Kinetic Operating Microarray Analyzer
User's Guide

Contents

1. Introduction

Kinetic Operating Microarray Analyzer (KOMA) is an easy-to-use software developed to perform calibration and analysis of quantitative microarray data collected using kinetic detection protocol. KOMA can be used to analyze data for all ELISA systems, and should be of interest for other bioanalytical applications in which signal amplification can be followed over time. The software possesses all important functions that are required for appropriate translating of raw data into resulting biological information: validation of signals from replicated spots, analysis of calibration raw data, estimation of assay quantification range according to FDA guidelines, calculation of unknown analyte concentration, normalization and visualization of results.

This guide is a step by step explanation of how to use the software and can be particularly helpful for people that are not familiar with R. The overall workflow scheme of the software is presented on **Figure 1**.

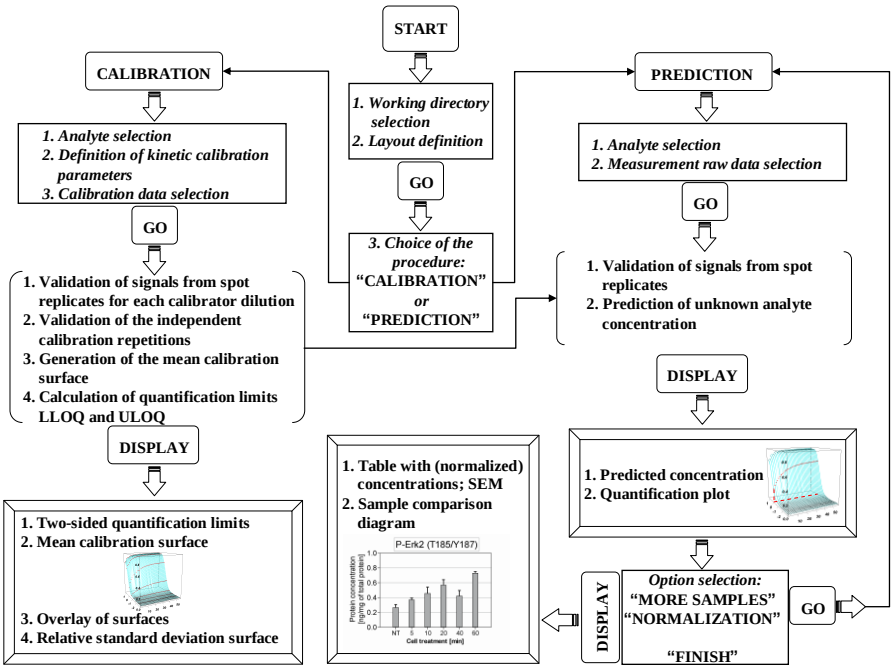


Figure 1. Overview on the workflow used by KOMA for analysis of kinetic microarray data.

2. How to install KOMA

Operating system Windows XP or its newer versions

1. Install Java Runtime Environment (Java Platform (JDK) includes JRE) from "http://www.oracle.com/technetwork/java/javase/downloads/index.html".
2. Install R by downloading from "http://cran.r-project.org/bin/windows/base" with default settings.
3. For R version from "*2.11.*" to <"*2.14.*" download KOMA package "*KOMA.win_1.0.zip*". For R versions ≥"*2.14.*" downlaod KOMA package "*KOMA.win_2.0.zip*".
4. Start R.
5. Go to "Packages/Install package(s) from local zip files" and search for downloaded KOMA_1.0.zip archive.
6. Go to "Packages/Install package(s)", choose a mirror near to your location and select in the next window all of the following packages: *epicalc*, *fields*, *gplots*, *magic*, *rgl*, *tcltk2*, *tkrplot* (to select more then one package, keep holding <Ctrl> and click on the package's name).
7. Close R (without saving the workspace).
8. Copy the "KOMA.bat" file after downloading in any arbitrary folder.
9. Start KOMA by activating "KOMA.bat".

Operating systems Mac/Linux/Unix

1. Install Java Runtime Environment (Java Platform (JDK) includes JRE) from "http://www.oracle.com/technetwork/java/javase/downloads/index.html".
2. Install R by downloading from "http://cran.r-project.org/bin/windows/base" with default settings.
3. For R versions from "*2.11.*" to <"*2.14.*" download KOMA package "*KOMA.mac_1.0.tar.gz*".
For R versions ≥"*2.14.*" downlaod KOMA package "*KOMA.mac_2.0.tar.gz*".
4. Unpack the downloaded KOMA archive file.
5. Start R.
6. Go to "Packages and Data"→"Install package(s)", select in the R package installation window "Local package (repository)" option and press the "Install" button. Browse for "KOMA.mac.1.0" or "KOMA.mac.2.0" folder, open it by a double click and press the "Open" button.
7. Go to "Packages and Data"→"Install package(s)", choose a mirror near to your location and select in the next window all of the following packages: *epicalc*, *fields*, *gplots*, *magic*, *rgl*, *tcltk2*, *tkrplot*.
8. Close R (without saving the workspace).
9. To start KOMA, open R and enter in the R GUI window:
library (KOMA.mac.1.0) or *library (KOMA.mac.2.0)* (depending on R version)
GUI ()
and press "Enter".

