Coffalyser.Net Reference Manual

Intended use

Coffalyser.Net is data analysis software for calculation of dosage quotients of MLPA probes that have been tested on DNA/RNA samples. The software assesses the quality of raw MLPA data as well as the analysis itself. Coffalyser.Net is intended to be used to analyse data obtained with SALSA MLPA probemixes and SALSA MLPA reagent kits as provided by MRC-Holland BV. Coffalyser.Net is intended to be used to analyse MLPA data obtained with the capillary electrophoresis devices as specified in the One-tube (MS-)MLPA protocol for DNA (and Methylation) detection and quantification. The results obtained with Coffalyser.Net should be interpreted by a clinical molecular geneticist or equivalent when used for diagnostic purposes.

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1. Introduction

The purpose of this document is to provide a step-by-step guide for the analysis of MLPA data with Coffalyser.Net™. It comprises the procedures for login, software set up, data analysis and result export, which are presented in a chronological order. Instructions for installation of Coffalyser.Net™ can be found in the installation manual.

IMPORTANT NOTES:

- Coffalyser.Net is registered for in vitro diagnostic (IVD) use in specific countries (see www.mlpa.com > Coffalyser.Net). In all other countries, this product is for research use only (RUO). Please note that when Coffalyser.Net is used with RUO MLPA products, diagnostic decisions based on the results obtained with this program are for the sole responsibility of the user.

- When Coffalyser.Net is used in a diagnostic setting, the quality scores FRSS, FMRS and CAS of all samples should have 4 green bars. The quality scores are explained in chapter **10. Quality scores** of this document.

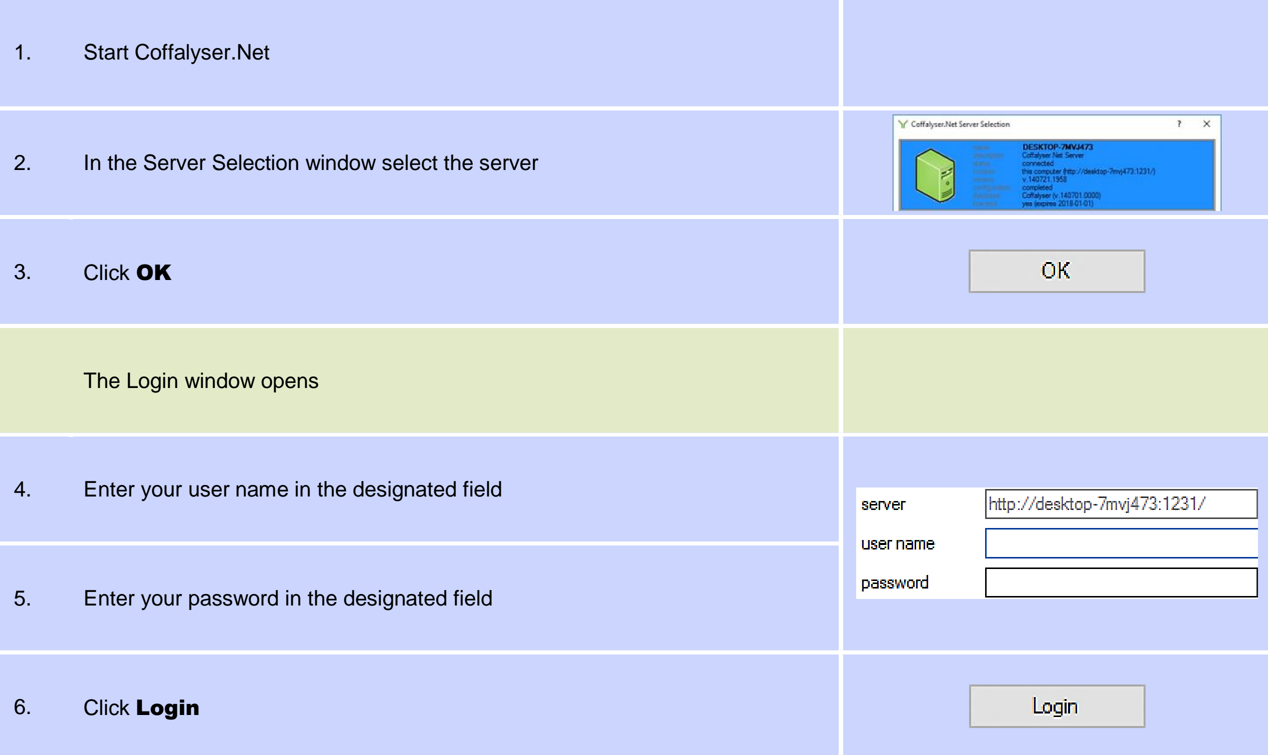
- Ensure to use the latest version of Coffalyser.Net and the Coffalyser.Net Reference Manual. The latest versions are available online at www.mlpa.com.

- For proper analysis it is necessary that the user knows which SALSA® MLPA® probemix version has been used, the type and fluorescent dye of the used size marker, and the model and type of the used capillary electrophoresis instrument. In addition, the user should have access to relevant sample information (e.g. which samples are references, positive controls, patient samples).

- For professional use only. Always consult the most recent product description AND the One-tube (MS-) MLPA protocol for DNA (and Methylation) detection and quantification before use. These are available online at www.mlpa.com. It is the responsibility of the user to be aware of the latest scientific knowledge of the application before drawing any conclusions from findings generated with this product.

2. Log in to Coffalyser.Net

For storage of data (e.g. raw data, experiments, analysis results etc.), Coffalyser.Net uses a database, which is hosted on an SQL server. This SQL server is shown in the Server Selection window, which is displayed when Coffalyser.Net is started. In most cases, only one SQL server is visible in this window. However, when more SQL servers are present that host a Coffalyser.Net database, these servers are displayed as well. In that case, make sure to select the appropriate server you wish to log in to.



1.	Start Coffalyser.Net	
2.	In the Server Selection window select the server	Coffalyser.Net Server Selection DESKTOP-7MVJ473
3.	Click **OK**	OK
	The Login window opens	
4.	Enter your user name in the designated field	server http://desktop-7mvj473:1231/ user name
5.	Enter your password in the designated field	password
6.	Click **Login**	Login

3. Sheet library & Coffalyser sheets

For reliable analysis and result interpretation, it is important that peaks in raw run data are properly recognised as signals coming from MLPA probes and fragments. Coffalyser.Net uses so-called Coffalyser sheets for this process. A Coffalyser sheet contains all necessary information that is specific for one lot of a probemix. Coffalyser sheets are stored in the sheet library. More information about the sheet library and Coffalyser sheets can be found in **Appendix III - Sheet library** on page 57 and **Appendix IV - Coffalyser sheets** on page 67).

Step	Action
1.	Right click on *Sheet Library*
2.	Select *Update (Internet Download)*
	The Download Updates (MRC-Holland) window opens
3.	Click **Start Update**
4.	In the Internet Permission window click **Yes** or **Always**
5.	Click **Close** to close the Download Updates (MRC-Holland) window
6.	Right click on *Sheet Library*
7.	Select *Open*
	The Manage Coffalyser Work Sheets window opens
8.	Right click and select *Add*
9.	In the Add Coffalyser Work Sheet window select create a work sheet based on a MRC Coffalyser sheet

10.	Select the appropriate probemix from the product drop-down menu	product <select a product> lot P002-BRCA1 P003-MLH1/MSH2 P004-ERBB2 P008-PMS2 P010-POLG
11.	Select the appropriate lot number from the lot drop-down menu	lot <select a lot> <select a lot> LOT0706 (B1) LOT0107 (B1) LOT0308 (B1)
12.	Click **OK**	OK
	The Coffalyser Work Sheet Editor window opens	
13.	Leave everything in the Coffalyser sheet on default	
14.	Click **OK** to save the Coffalyser sheet and close the Coffalyser Work Sheet Editor window	OK
15.	Repeat steps 8 to 14 to add more Coffalyser sheets if required	
16.	Click **Close** to close the Manage Coffalyser Work Sheets window	Close

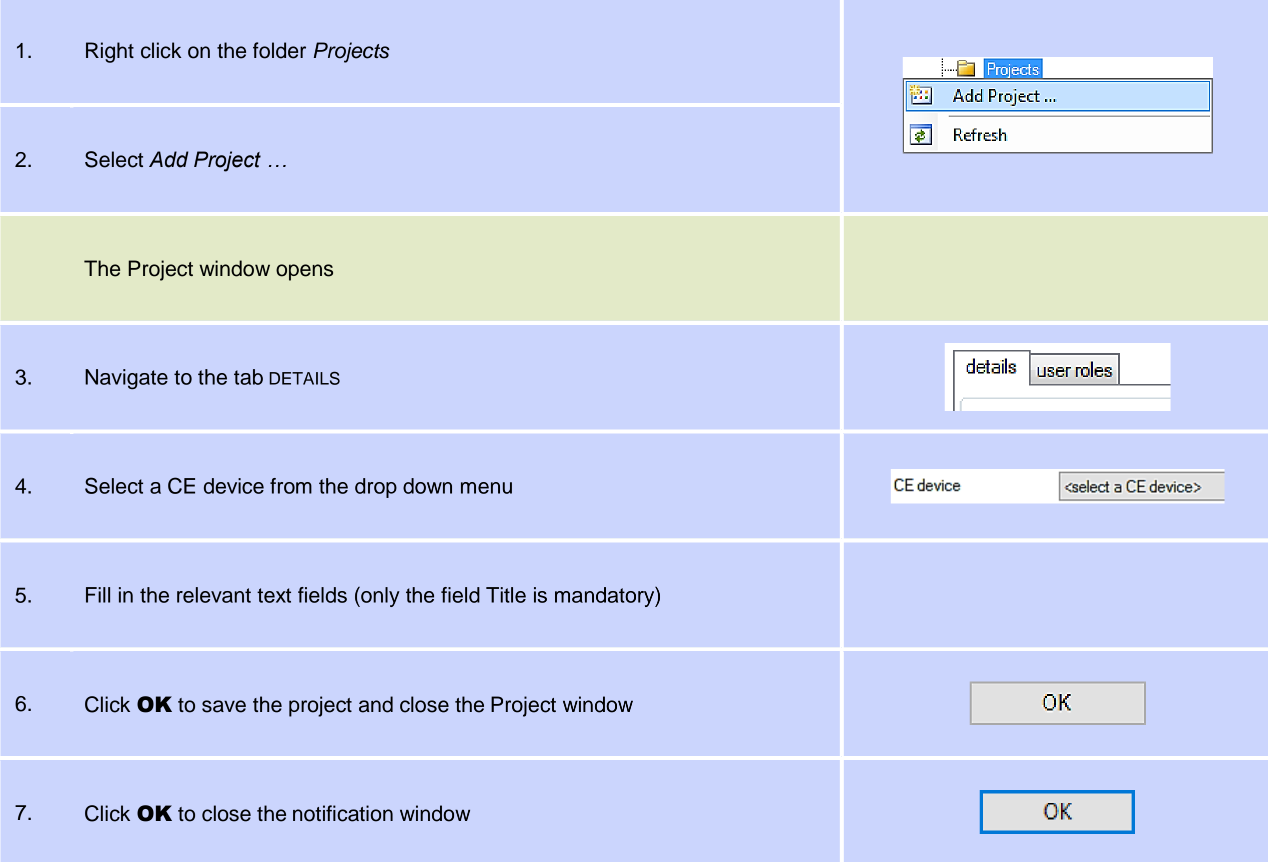
4. Create a Capillary Electrophoresis (CE) device

A CE device in Coffalyser.Net contains parameters for size-calling and peak recognition. Before data can be analysed, a CE device has to be created, which can then be linked to experiments later on. More information about CE devices in Coffalyser.Net can be found in **Appendix V - CE devices** on page 79.

