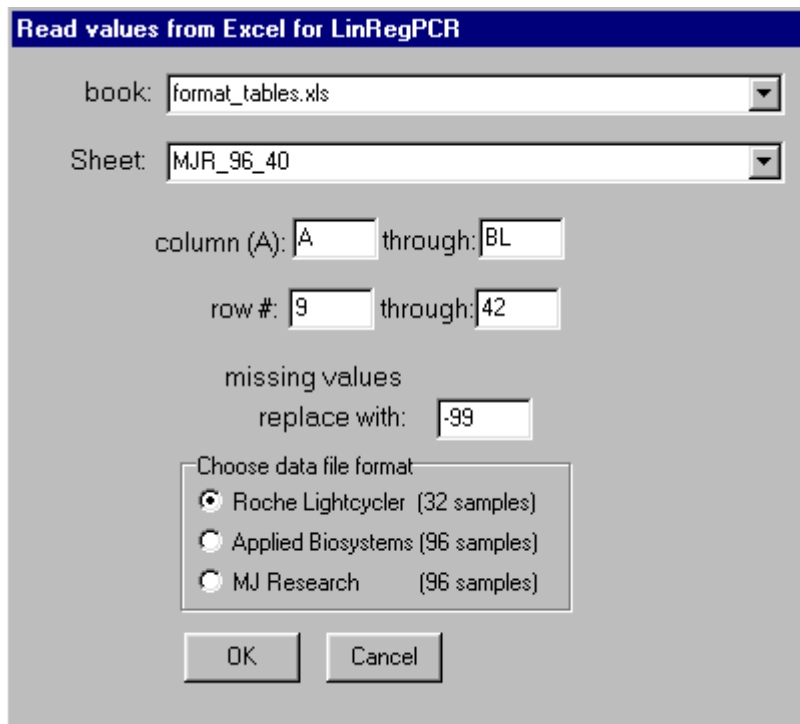


Figure 1: Read from Excel dialog. You have to choose the workbook and worksheet with your data (the currently open workbook and sheet are displayed on default), the range of cells that you want to read (see Tables 1 thru 3) and the format of your input file.

Trouble shooting:

- Sometimes the book or sheet does not show up in the listboxes. In that case check for typing errors in the file extension (*.TXT or *.CSV). You can almost always solve this problem by saving your data file as an Excel-workbook (*.XLS).
- Note that Excel may have 'hidden' sheets that it uses for storage of macros and functions. These sheets may turn up in the sheets-listbox as names that you do not recognize as your data files. NEVER choose one of these sheets.
- Depending on the range of fluorescence values in your data file the initial common window-of-linearity is set. This may turn out to be a bad choice because in some samples no points are selected. This will give a 'Floating point division by zero' error. Do not worry about this: make sure that your data have been imported and just press Fit all.

7. Description of the LinRegPCR userinterface

7.1 Screen layout and menu

The LinRegPCR user-interface consists of a menu and three parts on the screen:

- The upper-left panel to set the common window-of-linearity
- The upper-right tab-pages with sample graphs and data
- The bottom tab-pages with the fitting results

The **menu and sub-menu** options are:

- File menu
 - Read from Excel: use this menu option to read a new data set from Excel (chapter 5)
 - Save to Excel: use this option to save the results of the analysis to Excel (chapter 9)
 - eXit : exit LinRegPCR
- Y-axis
 - Log Axis. Use this option to toggle between normal and logarithmic display of the fluorescence data
 - Set Y-axis Maximum
 - Set Y-axis minimum
 - Reset: use this option to reset the Y-axis minimum and maximum to the values that were calculated after data import.
- Help
 - Help
 - Disclaimer (see chapter 13)
 - About

7.2 The upper-right tab-pages

The upper-right tab-pages consist of 6 pages with graphs, tables, fit option settings and the

tool to show or hide samples. Each page is described in the following sections.