3. Kinetic data requirements

KOMA can use output files from any microarray image analysis tool in the common format **.txt*. Raw data files should be generated for each measured time point of signal amplification time-course and organized in folders sorted in a chronological order (**Fig. 2a**). Each of the files should contain a tab-delimited table with following columns (**Fig. 2b**): "Spot ID" (spot ID numbers that correspond to chip layout), "Substance" (names of analytes encoded by the IDs), "Mean" (spot mean signal intensity values) and "Confidence" (confidence values evaluating signal goodness).

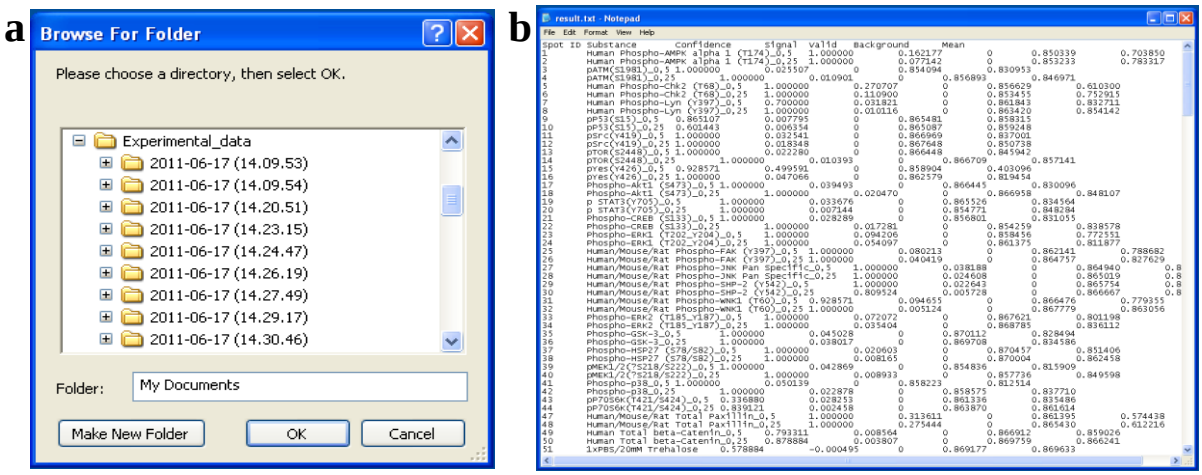


Figure 2. Organization of kinetic data. (a) Structure of chronologically sorted folders containing (b) *result.txt* files with raw data.

4. Analyzing data with KOMA

After executing "KOMA.bat", the "Main menu" window will appear. Any function of the software can be separately activated in this window.

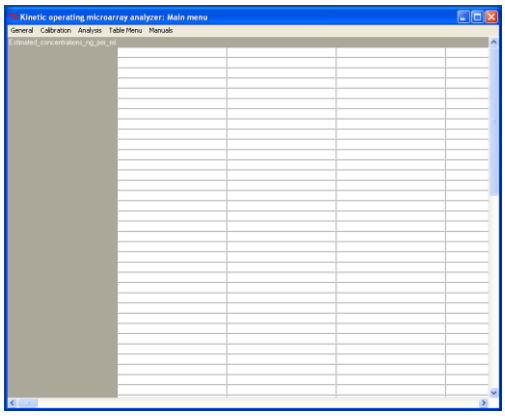


Figure 3. The main menu window.

4.1 General settings

Prior to calibration or data analysis:

1. Go to "General" → "Set working directory" and select a folder, where all results will be stored.
2. For layout definition, go to "General" → "Define array layout" and browse for any *result.txt* raw data file, which corresponds to the layout of interest (**Fig. 4**).