Step	Action
1.	Navigate to the default organisation in the tree structure at the right side of the screen or create a new organisation
2.	Right click on the folder *CE Devices*
3.	Select *Add CE Device …*
	The CE Device Properties window opens
4.	Navigate to the tab GENERAL
5.	Select the CE device type used for electrophoresis from the CE device drop-down menu
6.	Select the filter set used during electrophoresis from the CE device filter drop-down menu
7.	Fill in the Remarks text field when desired
8.	Click **OK** to save the CE device and close the window

5. Create a project

Organisations form the top layer in which data is stored in Coffalyser.Net. Data storage can be further refined by creating projects. Each organisation can hold an unlimited number of projects. This chapter describes how projects can be created.



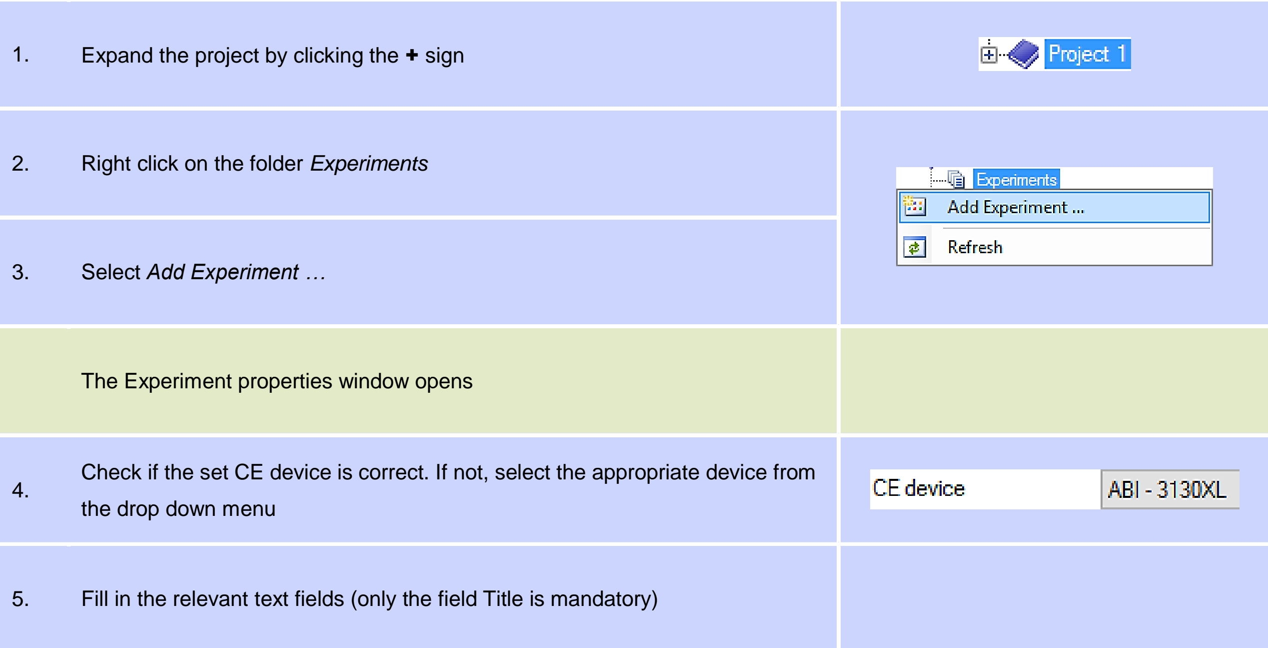
1.	Right click on the folder *Projects*
2.	Select *Add Project …*
	The Project window opens
3.	Navigate to the tab DETAILS
4.	Select a CE device from the drop down menu
5.	Fill in the relevant text fields (only the field Title is mandatory)
6.	Click **OK** to save the project and close the Project window
7.	Click **OK** to close the notification window

6.a Set up a copy number analysis experiment and analyse data

The deepest layer in the data storage structure is formed by experiments. Experiments hold the actual raw MLPA data as well as the analysis results. Per project an unlimited number of experiments can be created.
Two types of experiments exist in Coffalyser.Net: one for copy number analysis and one for the combined analysis of copy number and methylation status. In this chapter the procedure for copy number analysis is described. The combined analysis of copy number and methylation status is described in the next chapter.

IMPORTANT NOTES:

- The parameters for fragment analysis and comparative analysis have to be left on their default values in a diagnostic setting. These parameters have been determined after extensive testing and changing them might lead to inclusion of samples with a lower quality in the analysis. Adjusting these parameters should only be done in a research setting.
- When Coffalyser.Net is used in a diagnostic setting, the quality scores FRSS, FMRS and CAS of all samples should have 4 green bars.



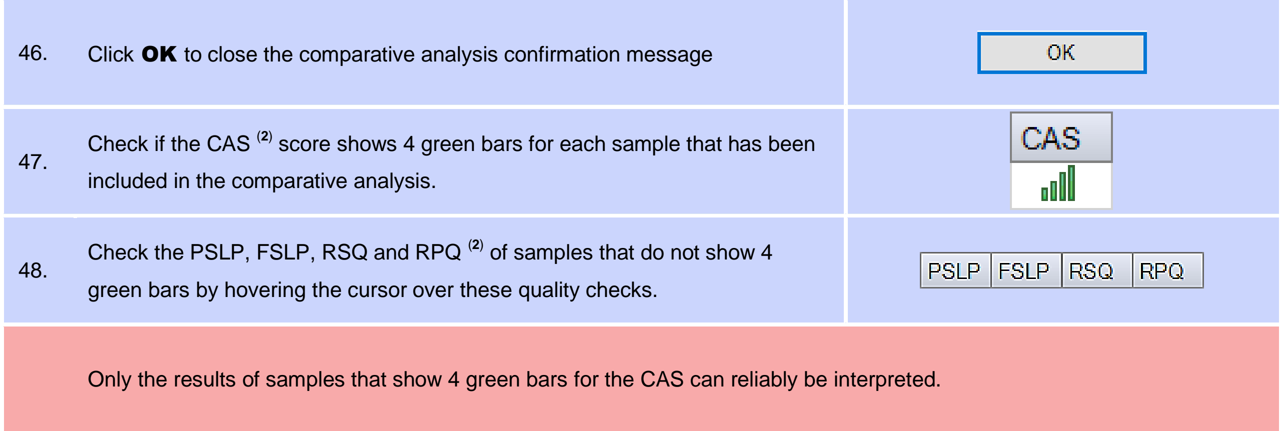
1.	Expand the project by clicking the + sign	Project 1
2.	Right click on the folder *Experiments*	Experiments Add Experiment ... Refresh
3.	Select *Add Experiment …*	
	The Experiment properties window opens	
4.	Check if the set CE device is correct. If not, select the appropriate device from the drop down menu	CE device ABI - 3130XL
5.	Fill in the relevant text fields (only the field Title is mandatory)	

6.	Click **OK** to save the experiment and close the Experiment properties window	
	The Experiment window opens	
7.	Navigate to the tab DETAILS in the window that opens	
8.	Select *DNA/MLPA [default]* as experiment type from the drop down menu	
9.	Check for each dye channel if the type of fragments is set correctly. If not, select the appropriate type from the drop down menu	
10.	Open the sheet library via the button with three dots in the column channel content for the dye channel that contains the MLPA probes	
11.	Select the applicable Coffalyser sheet from the list	
12.	Click **OK**	
13.	Select the applicable size marker from the drop down menu in the column CHANNEL CONTENT for the dye channel that contains the size marker	
14.	Leave the setting in the column ANALYSIS METHOD for the dye channel that contains the MLPA probes on default	
15.	Go to the tab FRAGMENT ANALYSIS by clicking **Next >>**	
16.	Right click in the FRAGMENT ANALYSIS tab and select *Add (From File)*	
	A dialog box opens	
17.	Navigate to the location of the raw data files	
18.	Select all raw data files that you want to analyse and click **Open**	

19.	Click **OK** to confirm the import	OK
20.	Click **Close** in the Import Files window	Close
21.	If the Manage Sample – O/S/E Connections window pops up, click **Close**	Close
22.	In the column sample type, click on the cell of a sample	sample type sample
23.	Click on the arrow head to expand the list with sample types	sample type sample sample reference positive reference no DNA digested sample
24.	Select a sample type from the list	
25.	Repeat steps 22 to 24 for the other samples	
26.	In case SALSA binning DNA has been included in the experiment, click the selection box of this sample in the column BIN SMPL.	bin smpl
27.	Click **Start Fragment Analysis**	Start Fragment Analysis
28.	Leave all settings on default in the Fragment Analysis Settings window and click **OK**	OK
29.	Click **OK** to close the fragment analysis confirmation message	OK
30.	Right click in window and select *Edit Manual Bin Set*	
31.	Select the channel that contains the MLPA probes	
	The Coffalyser Work Sheet Editor – Manual Bin Set window opens	
32.	Right click in the grid and select *Manual Bin Set Bounds*	Delete Ctrl+Del Manual Bin Set Bounds Replace Control Fragments With

33.	Select *Set Based On Current Experiment Results (All Rows)*	Set Based On Current Sample Results (All Rows) Set Based On Current Experiment Results (All Rows) Resolve Bin Set Overlap
34.	Check if probes fall inside their bin. For more information see the section **Inspect the bin set** in **Appendix VI - Bin set** on page 85	
35.	When probe signals fall outside their bin, adjust the bin set according to the procedure as described in the section **Adjust a bin set** in **Appendix VI - Bin set** on page 88	
36.	Click **OK** to close the Coffalyser Work Sheet Editor – Manual Bin Set window	OK
	A dialog box opens	
37.	Click **Yes** to set the probe recognition method to manual	Yes
38.	Click **Start Fragment Analysis**	Start Fragment Analysis
39.	Leave all settings on default in the Fragment Analysis Settings window and click **OK**	OK
40.	Click **OK** to close the fragment analysis confirmation message	OK
41.	Check if the FRSS and FMRS [1] scores show 4 green bars for every sample in the experiment	FRSS FMRS
	Only samples that show 4 green bars for the FRSS and FMRS should be used in the rest of the analysis. For troubleshooting purposes, inspect the individual quality checks and electropherograms of samples that do not show 4 green bars (right click on a sample and select Open)	
42.	Navigate to the tab COMPARATIVE ANALYSIS by clicking **Next >>**	nalysis comparative analysis
43.	Select samples that show 4 green bars for the FMRS to be included in the comparative analysis by clicking the selection box in the column ANALYSIS	analy...
44.	Click **Start Comparative analysis**	Start Comparative Analysis
45.	Leave all settings on default in the Comparative Analysis Settings window and click **OK**	OK

[1] See chapter 10. Quality scores for a description.