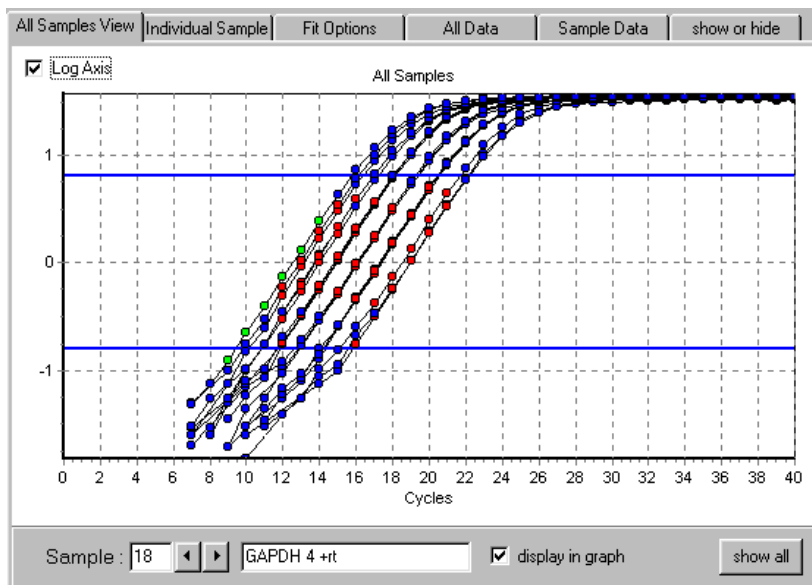


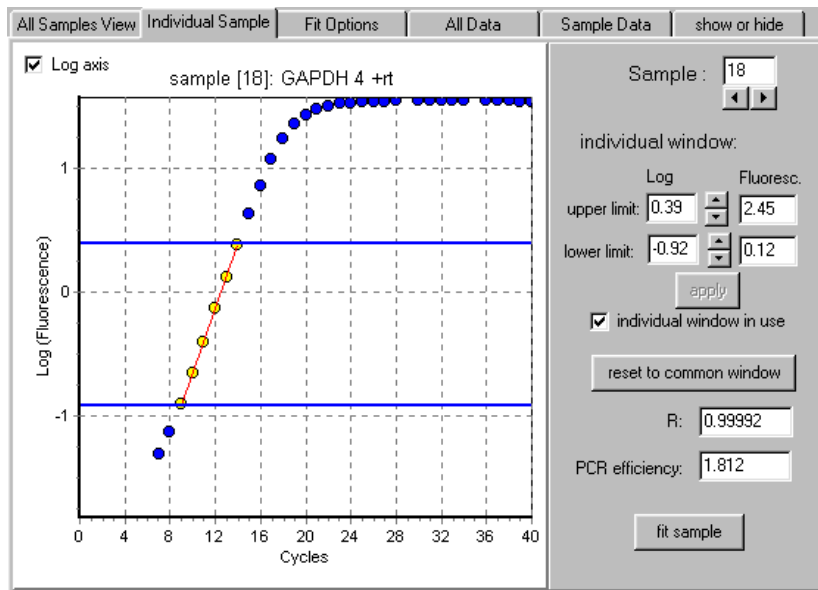
Figure 3: All Samples View. This graph displays the data points of all imported samples. With the arrow keys you can browse through the samples. Un-checking the check box hide the currently chosen graph from this view. Pressing 'show all' re-displays all graphs. The Log Axis check box toggles between logarithmic and normal Y-axis.

All Sample View: This window (Figure 3) shows the data points of all samples included in the input file. The blue horizontal lines are the limits of the common window of linearity. Data points included in this window-of-linearity are red, excluded points are blue. The left and right arrow buttons beneath the graph can be used to browse through the samples; the included points of the current

sample are highlighted in green.

The upper and lower limit of the common window-of-linearity can be set:

- Edit the fluorescence or Log(fluorescence) values and press **Apply**.
- Change these values by pressing the up and down arrow buttons.