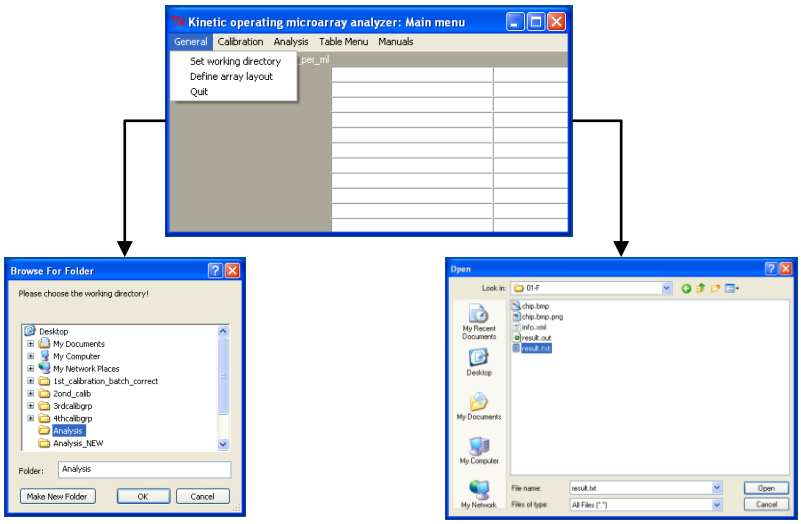


Figure 4. Designation of working directory and array layout definition.

4.2 Calibration data analysis

1. To define kinetic calibration parameters, go to "Calibration" → "Define calibration settings" and enter the number of calibrator concentrations in dilution series, the highest calibrator concentration, the dilution factor, the total time of kinetic detection and the time interval between each of measured time-points that were used for collecting of calibration data (**Fig. 5**).

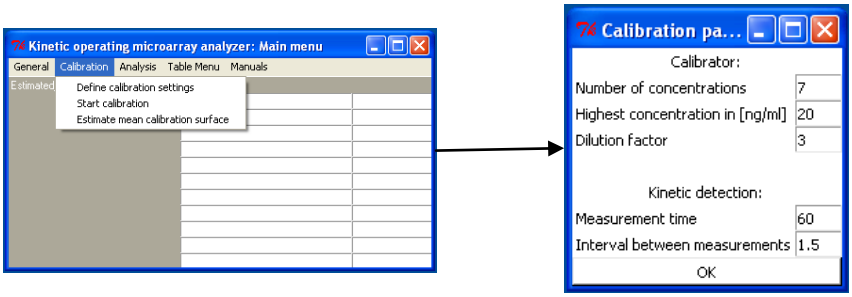


Figure 5. Definition of calibration parameters.

2. Go to "Calibration" → "Start calibration". KOMA will ask to browse for folder containing raw calibration data. Afterwards, in the opening windows select the analyte from the previously defined layout list, for which the calibration has to be performed, and enter an ID for the calibration replicate (**Fig. 6**).

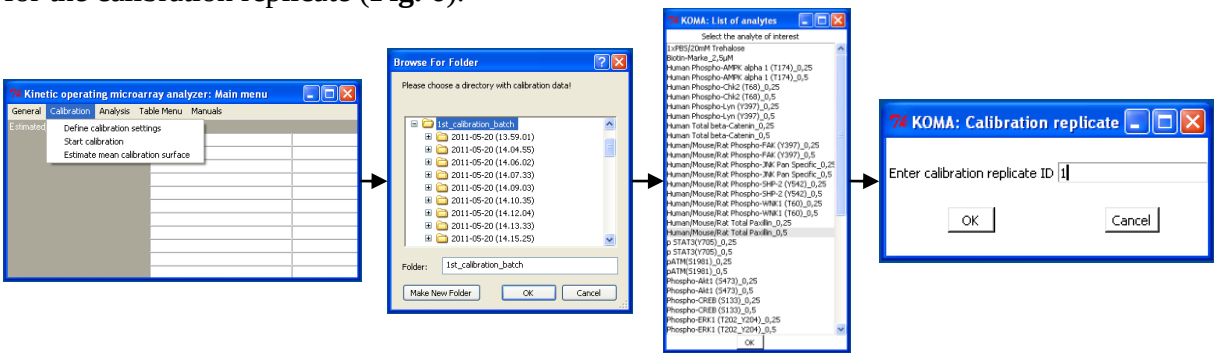


Figure 6. Selection of calibration data and analytes.

KOMA will automatically extract chosen calibration data. A subsequent window will display important diagnostic information: an overlay of signals versus amplification time from equivalent spot replicates, an overlay of signal confidence values versus amplification time, p-

values estimated by Kolmogorov-Smirnov normality test, as well as mean signal plots for every calibrator dilution (**Fig. 7**).
3. For each calibrator dilution, exclude, if necessary, failed spot intensity measurement(s) according to the visualized data and press the "OK" button to calculate the mean intensity value.