46.	Click **OK** to close the comparative analysis confirmation message	OK
47.	Check if the CAS [2] score shows 4 green bars for each sample that has been included in the comparative analysis.	CAS
48.	Check the PSLP, FSLP, RSQ and RPQ [2] of samples that do not show 4 green bars by hovering the cursor over these quality checks.	PSLP FSLP RSQ RPQ
	Only the results of samples that show 4 green bars for the CAS can reliably be interpreted.	

6.b Set up a methylation status analysis experiment and analyse data

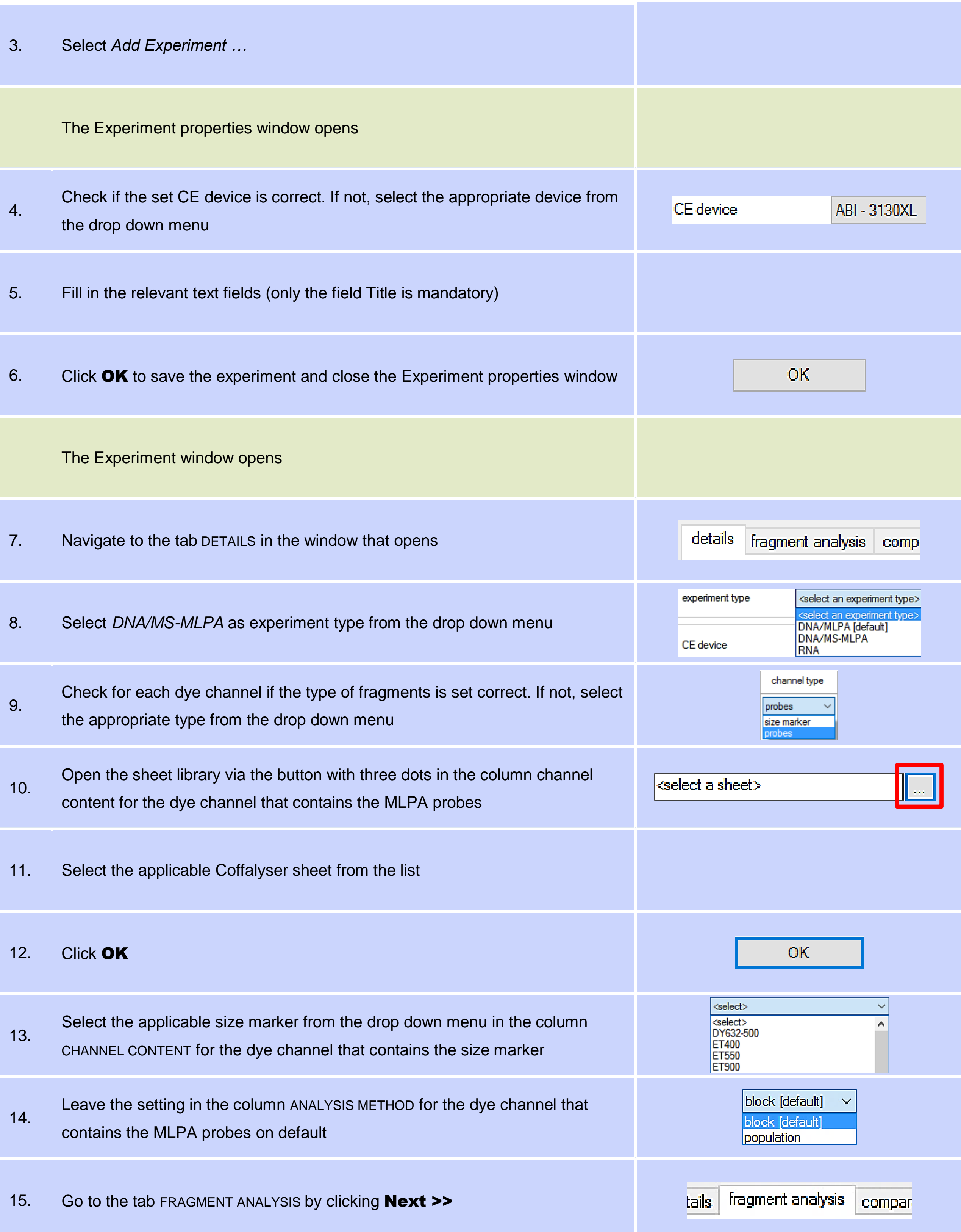
Two types of experiments exist in Coffalyser.Net: one for copy number analysis and one for the combined analysis of copy number and methylation status. In this chapter the combined analysis of copy number and methylation status is described. The procedure for copy number analysis is described in the previous chapter.

IMPORTANT NOTES:

- The parameters for fragment analysis and comparative analysis have to be left on their default values in a diagnostic setting. These parameters have been determined after extensive testing and changing them might lead to inclusion of samples with a lower quality in the analysis. Adjusting these parameters should only be done in a research setting.
- When Coffalyser.Net is used in a diagnostic setting, the quality scores FRSS, FMRS and CAS of all samples should have 4 green bars.

1.	Expand the project by clicking the + sign	Project 1
2.	Right click on the folder *Experiments*	Experiments Add Experiment ... Refresh

[2] See chapter 10. Quality scores for a description.



3.	Select *Add Experiment …*
	The Experiment properties window opens
4.	Check if the set CE device is correct. If not, select the appropriate device from the drop down menu
5.	Fill in the relevant text fields (only the field Title is mandatory)
6.	Click **OK** to save the experiment and close the Experiment properties window
	The Experiment window opens
7.	Navigate to the tab DETAILS in the window that opens
8.	Select *DNA/MS-MLPA* as experiment type from the drop down menu
9.	Check for each dye channel if the type of fragments is set correct. If not, select the appropriate type from the drop down menu
10.	Open the sheet library via the button with three dots in the column channel content for the dye channel that contains the MLPA probes
11.	Select the applicable Coffalyser sheet from the list
12.	Click **OK**
13.	Select the applicable size marker from the drop down menu in the column CHANNEL CONTENT for the dye channel that contains the size marker
14.	Leave the setting in the column ANALYSIS METHOD for the dye channel that contains the MLPA probes on default
15.	Go to the tab FRAGMENT ANALYSIS by clicking **Next >>**

16.	Right click in the FRAGMENT ANALYSIS tab and select *Add (From File)*	Open Ctrl+Space Add (From File) Ctrl+A Add (Folder) Ctrl+Shift+A Remove Ctrl+Del
	A dialog box opens	
17.	Navigate to the location of the raw data files	
18.	Select all raw data files that you want to analyse and click **Open**	Open
19.	Click **OK** to confirm the import	OK
20.	Click **Close** in the Import Files window	Close
21.	If the Manage Sample – O/S/E Connections window pops up, click **Close**	Close
22.	In the column sample type, click on the cell of a sample	sample type sample
23.	Click on the arrow head to expand the list with sample types	sample type sample sample reference positive reference no DNA digested sample
24.	Select a sample type from the list. All samples [3] to which the restriction enzyme HhaI has been added should be defined as digested sample	
25.	Repeat steps 22 to 24 for the other samples	
26.	In case SALSA binning DNA has been included in the experiment, click the selection box of this sample in the column BIN SMPL.	bin smpl
27.	Click **Start Fragment Analysis**	Start Fragment Analysis
28.	Leave all settings on default in the Fragment Analysis Settings window and click **OK**	OK

[3] This is also applicable for the digested counterparts of reference samples and positive control samples. These should be defined as 'digested sample'. No DNA samples to which the HhaI restriction enzyme is added should be defined as 'No DNA'.

29.	Click **OK** to close the fragment analysis confirmation message	OK
30.	Right click in the window and select *Edit Manual Bin Set*	
31.	Select the channel that contains the MLPA probes	
	The Coffalyser Work Sheet Editor – Manual Bin Set window opens	
32.	Right click in the grid and select *Manual Bin Set Bounds*	Delete Ctrl+Del Manual Bin Set Bounds Replace Control Fragments With
33.	Select *Set Based On Current Experiment Results (All Rows)*	Set Based On Current Sample Results (All Rows) Set Based On Current Experiment Results (All Rows) Resolve Bin Set Overlap
34.	Check if probes fall inside their bin. For more information see the section Inspect the bin set in Appendix VI - Bin set on page 85	
35.	When probe signals fall outside their bin, adjust the bin set according to the procedure as described in the section **Adjust a bin set** in **Appendix VI - Bin set** on page 88	
36.	Click **OK** to close the Coffalyser Work Sheet Editor – Manual Bin Set window	OK
	A dialog box opens	
37.	Click **Yes** to set the probe recognition method to manual	Yes
38.	Click **Start Fragment Analysis**	Start Fragment Analysis
39.	Leave all settings on default in the Fragment Analysis Settings window and click **OK**	OK
40.	Click **OK** to close the fragment analysis confirmation message	OK
41.	Check if the FRSS and FMRS [(4)] scores show 4 green bars for every sample in the experiment	FRSS FMRS

[4] See chapter 10. Quality scores for a description.