- Click on the blue lines until the \leftrightarrow cursor appears, drag the line and click again.

Individual Sample:

This window (Figure 4) shows the data points of the current sample. The blue horizontal lines are the limits of the common window-of-linearity. Data points included in this window-of-linearity are red, excluded points are blue. The up and down arrow buttons at the top right of the graph can be used to browse through

Figure 4: Individual Sample View. This graph displays the data points of the currently chosen sample samples. With the arrow keys you can browse through the samples. The Log Axis check box toggles between a logarithmic and a normal Y-axis.

the samples.

The upper and lower limit of the individual window-of-linearity can be set as in the All Sample View (see above) and:

- Click on an included point will shift the window limit to exclude the point
- Click on an excluded point will shift the window limit to include the point

When an individual window-of-linearity is set, the included data points are plotted as yellow dots.

Next to the graph the Individual Sample view shows the individual sample statistics. Note that the individual starting concentrations are not shown to avoid biased manipulation of the window-of-linearity

Fit Options: On this page the preferred fit option is chosen (Figure 5). The fit option determines how the program determines the linear regression line that best fits log-linear phase of the data points of each sample. With the default fit option, an iterative algorithm searches for lines consisting of at least 4 and no more than 6 data points with the highest R^2 value and a slope close to the maximum slope. Using lines with only 3 data points gives inconsistent N_0 values whereas longer lines may be biased by inclusion of below-detection-limit and / or plateau phase points. The slope criterion is included in the algorithm to avoid fitting in the plateau phase.

All Data: The table on this page shows the complete data file with two columns (cycle number and fluorescence) per sample. Always check this table to ascertain that the data have been imported in good order

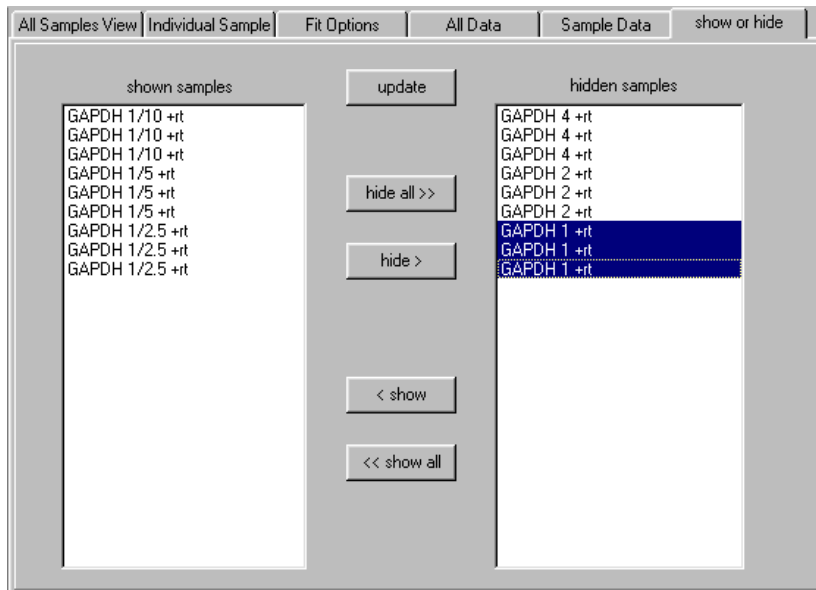


Figure 6: Show or hide samples in the All Samples view (Fig. 3) and the PCR efficiency, Correlation and Included Points graphs (Fig. 6,7 ,and 8). Start with pressing the ' update' button. Then move samples between ' shown' and ' hidden' by selecting them and pressing one the buttons. A multiple selection can be made by pressing the ' shift' button on the keyboard while selecting samples.