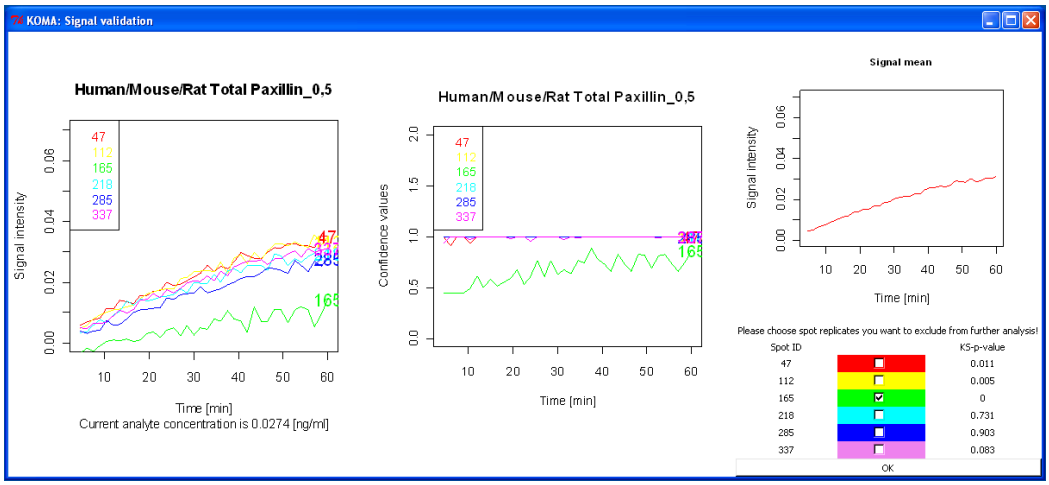


Figure 7. Validation of signals from equivalent spot replicates (6 replicates were used). In this example, the microarray spot with ID=165 was damaged and its signal has to be excluded from the mean calculation.

When the signals from every calibrator concentration are verified, a message window will inform that signal verification procedure for this particular calibration replicate is finished. An R file *Analyte_name/Calibration_replicate_ID*, containing validated mean signals will be saved in the working directory.
[*NOTE: A proper assay calibration, e. g. required for analysis of clinical samples, must be performed with at least 3 independent calibration replicates. The raw data from every replicate have to be analyzed as described above. After this analysis, the working directory will contain three R files with validated mean signal data.]*

4. In the final calibration step, the mean calibration surface and the limits of quantification are estimated. For this, go to "Calibration" → "Estimate mean calibration surface" and select the analyte of interest from the layout list. KOMA will load respective R files obtained from independent calibration replicates (at least 3 of them) and generate a 3D overlay of time-resolved signal increase for every calibrator concentration and every replicate. In a second window, failed calibration replicates can be excluded, if necessary, from the following analysis (**Fig. 8**).

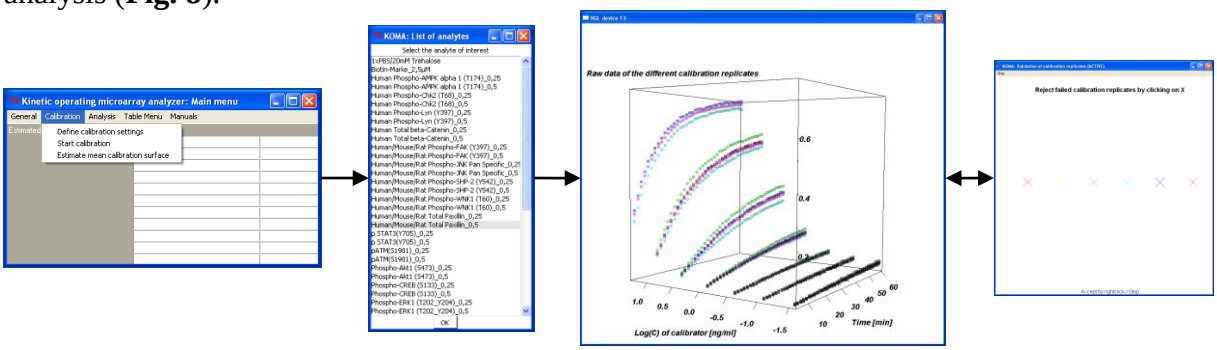


Figure 8. Verification of independent calibration replicates. In this example, data from 6 independent replicates were overlaid.

Validated calibration data must be accepted by right mouse click → Stop, and KOMA will load them to generate the mean calibration surface, calculate the upper and lower limits of quantification, as well as the relative standard deviation (**Fig. 9**).