Only samples that show 4 green bars for the FRSS and FMRS should be used in the rest of the analysis.
For troubleshooting purposes, inspect the individual quality checks and electropherograms of samples that do not show 4 green bars (right click on a sample and select Open)

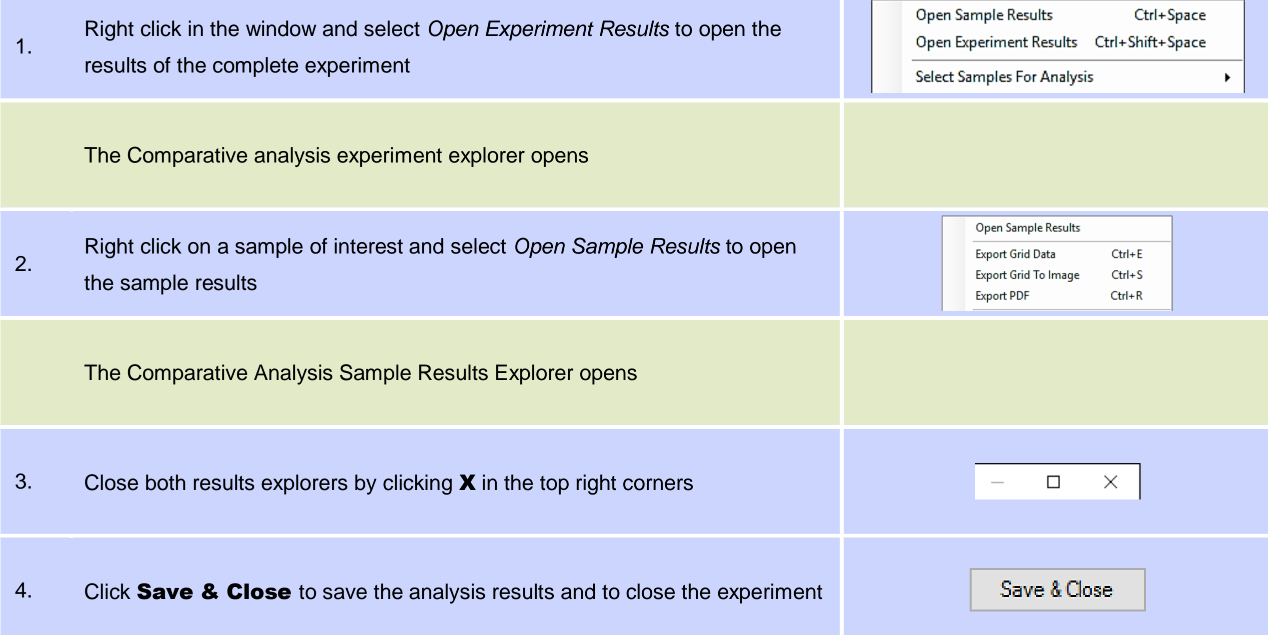
Step	Instruction	
42.	Navigate to the tab COMPARATIVE ANALYSIS by clicking **Next >>**	nalysis / comparative analysis
43.	In the column digested, click on the cell of a (undigested) sample	digested / (none)
44.	Click on the arrow head to expand the list with digested samples	digested / (none) / (none) / ME028-B2-0815-Reference1-D
45.	Select the digested sample corresponding to the undigested sample	
46.	Repeat steps 43 to 45 for the other samples	
47.	Select samples to be included in the comparative analysis by clicking the selection box in the column ANALYSIS	analy...
48.	Click **Start Comparative analysis**	Start Comparative Analysis
49.	Leave all settings on default in the Comparative Analysis Settings window and click **OK**	OK
50.	Click **OK** to close the comparative analysis confirmation message	OK
51.	Check if the CAS [5] score shows 4 green bars for each sample that has been included in the comparative analysis.	CAS
52.	Check the PSLP, FSLP, RSQ and RPQ [5] of samples that do not show 4 green bars by hovering the cursor over these quality checks.	PSLP / FSLP / RSQ / RPQ

Only the results of samples that show 4 green bars for the CAS can reliably be interpreted.

[5] See chapter 10. Quality scores for a description.

7. View analysis results

Coffalyser.Net offers the possibility to view the analysis results of all samples at once, but results of individual samples can also be viewed. It is recommended to first view the results of the complete experiment. In this way samples of interest can easily be spotted and subsequently be assessed in more detail by opening the results of these samples. Consult the most recent product description and the One-tube (MS-)MLPA protocol for DNA (and Methylation) detection and quantification for interpretation of the results. In addition, additional information about the display of results in Coffalyser.Net and interpretation can be found in **Appendix I – Normalisation** on page 24.

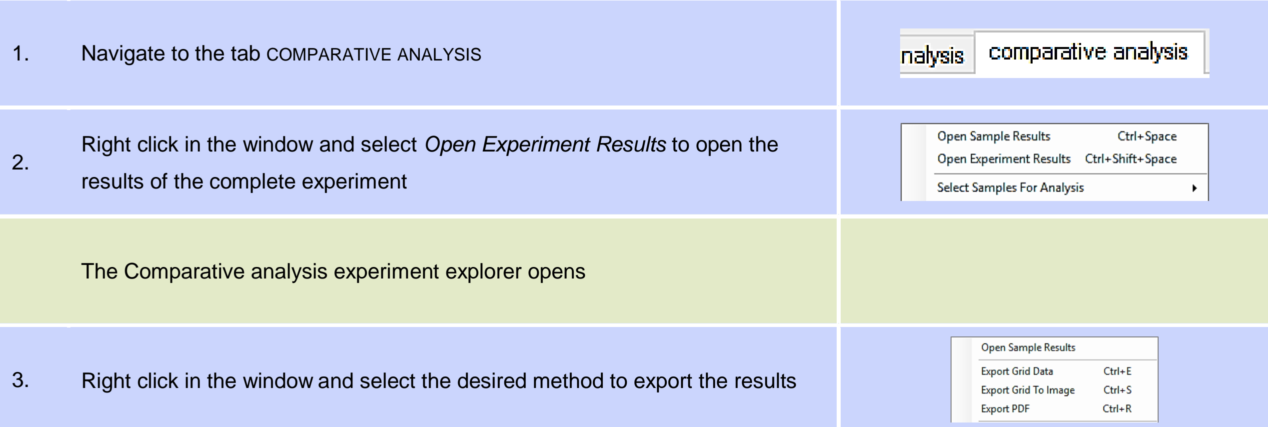


1.	Right click in the window and select *Open Experiment Results* to open the results of the complete experiment	Open Sample Results Ctrl+Space Open Experiment Results Ctrl+Shift+Space Select Samples For Analysis
	The Comparative analysis experiment explorer opens	
2.	Right click on a sample of interest and select *Open Sample Results* to open the sample results	Open Sample Results Export Grid Data Ctrl+E Export Grid To Image Ctrl+S Export PDF Ctrl+R
	The Comparative Analysis Sample Results Explorer opens	
3.	Close both results explorers by clicking **X** in the top right corners	
4.	Click **Save & Close** to save the analysis results and to close the experiment	Save & Close

8. Export results (optional)

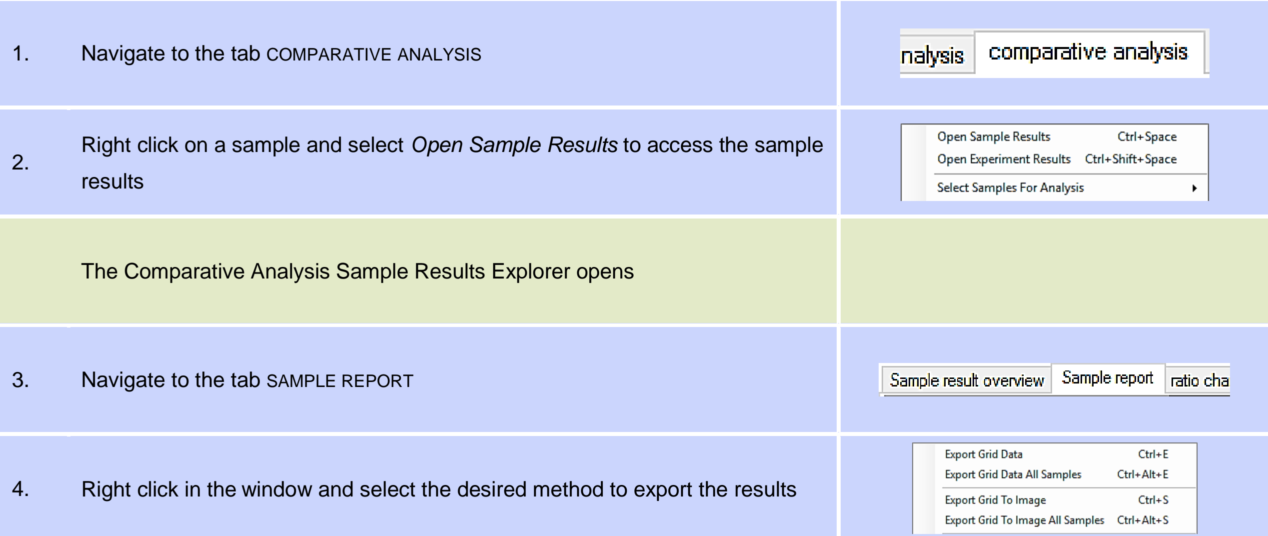
Results can be exported from Coffalyser.Net in several formats. Experimental results can be exported from the Comparative analysis experiment explorer, whereas results of individual samples can be exported from the Comparative Analysis Sample Results Explorer (see chapter **7. View analysis results**). The instructions below have the Comparative Analysis tab as starting point.

EXPORT RESULTS OF A COMPLETE EXPERIMENT



1.	Navigate to the tab COMPARATIVE ANALYSIS	nalysis / comparative analysis
2.	Right click in the window and select *Open Experiment Results* to open the results of the complete experiment	Open Sample Results Ctrl+Space Open Experiment Results Ctrl+Shift+Space Select Samples For Analysis
	The Comparative analysis experiment explorer opens	
3.	Right click in the window and select the desired method to export the results	Open Sample Results Export Grid Data Ctrl+E Export Grid To Image Ctrl+S Export PDF Ctrl+R

EXPORT RESULTS OF AN INDIVIDUAL SAMPLE



1.	Navigate to the tab COMPARATIVE ANALYSIS	nalysis / comparative analysis
2.	Right click on a sample and select *Open Sample Results* to access the sample results	Open Sample Results Ctrl+Space Open Experiment Results Ctrl+Shift+Space Select Samples For Analysis
	The Comparative Analysis Sample Results Explorer opens	
3.	Navigate to the tab SAMPLE REPORT	Sample result overview / Sample report / ratio cha
4.	Right click in the window and select the desired method to export the results	Export Grid Data Ctrl+E Export Grid Data All Samples Ctrl+Alt+E Export Grid To Image Ctrl+S Export Grid To Image All Samples Ctrl+Alt+S

5.	Right click in the window and select *Create PDF Report* to export the results to a pdf file when desired.	Create PDF Report Ctrl+R Create PDF Reports All Samples Ctrl+Alt+R

9. Open existing experiments

1.	Expand the organisation that holds the experiment by clicking the **+** sign	Organisation 1
2.	Expand the project that holds the experiment by clicking the **+** sign	Project 1
3.	Expand the folder Experiments by clicking the **+** sign	Experiments
4.	Right click on the experiment you want to open	Open Refresh Delete Properties Properties (Read Only)
5.	Select *Open*	

10. Quality scores

- The FRSS (Fragment Run Separation Score) indicates the quality of the capillary electrophoresis. It consists of several quality checks on the peak pattern of the size marker. Please see **Appendix II - Quality scores Fragment Analysis** on page 38 for more information.
- The FMRS (Fragment MLPA Reaction Score) indicates the quality of the MLPA experiment. It consists of several quality checks on the peak pattern of the MLPA probes. Please see **Appendix II - Quality scores Fragment Analysis** on page 38 for more information.
- The CAS (Coffalyser Analysis Score) indicates the quality of the normalisation. It consists of the PSLP, FSLP, RSQ, RPQ and FMRS.
- The PSLP (Preliminary Signal Sloping Probes) indicates if the difference in signal sloping between the sample and the reference samples is within limits.
- The FSLP (Final-normalisation Signal Sloping Probes) indicates if the correction for signal sloping could be applied successfully.
- The RSQ (Reference Sample Quality) indicates if the reference samples provide reproducible results.
- The RPQ (Reference Probe Quality) indicates if the reference probes provide reproducible results.