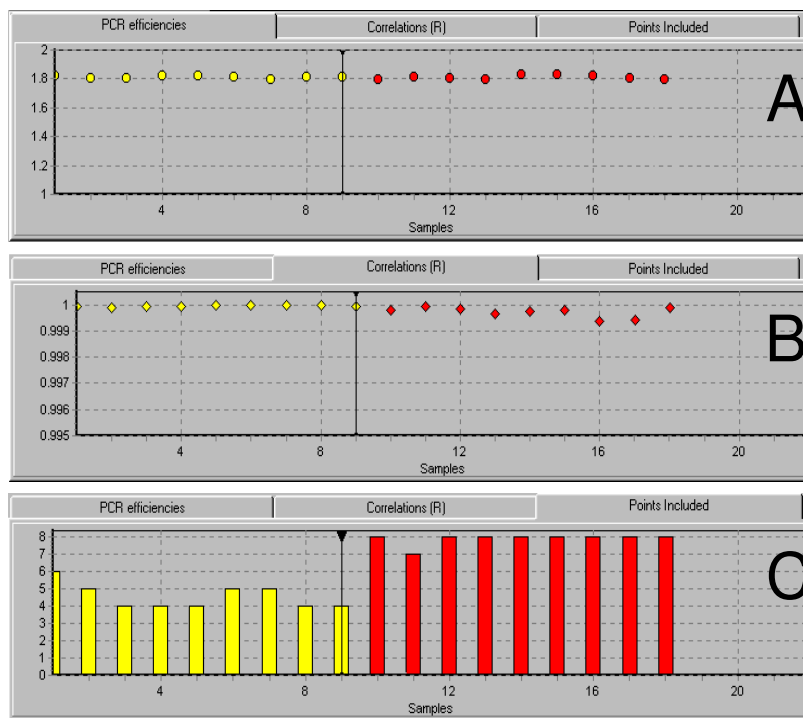


Figure 7: PCR efficiencies (A), Correlation Coefficients (B) and Number of points included in the currently fitted line (C) of each individual sample. The graph displays the samples halfway through the ' Fit all' procedure. The yellow spots represent fitted samples, the red spots still have to be fitted. A sample can be selected by clicking on its spot. This sample will then be displayed in the Individual Sample view.

individual sample (Figure 7A). Note that only efficiencies between 1 and 2 are plotted. When

Sample Data: The table on this page shows the data pairs of the currently chosen sample. Note that negative values have been removed and that, when two adjacent cycles have the same fluorescence value, only the first of these two has been retained. The 'Y' and 'N' in the third column indicate whether or not the data pair is included in the window-of-linearity of the current sample.

Show or Hide: With the tool on this page you can chose which samples you want to display in the 'All Sample View'. The samples in the listbox on the left are displayed, those on the left are hidden (Figure 6). Pressing *Update* refreshes the listboxes with the samples in their original order. Then use the show, show all, hide and hide all buttons to move samples. You can select a group of sample by pressing shift while selecting. With Ctrl you can make discontinuous multiple selections

7.3 The bottom tab-pages

The three bottom tab-pages display the results of the fitting of a straight line to each of the samples.

PCR Efficiencies

This graph shows the PCR efficiency of each

a sample does not appear in this graph it may have an deviant efficiency. Check the Individual Sample view to see the PCR efficiency value.

Correlations (R)

This graph shows the individual correlation coefficients of the fitted lines (Figure 7B). The graph only shows correlation coefficients of better than 0.995. The Individual Sample view gives the exact value of each correlation coefficient. For a good fit the R should be at least 0.999.

Included Points

In this graph (Figure 7C) the number of points that have been included into each fitted line is plotted. Preferably the number of points should be between 4 and 6.

8. Fitting the linear regression lines

Immediately after importing the data LinRegPCR calculates a linear regression to all data using a common window-of-linearity that is based on the minimum and maximum fluorescence values. These first results are crap. After inspection of your data, to see whether all data have been imported correctly, you can perform a proper linear regression fit:

1. Press the **Fit all** button on the left of the screen

The program will walk through all your samples and calculate the best fitting line (according to the chosen fit option) to your data. The correlation coefficients and PCR efficiencies are given in the graphs on the pages of the bottom notebook.