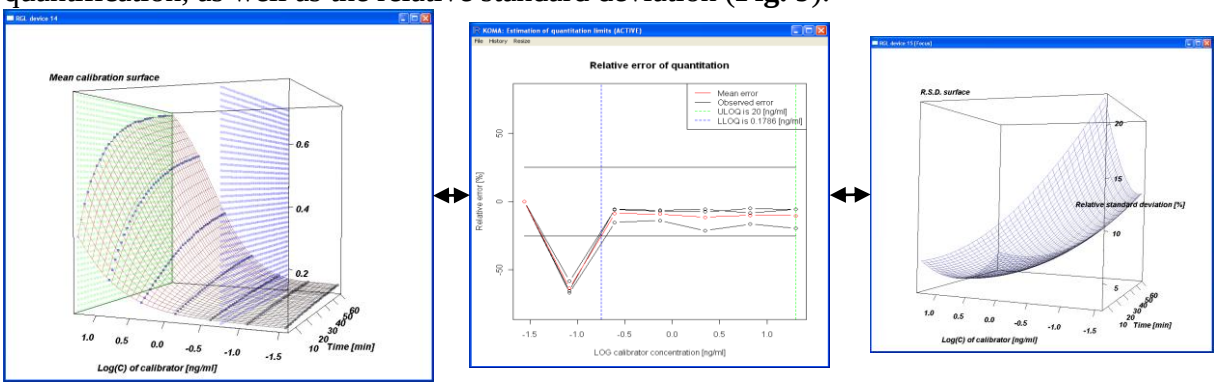


Figure 9. Mean calibration surface and limits of quantification. The mean calibration surface (red) is generated by GRPR on kinetic data from the batch of 7 calibrator concentrations (blue points). The upper and lower limits of quantification are indicated by green and blue planes (left window) or lines (mid window), respectively. The right diagnostic window shows a 3D plot of relative standard deviation, representing the exactness of assay calibration.

Once generated for every analyte separately, the mean calibration surfaces will be saved as R files *Analyte_name/Mean_calibration_surface* in the working directory and can be used for calculation of unknown concentrations.

4.3 Calculation of unknown concentrations

Calculation of unknown analyte concentrations in samples can be performed in two ways.
If the number of samples to be analyzed is small, or kinetic data must be loaded from different folders, use the regular analysis format. For this,
1. go to "Analysis" → "Analyze single sample", select analyte of interest from the layout list and browse for the folder with experimental data of interest.
2. If several samples were measured simultaneously, select (left mouse click) in the opening window the subdirectory, which contains readout data of the sample of interest, enter sample name (ID) and press "OK" button (**Fig. 10**).

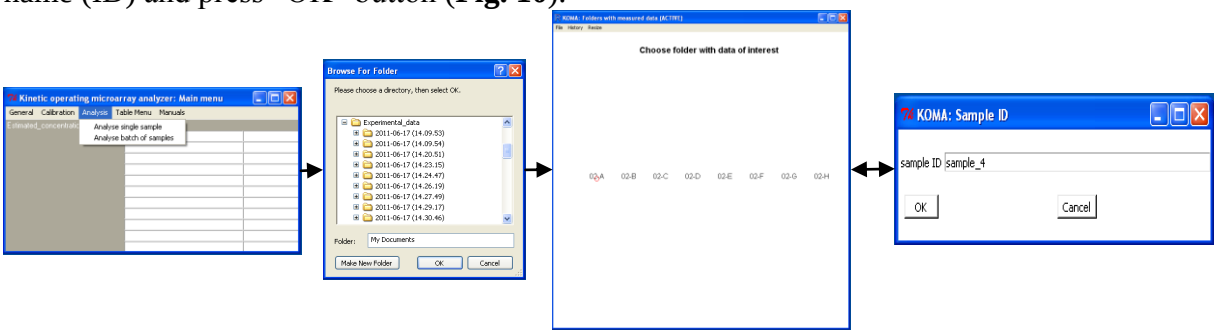


Figure 10. Workflow of single sample analysis. In the shown example, 8 samples were measured in parallel, so the main directory "Experimental data" contains subdirectories for each measured time-point; each of them contains 8 folders ("02-A" … "02-D") with *result.txt* files corresponding to each of the 8 samples.

KOMA will extract selected sample data and generate a diagnostic figure for signal validation (**Fig. 11**). Signal validation procedure is the same as described in 4.2.