The scores of the individual quality checks on the peak patterns of the size marker (FRSS) and MLPA probes (FMRS) can be assessed by opening the samples from the Fragment analysis tab.

1.	Navigate to the tab FRAGMENT ANALYSIS	tails | fragment analysis | compar
2.	Right click on a sample and select *Open*	Open Ctrl+Space Add (From File) Ctrl+A Add (Folder) Ctrl+Shift+A
	The Sample Results Explorer opens	
3.	Click the **+** sign next to FRSS and FMRS to display the individual quality checks	
4.	Under FMRS, click the **+** sign for more detailed information about the quality checks	
5.	Hover over a quality score to see the set thresholds for the quality check	

Appendix I – Normalisation and result interpretation

MLPA is a relative technique that is based on the analysis of relative changes in probe signals. Absolute fluorescent signal intensities of MLPA probes, as measured by the capillary electrophoresis instrument, can therefore not directly be used for data analysis.

Normalisation

Copy number analysis

Coffalyser.Net uses a series of normalisation steps and calculations to compute final probe ratios.
In a process called intra-normalisation, Coffalyser.Net converts absolute signal intensities into relative values by normalising probe signals against the signals of the reference probes in one sample. This is done for each sample. During inter-normalisation, Coffalyser.Net compares each sample to the reference samples.

A simplified version of the normalisation process is as follows:

Step 1

The signal intensity of target probe 1 (Tp1) is divided by the signal intensity of reference probe 1 (Rp1) in sample 1. The same is done in reference sample 1. The first value is then divided by the second value.

This is then computed with every **reference probe** included in the probemix for the target probe. This results in the same number of intermediate ratios for target probe 1 as there are reference probes in the probemix. Next, the median value is taken over these intermediate ratios. See the equation below.

$$Median\left(\frac{(Tp1\ in\ sample\ 1/Rp1\ in\ sample\ 1)}{(Tp1\ in\ reference\ sample\ 1/Rp1 in\ reference\ sample\ 1)},\ldots,\frac{(Tp1\ in\ sample\ 1/Rp_n\ \ in\ sample\ 1)}{(Tp1\ in\ reference\ sample\ 1/Rp_n\ in\ reference\ sample\ 1)}\right)$$

Step 2

Step 1 is repeated using every **reference sample** included in the analysis. This results in as many median values for target probe 1 in sample 1 as there are reference samples in your analysis.

Coffalyser.Net then calculates the average value over these median values. This results is the final ratio of target probe 1 in sample 1.

This procedure is repeated for all probes in a probemix and samples in an experiment.

No reference samples defined in analysis

When no reference samples are defined in an analysis, all samples are used for normalisation. This will result in the same number of median values for a probe as there are samples. The median value over these medians is the final ratio for a probe in a sample. This procedure is repeated for all probes and samples.

The exact procedures and algorithms as used in Coffalyser.Net for normalisation of MLPA data have been described by the developers of Coffalyser.Net (Jordy Coffa and Joost van den Berg (2011). Analysis of MLPA Data Using Novel Software Coffalyser.NET by MRC-Holland, Modern Approaches To Quality Control, Dr. Ahmed Badr Eldin (Ed.), InTech, DOI: 10.5772/21898. Available from: http://www.intechopen.com/books/modern-approaches-to-quality-control/analysis-of-mlpa-data-using-novel-software-coffalyser-net-by-mrc-holland).

Methylation-Specific MLPA analysis

The analysis of MS-MLPA data is divided into two parts. In the first part copy numbers are determined by normalising the undigested patient samples to the undigested reference samples, like in copy number analysis.
In the second part the methylation status of a sample is determined. This is done by comparing the digested sample to its undigested counterpart.

Simplified, the second part of the normalisation works as follows:
The signal intensity of target probe 1 (Tp1) is divided by the signal intensity of reference probe 1 (Rp1) in the digested sample 1. The same is done in the undigested sample 1. The first value is then divided by the second value.

This is done with every reference probe included in the probemix, which will result in as many intermediate ratios for target probe 1 as there are reference probes in the probemix. Next, the median value is taken over these intermediate ratios. See the equation below.

$$Median\left(\frac{(Tp1\ in\ digested\ sample\ 1/Rp1\ in\ digested\ sample\ 1)}{(Tp1\ in\ undigested\ sample\ 1/Rp1 in\ undigested\ sample\ 1)},\ldots,\frac{(Tp1\ in\ digested\ sample\ 1/Rp_n\ \ in\ digested\ 1)}{(Tp1\ in\ undigested\ sample\ 1/Rp_n\ in\ undigested\ sample\ 1)}\right)$$

The median value is multiplied by 100%. This results in the methylation percentage of target probe 1 in digested sample 1.

This procedure is repeated for all probes and samples.

Results and interpretation

IMPORTANT NOTES:

The following results should always be confirmed visually in the size called peak pattern and / or raw run data:

- Homozygous deletions
- Single probe deletions and gains
- MS-MLPA results
- Mosaicisms
- Aberrant and unexpected results

It is recommended to visually confirm other results as well, but this is not required.

Copy number analysis

95% confidence interval

Besides calculating probe ratios, Coffalyser.Net also makes use of statistics to determine if a result is reliable or not. To do so, it calculates a 95% confidence interval over the reference samples for each probe. This represents the range in which the probe's ratio is expected to fall in 95 out of 100 reference samples. The 95% confidence interval of a probe over the reference samples is depicted as a coloured bar in the ratio chart (see Figure 1).

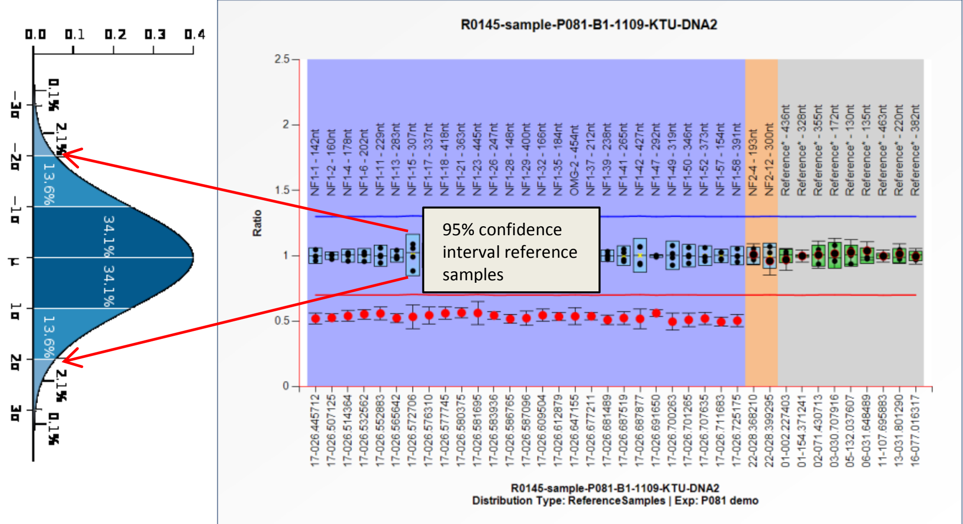


Figure 1. 95% confidence intervals over the reference samples.

In addition, it also calculates a 95% confidence interval estimate for each probe in a sample. This represents the range in which the probe's ratio is expected to fall in 95 out of 100 experiments on this sample. The 95% confidence interval of a probe in a sample is depicted as error bars in a ratio chart that surround the calculated probe ratio which is represented as a dot (see Figure 2).

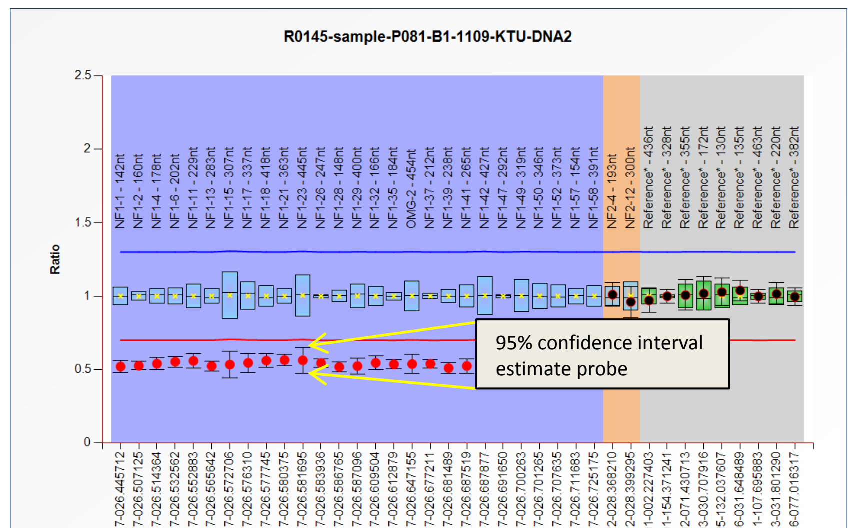


Figure 2. 95% confidence interval estimates of probes in a sample.

When these two 95% confidence intervals do not overlap, it can be concluded with a high degree of certainty that the result in a sample is significantly different from the reference samples. In case there is an overlap, the result is less clear and it can therefore not be concluded that the result is different from the reference samples.

Arbitrary borders

Coffalyser.Net also displays arbitrary borders in ratio charts as red (lower arbitrary border) and blue (upper arbitrary border) lines. By default the borders are placed -/+ 0.3 from the average probe value of a probe over the reference samples (indicated by a yellow 'x' in the ratio chart). For example, when the average value of a probe over the reference samples is 0.95, the lower arbitrary border is set at 0.65 (0.95 - 0.3) and the upper arbitrary border at 1.25 (0.95 + 0.3). Because the average value of the probes over the reference samples is different for every probe (it is not ratio 1 for all probes), the arbitrary borders are not straight lines.

When a probe ratio crosses these borders, it is indicative for a duplication or deletion (assuming that the normal copy number of the sequence targeted by the probe is two). However, crossing an arbitrary border does not necessarily mean that the probe's target sequence is indeed deleted or duplicated! For instance, it could be that the 95% confidence interval of the same probe over the reference sample also crosses the arbitrary borders. In that case, the probe result in a sample might not be different from the reference samples.

Display of results in Coffalyser.Net

Coffalyser.Net offers the possibility to display probe results in grids, ratio charts and pdf reports. Figure 3 presents an overview of possible probe results and how they are displayed in the different areas of Coffalyser.Net.