2. Inspect all your samples by browsing through the individual graphs and adjust the individual window-of-linearity if necessary.

Trouble shooting:

- Note that you only see the correlation coefficient and the PCR efficiency of each sample. Therefore manually adjusting the window-of-linearity is no direct bias of the result of the analysis. However, you are no longer allowed to do this when you have saved the results to Excel and have inspected the calculated starting concentrations. LinRegPCR cannot prevent you from doing so, your sense of fair play should.

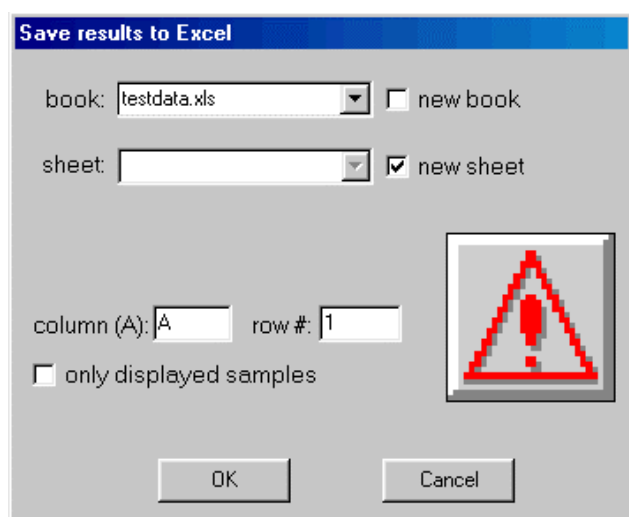


Figure 8: Save results to Excel dialog. Choose the name of the Excel workbook where you want the results saved (default the current workbook is given). It is advised to save to a new sheet. Always give the cell coordinates (A1) where you want to start writing the results.

9. Saving the results to Excel

When you are satisfied with the correlation coefficients and PCR efficiencies you can save the analysis results to Excel:

1. Choose **File – Save to Excel** from the menu

The Save dialog (Figure 8) opens.

2. Choose the book and sheet that you want to save your results to.

It is advised to save to a new sheet.

LinRegPCR does not check whether the

cells that you write to are empty: the target sheet will be overwritten without warning!!

| | A | B | C | D | E | F | G | H |
|----|--|----------|-------------|-------------|------------|----------|----------|----------|
| 1 | Analysis of Real Time PCR data version:4.0 | | | | | | | |
| 2 | analysis date:22-10-2002 | | | | | | | |
| 3 | fit option 2: n between 4 and 6 and best R | | | | | | | |
| 4 | sample | name | lower limit | upper limit | n included | No | PCR eff | R2 |
| 5 | 1 | GAPDH 1/ | 0.15 | 3.86 | 6 | 1.14E-05 | 1.824637 | 0.999869 |
| 6 | 2 | GAPDH 1/ | 0.38 | 4.88 | 5 | 1.90E-05 | 1.800637 | 0.999804 |
| 7 | 3 | GAPDH 1/ | 0.51 | 3.77 | 4 | 1.43E-05 | 1.802323 | 0.999914 |
| 8 | 4 | GAPDH 1/ | 0.7 | 5.32 | 4 | 2.98E-05 | 1.820735 | 0.999917 |
| 9 | 5 | GAPDH 1/ | 0.42 | 3.22 | 4 | 3.22E-05 | 1.821865 | 1.000000 |
| 10 | 6 | GAPDH 1/ | 0.41 | 5.53 | 5 | 3.37E-05 | 1.812477 | 0.999985 |
| 11 | 7 | GAPDH 1/ | 0.46 | 6.03 | 5 | 8.01E-05 | 1.795349 | 0.999969 |
| 12 | 8 | GAPDH 1/ | 0.45 | 3.37 | 4 | 7.05E-05 | 1.808414 | 0.999943 |
| 13 | 9 | GAPDH 1/ | 0.48 | 3.56 | 4 | 7.09E-05 | 1.813536 | 0.999905 |
| 14 | 10 | GAPDH 1 | 0.5 | 3.81 | 4 | 1.26E-04 | 1.822448 | 0.999971 |
| 15 | 11 | GAPDH 1 | 0.16 | 1.19 | 4 | 1.51E-04 | 1.80586 | 0.999919 |
| 16 | 12 | GAPDH 1 | 0.55 | 4.14 | 4 | 1.43E-04 | 1.817973 | 0.999935 |
| 17 | 13 | GAPDH 2 | 0.26 | 2.06 | 4 | 1.89E-04 | 1.844092 | 0.999975 |
| 18 | 14 | GAPDH 2 | 0.57 | 4.33 | 4 | 2.68E-04 | 1.819547 | 0.999964 |
| 19 | 15 | GAPDH 2 | 0.55 | 4.31 | 4 | 2.26E-04 | 1.838961 | 0.999893 |
| 20 | 16 | GAPDH 4 | 0.44 | 3.4 | 4 | 3.28E-04 | 1.839274 | 0.999837 |
| 21 | 17 | GAPDH 4 | 0.52 | 3.89 | 4 | 4.66E-04 | 1.811891 | 0.999933 |
| 22 | 18 | GAPDH 4 | 0.11 | 2.69 | 6 | 5.76E-04 | 1.812228 | 0.999852 |