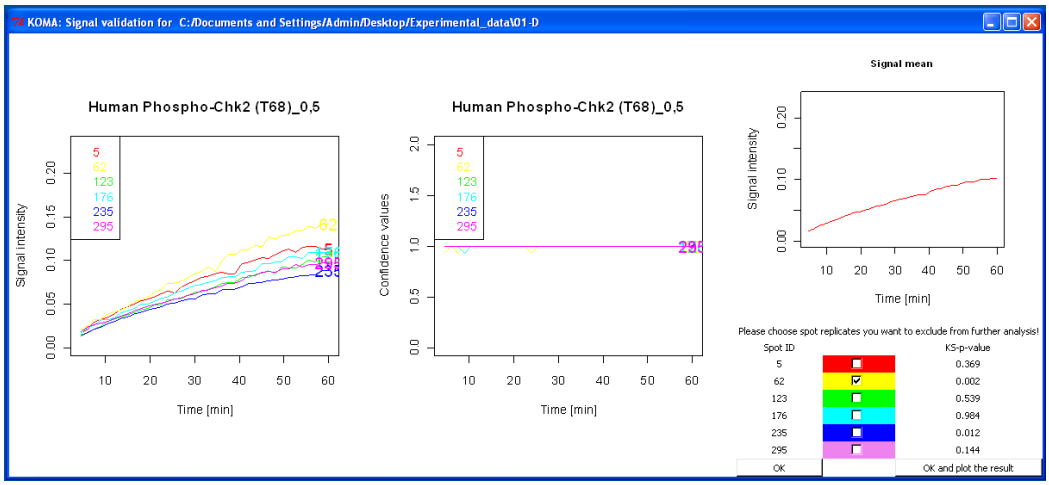


Figure 11. Validation of signals from equivalent spot replicates for the calculation of an unknown analyte concentration.

If the button "OK and plot the result" is pressed, KOMA will import two-dimensional time-resolved mean signal curve (**Fig. 11**, right plot) and calculate the minimal Euclidian distance from this curve to the respective mean calibration surface, loaded from R-file. The curve is then plotted in 3D space using the criterion of the minimal Euclidian distance versus the calibration surface and its projection on the concentration-time plane will give the exact analyte concentration (**Fig. 12**). Returned analyte concentration will be automatically saved in the Main menu table.

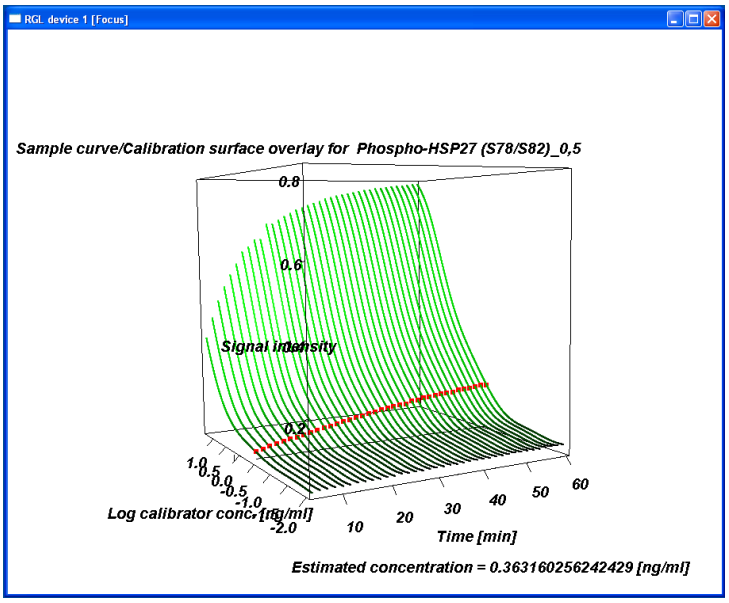
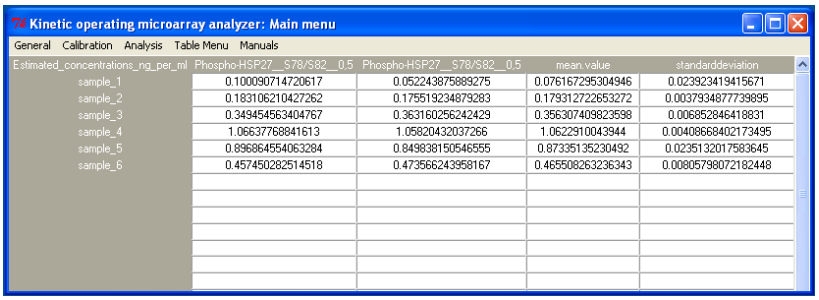


Figure 12. Calculation of unknown analyte concentration.

By clicking on the "OK" button in the diagnostic window (**Fig. 11**), the plot generation will be skipped.