	1	2	3	4	5	6	7	8
Report	=	<<	>>	<<*	>>*	<*	>*	?
Grids								
Charts								

Figure 3. Probe results in Coffalyser.Net.

Situation 1: Probe results do not indicate a copy number change: equal to (=) sign in pdf reports, grey cells in the grids and probe ratio dots fall well within the reference sample distribution in the ratio charts.

Situation 2: Probe results indicate significantly decreased signals compared to the reference samples as a decrease of more than two standard deviations has been calculated. In pdf reports this is indicated by two less-than brackets (<<). In the grids, cells are coloured pink to indicate that the lower arbitrary border of 0.7 has not been crossed (ratios are between 0.7 and 1). In ratio charts, the error bars do not overlap with the 95% confidence intervals of the same probes over the reference samples (the blue boxes).

Situation 3: Probe result indicates a significantly increased signal compared to the reference samples as an increase of more than two standard deviations has been calculated. In pdf reports this is indicated by two greater-than brackets (>>). In the grids, cells are coloured lilac to indicate that the upper arbitrary border of 1.3 has not been crossed (ratio is between 1 and 1.3). In ratio charts the error bars do not overlap with the 95% confidence interval of the same probe over the reference samples (the blue box).

Situation 4: Probe results indicate a heterozygous deletion (assuming that the probe normally targets two copies). A decrease of more than two standard deviations has been calculated and the lower arbitrary border of 0.7 has been crossed. In pdf reports this is indicated by two less-than brackets with an asterisk (<<*). In the grids, cells are coloured bright red. In ratio charts the error bars do not overlap with the 95% confidence intervals of the same probes over the reference samples (the blue boxes), and the probe ratios cross the lower arbitrary border of 0.7 (the red line).

Situation 5: Probe results indicate a heterozygous duplication (assuming that the probe normally targets two copies). An increase of more than two standard deviations has been calculated and the upper arbitrary border of 1.3 has been crossed. In pdf reports this is indicated by two greater-than brackets with an asterisk (>>*). In the grids, cells are coloured deep blue. In ratio charts the error bars do not overlap with the 95% confidence intervals of the same probes over the reference samples (the blue boxes), and the probe ratios cross the upper arbitrary border of 1.3 (the blue line).

Situation 6: The probe result indicates a non-significantly decreased signal compared to the reference samples as a decrease of only one standard deviation has been calculated. However, the lower arbitrary border of 0.7 has been crossed. In pdf reports this is indicated by one less-than bracket with an asterisk (<*). In the grids, cells are coloured light red. In ratio charts the error bars overlap with the 95% confidence interval of the same probe over the reference samples (the blue box), and probe ratio crosses the lower arbitrary border of 0.7 (the red line).

Situation 7: The probe result indicates a non-significantly increased signal compared to the reference samples as an increase of only one standard deviation has been calculated. However, the upper arbitrary border of 1.3 has been crossed. In pdf reports this is indicated by one greater-than bracket with an asterisk (>*). In the grids, cells are coloured light blue. In ratio charts the error bar overlaps with the 95% confidence interval of the same probe over the reference samples (the blue box) and the probe ratio crosses the upper arbitrary border of 1.3 (the blue line).

Situation 8: The probe result is inconclusive, although the lower arbitrary border of 0.7 has been crossed. In pdf reports this is indicated by a question mark (?). In grids, cells are white. In ratio charts the probe ratio dot is coloured yellow.

NOTE: in the situations above it is assumed that the average probe value of a probe over the reference samples is 1.0.

Methylation-Specific MLPA analysis

Although in MS-MLPA analysis a methylation percentage is calculated for every probe, only the percentages of the methylation-specific probes (containing an HhaI site) are indicative for the methylation status of a sample. In Coffalyser.Net these probes are marked with [HHA1] in their names.

The calculated methylation percentage of a methylation-specific probe indicates the percentage of copies that are methylated in a sample. Therefore, to understand the methylation status of a probe in a sample, it is important to know its copy number. Some examples are listed in Table 1.

Table 1 MS-MLPA results

Copy number analysis		Methylation status analysis	
Final ratio	Number of copies	Methylation result	Number of methylated copies
1.0	2	50%	1
1.0	2	100%	2
2.0	4	50%	2
0.5	1	100%	1

To determine whether the obtained results indicate an aberration, they should be compared to the results obtained for the reference samples, which are expected to have a have a normal copy number and methylation status for the regions of interest.

Coffalyser.Net calculates a 95% confidence interval over the reference samples for the methylation status of each probe (the blue boxes in in the lower ratio chart in Figure 5). This represents the range in which the probe's methylation value is expected to fall in 95 out of 100 reference samples. A 95% confidence interval estimate is also calculated for each probe in a sample, this represents the range in which the probe's methylation status is expected to fall in 95 out of 100 experiments on this sample (the error bars related to the probe results in the lower ratio chart in Figure 5).

Arbitrary borders

Coffalyser.Net also displays arbitrary borders in both the copy number and methylation ratio charts as red (lower arbitrary border) and blue (upper arbitrary border) lines. By default the borders are placed -/+ 0.3 from the average probe value of a probe over the reference samples. For example, when the average value of a probe over the reference samples is 0.95, the lower arbitrary border is set at 0.65 (0.95 - 0.3) and the upper arbitrary border at 1.25 (0.95 + 0.3). In the methylation chart the average value of the probes over the reference samples is different for every probe (e.g. due to the presence of a HhaI restriction site, experimental variation etc.), therefore, the arbitrary borders are not straight lines.

When the methylation status of a probe crosses these borders, it is an indication of a difference in methylation status compared to the reference samples. However, crossing an arbitrary border does not necessarily mean that the methylation status of the probe's target sequence is indeed aberrant! For instance, it could be that the 95% confidence interval of the same probe over the reference samples also crosses the arbitrary borders. In that case, the probe result in a sample might not be different from the reference samples.

Display of results in Coffalyser.Net

Although Coffalyser.Net calculates the methylation percentage of probes in digested samples, it displays these results as ratios in screens and reports. Only in the *Comparative analysis experiment explorer* (see Figure 4) percentages are shown.

In the *Comparative analysis experiment explorer* Coffalyser.Net by default displays the results of the undigested and digested counterparts of a sample directly adjacent to each other (see Figure 4).

			A		B	
		Probe target info	AllSamples			
			R0093-sample-U-...	R0093-s...	R0400-sample-U-...	R0400-s...
FRSS (n=7)		n/a	100%	100%	100%	100%
CAS (n=5)	FMRS	n/a	100%	100%	100%	100%
	PSLP - Relative preliminary...	n/a	OK	n/a	OK	n/a
	FSLP - Relative final signal s...	n/a	OK	n/a	OK	n/a
	RSQ - Reference sample qu...	n/a	OK	OK	OK	OK
	RPQ - Reference probe qual...	n/a	OK	OK	OK	OK
15q (n=32)	TUBGCP5-8	15-020.398303	0.95	109%	0.49	102%
	NIPA1-4	15-020.612289	1.09	92%	0.5	106%
	MKRN3-1	15-021.362818	1.07	98%	0.48	106%
	MAGEL2-1	15-021.440355	1.08	97%	0.46	100%
	NDN-1	15-021.482381	1.06	91%	0.45	111%
	NDN-1 [HHA1]	15-021.483412	1.05	88%	0.47	33%
	SNRPN-u1b	15-022.619902	1.05	97%	0.52	96%
	SNRPN-u1b*	15-022.626072	1.03	102%	0.5	98%
	SNRPN-Intr.u2	15-022.690980	1.04	100%	0.52	93%
	SNRPN-Intr.u2	15-022.703328	1.05	98%	0.48	103%
	SNRPN-u5	15-022.716714	1.08	95%	0.48	102%
	SNRPN-u5	15-022.717321	0.98	102%	0.42	116%
	SNRPN-CpG isl [HHA1]	15-022.751105	1.08	93%	0.45	0
	SNRPN-CpG isl [HHA1]	15-022.751214	1.05	95%	0.47	0
	SNRPN-CpG isl [HHA1]	15-022.751480	1.06	101%	0.48	0
	SNRPN-CpG isl [HHA1]	15-022.751773	1.04	89%	0.5	0
	SNRPN-3	15-022.764248	1	100%	0.45	102%
	SNRPN-7	15-022.772555	1.05	102%	0.42	108%
	SNRPN-HB2-85-SNO	15-022.848250	0.97	104%	0.48	104%
	SNRPN-HB2-85-SNO	15-022.872658	1	102%	0.41	105%
	SNRPN-HB2-85-SNO [HHA1]	15-022.888662	1.02	75%	0.44	103%
	UBE3A-13	15-023.136395	1	108%	0.44	112%
	UBE3A-8	15-023.156677	0.98	116%	0.51	90%
	UBE3A-7	15-023.167740	1	103%	0.49	101%
	UBE3A-6	15-023.171919	1	106%	0.47	100%
	UBE3A-5	15-023.201674	1.17	93%	0.45	100%
	UBE3A-1 [HHA1]	15-023.235184	1.02	0	0.49	0
	ATP10A-5	15-023.522207	1.1	91%	0.48	101%
	ATP10A-1	15-023.659906	1.04	106%	0.53	93%
	GABRB3-12	15-024.344242	1.02	97%	0.47	107%
	GABRB3-10	15-024.363881	1.06	99%	0.48	100%
	APBA2-14 [HHA1]	15-027.196749	0.95	106%	0.86	113%
03p (n=1)	MLH1-1 [HHA1]	03-037.009621	1.01	0	0.99	0
15q (n=1)	BLM-1 [HHA1] (Dig)	15-089.061432	1.04	0	1.05	0

Figure 4. Ratio overview of the Comparative analysis experiment explorer in which the results of the copy number analysis (as ratios) and the methylation status analysis (as percentages) are grouped per sample. Probes containing an HhaI site have the label [HHA1] in their names.
A: Sample R0093 **B:** Sample R0400

In the tab ratio chart of the Comparative Analysis Sample Results Explorer, Coffalyser.Net normally presents two ratio charts. For the selected sample the upper chart shows the results of the undigested counterpart and thus copy number analysis, and the lower chart shows the results of the digested counterpart of the MLPA reaction and thus the methylation status analysis (see Figure 5).