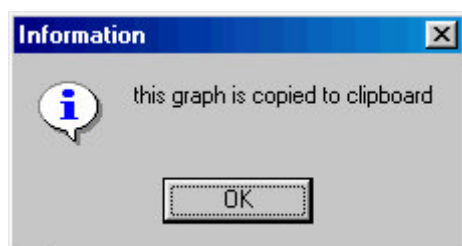
Figure 9: Overview of an Excel sheets with the saved results of the 18 samples displayed in Fig. 3. Following a three lines header you find eight columns with sample number, sample name, lower and upper limit of the window -of-linearity, number of included points, **starting concentration N_0** , PCR efficiency and correlation coefficient-squared.

3. Give the coordinates of the top-left cell where you want to write to
4. When you are looking at a selection of your samples you can choose to write only the results for this selection by clicking the checkbox
5. Press **OK**

LinRegPCR will write the results of the analysis to a new or the chosen Excel sheet. The resulting table (Figure 9) consists of 7 columns:

- A. Sample number
- B. Sample name
- C. Lower limit of window-of-linearity
- D. Upper limit of window-of-linearity
- E. N_0 = starting concentration
- F. PCR efficiency
- G. R^2 = correlation coefficient squared

Note that initially all output will be shown in numeric format with 3 decimal places. Select a column and choose **Format – Cells – Number** to change this the format you wish. The result you were most interested in, the starting concentration of your mRNA is in column E. This value is expressed in ‘fluorescence units’. The variation in PCR efficiency can be used as a quality control. This is also the case for the correlation coefficient.



10. Exporting graphs

When you click the right mouse button on a graph, the graph is copied to the windows clipboard as a bitmap. This bitmap can be pasted into your presentation.

11. Recommendations

1. It is necessary to do a visual inspection of the fit to each sample by browsing through the samples in the Individual Sample view after an automatic fit has been done.
2. Samples with strongly deviating PCR efficiencies have to be considered unreliable.
3. Samples with correlation coefficients below 0.999 have to be regarded as suspicious.

12. Frequently asked questions

Questions asked before this manual was written are included in the *Trouble shooting* sections of each chapter. Please feel free to contact us with problems encountered with the use of this program

13. Disclaimer

LinRegPCR is based on no less and no more than the information given in the paper by Ramakers et al., Neuroscience Letters 339, 2003.

By opening and using this software you acknowledge that you have read this paper, understand it, and agree with its conclusions.

Therefore, you assume all responsibility and liability for the selection of this software program to achieve your intended results, and for the conclusions you draw from these results. The authors can not be hold responsible for any consequences of the use of this program.

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