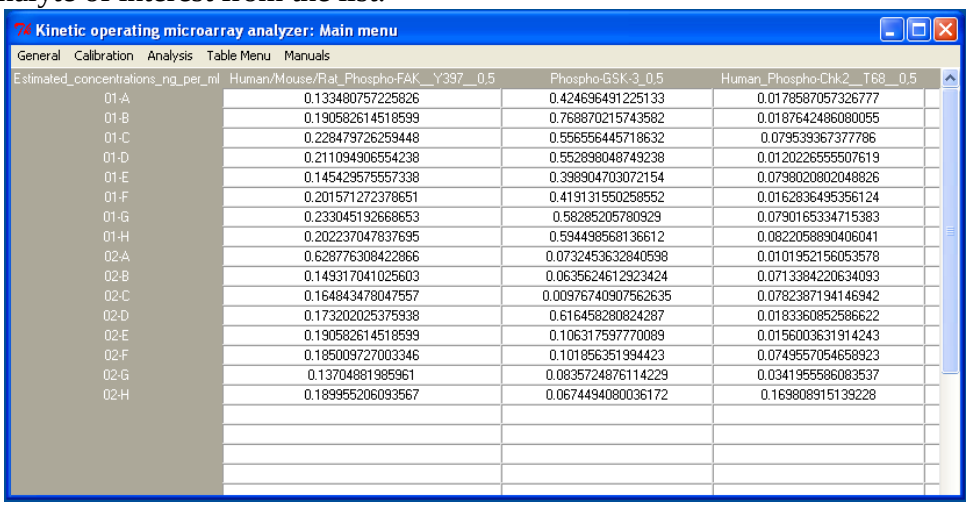
3. To analyze the next sample, repeat the procedure described above. Calculated concentrations will be saved in the Main menu table. In the analysis of biological or technical replicates, KOMA will also calculate mean concentrations and standard deviations in repeated measurements, when the same analyte is consequently analyzed (**Fig. 13**).



Estimated_concentrations_ng_per_ml	Phospho-HSP27__S78/S82__0,5	Phospho-HSP27__S78/S82__0,5	mean.value	standarddeviation
sample_1	0.100090714720617	0.052243875889275	0.076167295304946	0.023923419415671
sample_2	0.183106210427262	0.175519234879283	0.179312722653272	0.003793487739895
sample_3	0.349454563404767	0.363160256242429	0.356307409823598	0.006852846418831
sample_4	1.06637768841613	1.05820432037266	1.0622910043944	0.004086840217349 5
sample_5	0.896864554063284	0.849838150546555	0.87335135230492	0.023513201758364 5
sample_6	0.457450282514518	0.473566243958167	0.465508263236343	0.008057980721824 48

Figure 13. Main menu table containing calculated concentrations of *Phospho-HSP27* in 5 samples measured twice. Mean values and standard deviations are calculated automatically.

For high-throughput analysis of many samples,
1. go to "Analysis" → "Analyze batch of samples", select an analyte from the layout list and browse for the folder with the respective experimental data of interest. After this, diagnostic figures will be generated and the concentration calculation performed for all samples (one by one) in the chosen directory. The resulting data will be automatically saved in the Main menu table, sample names will be the same as names of subdirectories containing *result.txt* files (**Fig. 14**).
2. To analyze the next analyte, go to "Analysis" → "Analyze batch of samples" and select another analyte of interest from the list.



Estimated_concentrations_ng_per_ml	Human/Mouse/Rat_Phospho-FAK__Y397__0,5	Phospho-GSK-3_0,5	Human_Phospho-Chk2__T68__0,5
01-A	0.133480757225826	0.424696491225133	0.0178587057326777
01-B	0.190582614518599	0.768870215743582	0.0187642486080055
01-C	0.228479726259448	0.556556445718632	0.079539367377786
01-D	0.211094906554238	0.552898048749238	0.0120226555507619
01-E	0.145429575557338	0.398904703072154	0.0798020802048826
01-F	0.201571272378651	0.419131550258552	0.0162836495356124
01-G	0.233045192668653	0.58285205780929	0.0790165334715383
01-H	0.202237047837695	0.594498568136612	0.0822058890406041
02-A	0.628776308422866	0.0732453632840598	0.0101952156053578
02-B	0.149317041025603	0.0635624612923424	0.0713384220634093
02-C	0.164843478047557	0.00976740907562635	0.0782387194146942
02-D	0.173202025375938	0.616458280824287	0.0183360852586622
02-E	0.190582614518599	0.106317597770089	0.0156003631914243
02-F	0.185009727003346	0.101856351994423	0.0749557054658923
02-G	0.13704881985961	0.0835724876114229	0.0341955586083537
02-H	0.189955206093567	0.0674494080036172	0.169808915139228

Figure 14. Main menu table with results of a high-throughput analysis. In the shown example, high-throughput data analysis allows to calculate precise concentrations of 20 analytes in 16 samples within few minutes. However, this option does not allow for automatic calculation of mean and SD values as well as for renaming samples.