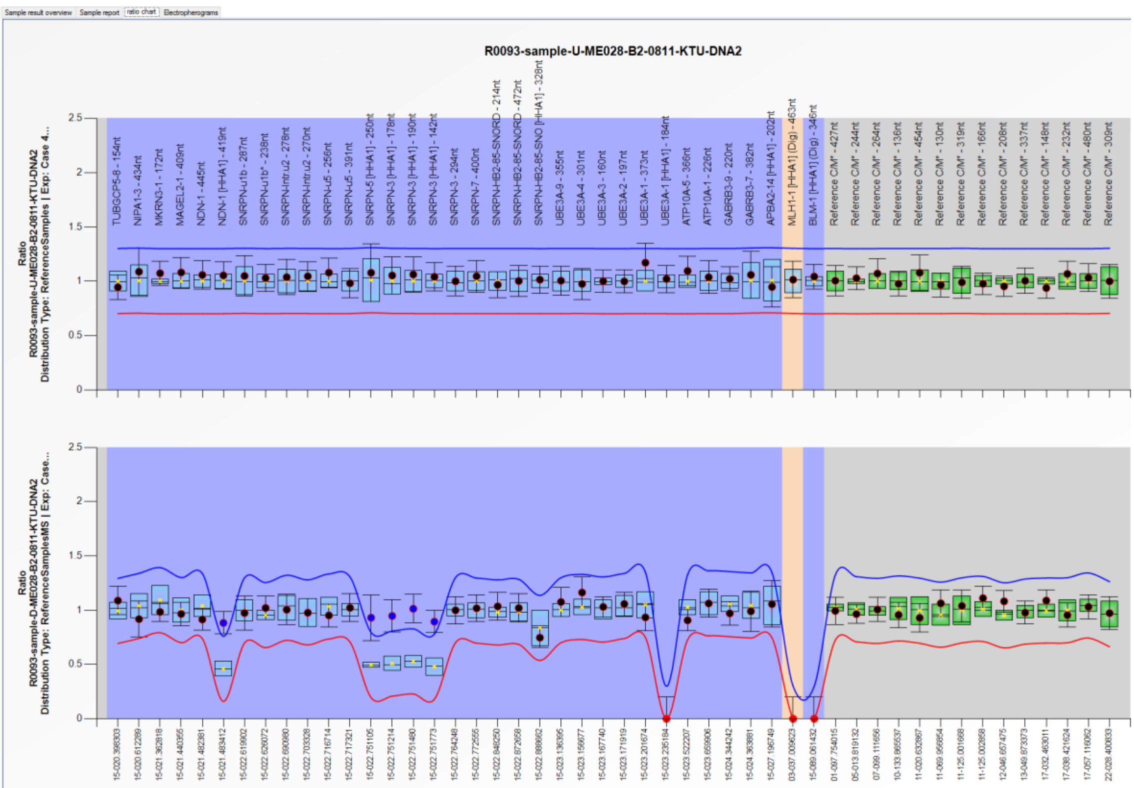


Figure 5 Ratio charts of a sample in the Comparative Analysis Sample Results Explorer. The upper chart displays the results of the undigested counterpart / the copy number analysis and the lower chart displays the results of the digested counterpart / the methylation status analysis.

It is also possible to separately view the results of the undigested or digested samples in Coffalyser.Net in the tabs Ratio overview of the Comparative analysis experiment explorer and ratio chart of the Comparative Analysis Sample Results Explorer

PROCEDURE: CHANGE THE DISPLAY OF RESULTS IN THE TAB RATIO OVERVIEW OF THE COMPARATIVE ANALYSIS EXPERIMENT EXPLORER

1.	In the tab ratio overview of the Comparative analysis experiment explorer right click and select *Show Data Type*	Open / Close Regions Show Data Type Show Probe Ratio Type
2.	From the appearing list select *DNA* or *MS* to only display the results of the undigested samples or digested samples, respectively	RNA DNA MS DNA/MS
3.	To see the combined results again, follow steps 1 and 2 and select *DNA/MS*	

PROCEDURE: CHANGE THE DISPLAY OF RESULTS IN THE TAB RATIO CHART OF THE COMPARATIVE ANALYSIS SAMPLE RESULTS EXPLORER

1.	In the tab ratio chart of the Comparative Analysis Sample Results Explorer right click on a chart and select *Sample Results Display Mode*	Distribution Type Sample Results Display Mode Show all series
2.	From the appearing list select *Single*	Single Coupled
3.	To see the combined results again, follow steps 1 and 2 and select *Coupled*	

Mutation specific probes

Several MLPA probemixes contain one or more mutation specific probes. A mutation specific probe is a probe that has been designed to detect a specific mutation. A mutation specific probe can only be ligated and amplified when the mutation for which it is designed, is present in a sample. Consequently, only these samples will contain a signal of this probe.

Probe counter

The probe counter in the Fragment analysis tab is slightly different for probemixes containing mutation specific probes. Instead of displaying the number of probes found / total number of probes in the probemix (for probemixes without mutation specific probes, see Figure 6), it displays the number of probes found / a range of probes. The start of the range indicates the number of 'normal' probes, and the end of the range the total number of probes in the probemix ('normal' probes + mutation specific probes). Figure 7A and B show examples of probe counters for probemixes with mutation specific probes.

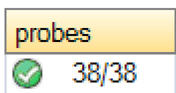


Figure 6. Probe counter of a probemix without mutation specific probes. 38 probes have been found and 38 probes are expected.

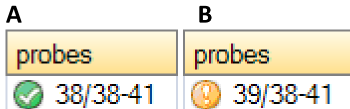


Figure 7. Probe counters of a probemix with mutation specific probes. This probemix contains a total of 41 probes (38 'normal' probes and 3 mutation specific probes) **A.** 38 probes have been found; no mutation specific probe has been detected. **B.** 39 probes have been found: 38 'normal' probes and 1 mutation specific probe.

Sample DNA SD

Because signals of mutation specific probes are usually absent in normal samples, the bins for these probes might not be correct. Consequently, they will not be properly recognised. To avoid this problem, a manual bin set has to be created.

For most MLPA probemixes that contain mutation specific probes, a special Sample DNA (SD) will be supplied with the probemix. In this SD, DNA target sequences for all probes are present (including mutation specific sequences). The MLPA data of the SD sample is extremely useful for creating a manual bin set. See section **6.a Set up a copy number analysis experiment and analyse data** (step 26) and section **6.b Set up a methylation status analysis**

experiment and analyse data (step 26) for instruction on the use of an SD sample in Coffalyser.Net. **Appendix VI - Bin set** on page 85 contains instructions for creating a manual bin set.

Normalisation

Mutation specific probes are a special type of probes and are therefore not always treated the same as normal probes during normalisation. This is due to the fact that it is not always possible to calculate ratios for these probes, because a signal is not always present in reference samples, or because the signal of the mutation specific probe is very low in a sample. This section describes how Coffalysers.Net deals with mutation specific probes in different situations.

Reference samples included in experiment

- **No signal for mutation specific probe present in reference samples**

When none of the reference samples have a signal of the mutation specific probe, it is not possible to calculate the final ratio for this probe in a sample. Coffalyser.Net can only calculate an intra-normalised ratio. It does that by dividing the signal of the mutation specific probe against the signal of every reference probe in the same sample. This results in the same number of intermediate values as there are reference probes. The median value over these intermediate values is the intra-normalised ratio.

$$Intra\text{-}normalised\ ratio = Median\ \left(\frac{Mutation\ specific\ probe\ in\ sample\ 1}{Reference\ probe\ 1\ in\ sample\ 1}, \ldots, \frac{Mutation\ specific\ probe\ in\ sample\ 1}{Reference\ probe_n\ in\ sample\ 1}\right)$$

To distinguish this from the final ratios of the other probes, Coffalyser.Net displays the intra-normalised ratio of the mutation specific probe in the Experiment results window and pdf report as percentage[6]. In the ratio chart the intra-normalised ratio is presented as an orange box.

- **Signal for mutation specific probe present in reference samples**

When a signal for a mutation specific probe is found in at least one reference sample, Coffalyser.Net can calculate the final ratio for the mutation specific probe like for regular probes. The final ratio will be displayed in all results windows and pdf reports.

However, it can happen that a mutation specific probe generates a small signal when the actual mutation is not present in the sample. It is therefore not always clear whether a sample contains the mutation or not. In case the signal of the mutation specific probe is lower than 10% of the median signal of the reference probes in the same sample, Coffalyser.Net displays the intra-normalised ratio of the mutation specific probe in the Experiment results window and PDF report as percentage[6]. In the ratio chart the intra-normalised ratio is presented as an orange box.

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[6] Note that this percentage does not indicate the percentage of cells carrying the mutation!

Because the final ratio can be calculated, this value is noted in the column Final ratio in the sample report tab of the Sample results explorer.

Reference samples	Patient sample	Experimental results	Sample results final ratio	Ratio chart	pdf report
No signal for mutation specific probe	Signal of mutation specific probe present in the sample	Intra-normalised ratio as percentage	Infinite	Intra-normalised ratio (orange box)	Intra-normalised ratio as percentage
Signal for mutation specific probe present in at least one reference sample	Signal of mutation specific probe below 10% of median signal of the reference probes	Intra-normalised ratio as percentage	Final ratio	Intra-normalised ratio (orange box)	Intra-normalised ratio as percentage
Signal for mutation specific probe present in at least one reference sample	Signal of mutation specific probe above 10% of median signal of the reference probes	Final ratio	Final ratio	Final ratio	Final ratio

No reference samples included in experiment

When no reference samples are defined, all other samples are used to calculate the ratio of a mutation specific probe in a sample. This will result in the same number of median values for a mutation specific probe as there are samples. The median value over these medians is the final ratio for the mutation specific probe in the sample. This procedure is repeated for all samples.

In samples where the signal of the mutation specific probe is lower than 10% of the median signal of the reference probes, Coffalyser.Net displays the intra-normalised ratio of the mutation specific probe in the Experiment results window and PDF report as percentage[7]. In the ratio chart the intra-normalised ratio is presented as an orange box. Because the final ratio can be calculated, this value is noted in the column Final ratio in the sample report tab of the Sample results explorer. In case only one sample contains a signal of the mutation specific probe the final ratio will be 1.

Number of samples with mutation	Patient sample	Experimental results	Sample results final ratio	Ratio chart	pdf report
1	Signal of mutation specific probe below 10% of median signal of the reference probes	Intra-normalised ratio as percentage	Final ratio (=1)	Intra-normalised ratio (orange box)	Intra-normalised ratio as percentage

[7] Note that this percentage does not indicate the percentage of cells carrying the mutation!

Number of samples with mutation	Patient sample	Experimental results	Sample results final ratio	Ratio chart	pdf report
1	Signal of mutation specific probe above 10% of median signal of the reference probes	Final ratio (=1)	Final ratio (=1)	Final ratio (=1)	Final ratio (=1)
Multiple	Signal of mutation specific probe below 10% of median signal of the reference probes in all samples	Intra-normalised ratio as percentage	Final ratio	Intra-normalised ratio (orange box)	Intra-normalised ratio as percentage
Multiple	Signal of mutation specific probe above 10% of median signal of the reference probes in all samples	Final ratio	Final ratio	Final ratio	Final ratio

IMPORTANT NOTE:

The results of mutation specific probes indicating the presence or absence of a mutation, should always be confirmed visually in the size called peak pattern and / or raw run data.