4.4 Visualization of results

When the calculation of concentrations is finished go to "Table menu" → "Save table" (**Fig. 15**). Calculation of mean values and standard deviations (for the regular analysis only) will be completed and the resulting table will be saved as an Excel document in the working directory.

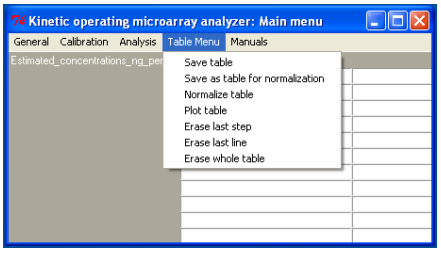


Figure 15. Table menu.

For the visualization of results, go to “Table menu” → “Plot table”. If the resulting table is generated in a regular format analysis, KOMA will plot mean concentration values together with respective SD values (**Fig. 16**):

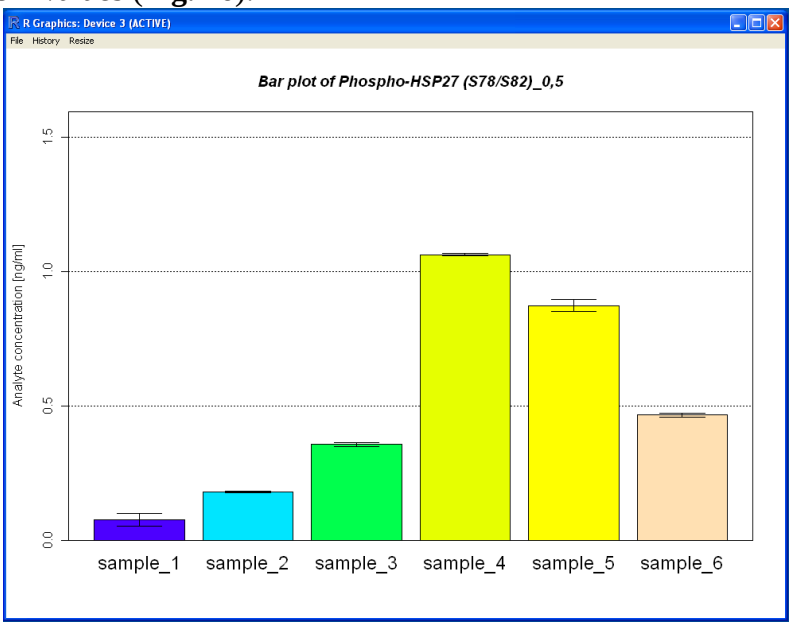


Figure 16. Bar plot of results table depicted in Fig. 13.

If the resulting table is generated in a high-throughput format analysis, KOMA will plot the calculated concentrations for each analyte in a separate window (**Fig. 17**).

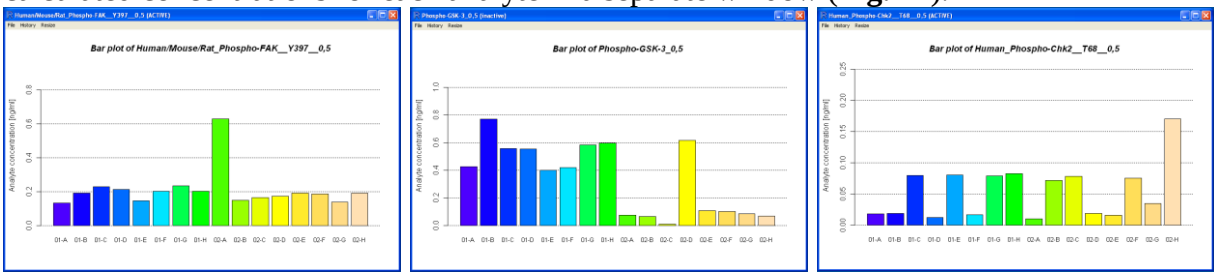


Figure 17. Bar plot of results table depicted in Fig. 14.

The table of results can be saved as normalization table, e. g. if it contains data of some internal standard or a housekeeping protein. For this, go to “Table menu” → “Save as table for normalization”. KOMA will save this table in the working directory as R file “*Normalization table/Analyte*”. Every next table of results can be normalized by selecting “Table menu” → “Normalize table” and choosing the normalization R file of interest.
Additional functions of “Table menu”, “Erase last step” and “Erase last line” can be used to remove the last input or the last sample respectively.
“Erase whole table” function has to be used after saving current results table and prior to performing a new round of data analysis.

5. Help

Function “Manuals” in Main menu allows to access PDF files containing this user’s guide and a detailed description of R functions used by KOMA.