Appendix II - Quality scores Fragment Analysis

During fragment analysis Coffalyser.Net assesses the quality of both the fragment separation / electrophoresis and the MLPA reaction itself using the information from the raw data files. This is of great importance as low quality data has a negative effect on the analysis. It may complicate result interpretation and lead to false calls, or even block the analysis completely.

The outcome of the quality assessment is summarized in two main scores: one for the fragment separation and one for the MLPA reaction. This enables you to quickly distinguish samples with high quality raw data from those with low quality raw data.

Fragment Run Separation Score (FRSS)

The Fragment Run Separation Score (FRSS) is a measure for the quality of the fragment separation and peak size-calling. This score is the result of seven different evaluations of the peak pattern of the size marker. The maximum score is 100 points (or 100%). For every quality criterion that is not met, points are subtracted from the FRSS.

Table 2. Overview FRSS scores

FRSS	Points
	≥ 90
	≥ 75 and < 90
	≥ 45 and < 75
	> 25 and < 45
	≤ 25

Most FRSS evaluations depend on one or more thresholds, which are specific for the (type of) capillary electrophoresis device that is used. Table 3 presents an overview of the thresholds per capillary electrophoresis device.

Table 3. Overview thresholds size marker signal intensities

	Size marker maximum signal of the baseline (in RFU)	Size marker minimum median signal (in RFU)	Size marker maximum signal (in RFU)
ABI 310(0)	700	100	7,000
ABI 3130(xl)	700	100	7,000
ABI 3700	1,000	100	30,000
ABI 3730(xl)	1,000	100	30,000
ABI 3500(xl)	1,000	100	30,000
Beckman CEQ 2000	12,000	1,000	160,000
Beckman CEQ 8000	12,000	1,000	160,000
Beckman CEQ 8800/GEXP	12,000	1,000	160,000
MegaBACE 1000	1,000	100	30,000

Diagram calculation Fragment Run Separation Score (FRSS)
FRSS 100%
Correlation (R2)
Above 0.999
No
Yes
Notification: Bad
FRSS penalty
Notification: OK
No FRSS penalty
Correlation (R2) penalty
Baseline size marker
Below 80% of maximum signal intensity for the baseline
No
Yes
Above the maximum signal intensity for the baseline
No
Yes
Notification: Warning
FRSS penalty
Notification: Bad
FRSS penalty
Notification: OK
No FRSS penalty
Baseline size marker penalty
Median signal height size marker
Below the minimum median signal intensity
No
Yes
Below 125% of minimum median signal intensity
No
Yes
Notification: OK
No FRSS penalty
Notification: Warning
FRSS penalty
Notification: Bad
FRSS penalty
Median height size marker penalty
Maximum signal height size marker
Above 80% of maximum signal intensity
Yes
No
Above the maximum signal intensity
No
Yes
Notification: Warning
FRSS penalty
Notification: Bad
FRSS penalty
Notification: OK
No FRSS penalty
Maximum height size marker penalty
Size marker signal sloping
Above 40%
No
Yes
Notification: OK
No FRSS penalty
Above 60%
No
Yes
Size marker signal widening above 50%
No
Yes
Notifications: Sloping: Warning Widening: OK
FRSS penalty
Notifications: Sloping: Warning Widening: Warning
FRSS penalty
Size marker signal widening above 50%
No
Yes
Notifications: Sloping: Bad Widening: OK
FRSS penalty
Notifications: Sloping: Bad Widening: Warning
FRSS penalty
Sloping size marker penalty
Size marker complete
No
Yes
Notification: Bad
FRSS penalty
Notification: OK
No FRSS penalty
Size marker complete penalty
Final FRSS

FRSS evaluations

Correlation (R2)

Background:

This indicates the correlation of the standard curve based on the size marker. A higher correlation implies a more even electrophoresis run and better size-calling of the MLPA peaks.

Conditions:

		Notification	Penalty
✓	Correlation above 0.999	Ok	No penalty
✗	Correlation below 0.999	Bad	80 points

Related issues and solutions:

A low correlation may suggest variable conditions during the electrophoresis run. As a result, it becomes more difficult to properly identify the MLPA peaks. The raw data should be visually inspected for run artefacts. It often helps to check the CE device for flaws as well, and to replace buffer, water, polymer and the capillary array when needed. A rerun of the MLPA samples is required before the data can be interpreted.

Baseline size marker

Background:

This is a measure for the average signal intensity in the size marker channel when no peaks are passing the detector (the baseline). In a calibrated system this value should usually be close to 0.

Conditions:

		Notification	Penalty
✓	Below 80% of the Size marker maximum signal of the baseline specified for the Capillary Electrophoresis instrument (see **Table 3**)	Ok	No penalty
!	Between 80% and 100% of the Size marker maximum signal of the baseline specified for the Capillary Electrophoresis instrument (see **Table 3**)	Warning	10 points
✗	Above 100% of the Size marker maximum signal of the baseline specified for the Capillary Electrophoresis instrument (see **Table 3**)	Bad	15 points

Related issues and solutions:

An elevated baseline can lead to erroneous size-calling of peaks. A high baseline also decreases the dynamic range of the channel. The CE device performs optimally when the baselines for all channels are lower than 5% of the maximum intensity of the device.

In case of an elevated baseline with an ABI device, remove the capillary array at the manifold end and clean the detection cell by applying a little bit of ethanol. Remove the ethanol by holding a lint-free lab wipe on the side of the detection and blow it dry with compressed air. Ensure that no air bubbles are present in the capillary array and tubing after reinstalling the array.

Median signal height

Background:

This indicates the median height of the peaks of the size marker. Signal intensities of the fragments of the size marker should be sufficiently high to allow accurate size-calling. In addition, these fragments should be at least 3x the signal of the baseline.

Conditions:

		Notification	Penalty
	Above 125% of the Size marker minimum median signal specified for the Capillary Electrophoresis instrument (see **Table 3**)	Ok	No penalty
	Between 100% and 125% of the Size marker minimum median signal specified for the Capillary Electrophoresis instrument (see **Table 3**)	Warning	10 points
	Below 100% of the Size marker minimum median signal specified for the Capillary Electrophoresis instrument (see **Table 3**)	Bad	20 points

Related issues and solutions:

Depending on the overall signal intensity of the MLPA probe peak pattern, the signals of the size marker can be increased by adjusting the injection settings or by using more size marker in the injection mixture.

Maximum signal height

Background:

This indicates the highest peak in the pattern of the size marker.

Conditions:

		Notification	Penalty
	Below 80% of the Size marker maximum signal specified for the Capillary Electrophoresis instrument (see **Table 3**)	Ok	No penalty
	Between 80% and 100% of the Size marker maximum signal specified for the Capillary Electrophoresis instrument (see **Table 3**)	Warning	10 points
	Above 100% of the Size marker maximum signal specified for the Capillary Electrophoresis instrument (see **Table 3**)	Bad	15 points

Related issues and solutions:

MLPA amplification products and size marker are simultaneously injected in a competitive fashion. High signals of the size marker indicate that more size marker is injected than necessary. Consequently, less MLPA amplification products have been injected, which might result in less accurate measurement of these products.

Depending on the overall signal intensity of the MLPA probe peak pattern, the signals of the size marker can be decreased by adjusting the injection settings or by using less size marker in the injection mixture.

Size marker signal sloping

Background:

This is a measure of the drop in signal intensity of the fragments in the peak pattern of the size marker that is proportional to the length (also known as signal to size drop or, simply, sloping). Sloping of the size marker is introduced during capillary electrophoresis and it will have a similar effect on the MLPA probes.

Conditions:

		Notification	Penalty
	Sloping lower than 40%	Ok	No penalty
	Sloping between 40% and 60%	Warning	15 points
	Sloping above 60%	Bad	30 points

Related issues and solutions:

Sometimes signal sloping occurs randomly and by rerunning the samples this problem may be solved. In case it persists and/or when it is accompanied by signal widening, check the capillary electrophoresis instrument for flaws and check the age of the capillary array and polymer. These may have to be replaced.

Size marker signal widening

Background:

Like sloping of the size marker, signal widening of the size marker is introduced during capillary electrophoresis. It is therefore also seen in the MLPA probes. The phenomenon of signal widening appears in the electropherogram as peaks being broader at their base and less sharp than usual.

Conditions:

		Notification	Penalty
✓	Signal widening lower than 50%, but no sloping	Ok	No penalty
!	Signal widening above 50%, but no sloping	Warning	No penalty
!	Signal widening above 50%, and sloping between 40% and 60%	Warning	50 points
!	Signal widening above 50%, and sloping above 60%	Warning	75 points

Related issues and solutions:

Sometimes signal widening occurs randomly and by rerunning the samples this problem may be solved. In case it persists and/or when it is accompanied by signal sloping, check the capillary electrophoresis instrument for flaws as well as the age of the capillary array and polymer. These may have to be replaced.

Size marker complete

Background:

This indicates whether all fragments of the size marker have been detected by the software. For accurate size-calling of the MLPA probe fragments, it is important that all fragments of the size marker are present and detectable.

Conditions:

		Notification	Penalty
✓	All fragments of the size marker detected	Ok	No penalty
✗	Not all fragments of the size marker detected	Bad	60 points

Related issues and solutions:

In case a notification is given, it is important to visually examine the raw data to determine the cause of the problem. When the last fragments of the size marker are not visible, the runtime was too short. The solution for this situation is elongating the runtime and rerunning the samples.

If one or more fragments are too low to be detected by the software, the samples can be rerun with adjusted injection settings. Alternatively, samples can be reloaded with more size marker added to the injection mixture.

Fragment MLPA Reaction Score (FMRS)

The Fragment MLPA Reaction Score (FMRS) is a measure for the quality of the MLPA reaction. This score is the result of twelve different evaluations of the peak pattern of the MLPA probes. The maximum score is 100 points (or 100%). For every quality criterion that is not met, points are subtracted from the FMRS.

Table 4. Overview FMRS

FMRS	Points
	≥ 90
	≥ 75 and < 90
	≥ 45 and < 75
	> 25 and < 45
	≤ 25

Most FMRS evaluations depend on one or more thresholds, which are specific for the (type of) capillary electrophoresis device that is used. Table 5 presents an overview of the thresholds per capillary electrophoresis device.

Table 5. Overview probe peak signal intensities

	Probes maximum signal of the baseline (in RFU)	Probes minimum median signal (in RFU)	Probes maximum median signal (in RFU)	Probes maximum signal (in RFU)
ABI 310(0)	700	300	5,000	7,500
ABI 3130(xl)	700	300	5,000	7,500
ABI 3700	1,000	300	26,000	31,000
ABI 3730(xl)	1,000	300	26,000	31,000
ABI 3500(xl)	1,000	300	26,000	31,000
Beckman CEQ 2000	12,000	3,000	150,000	170,000
Beckman CEQ 8000	12,000	3,000	150,000	170,000
Beckman CEQ 8800/GEXP	12,000	3,000	150,000	170,000
MegaBACE 1000	1,000	300	26,000	31,000

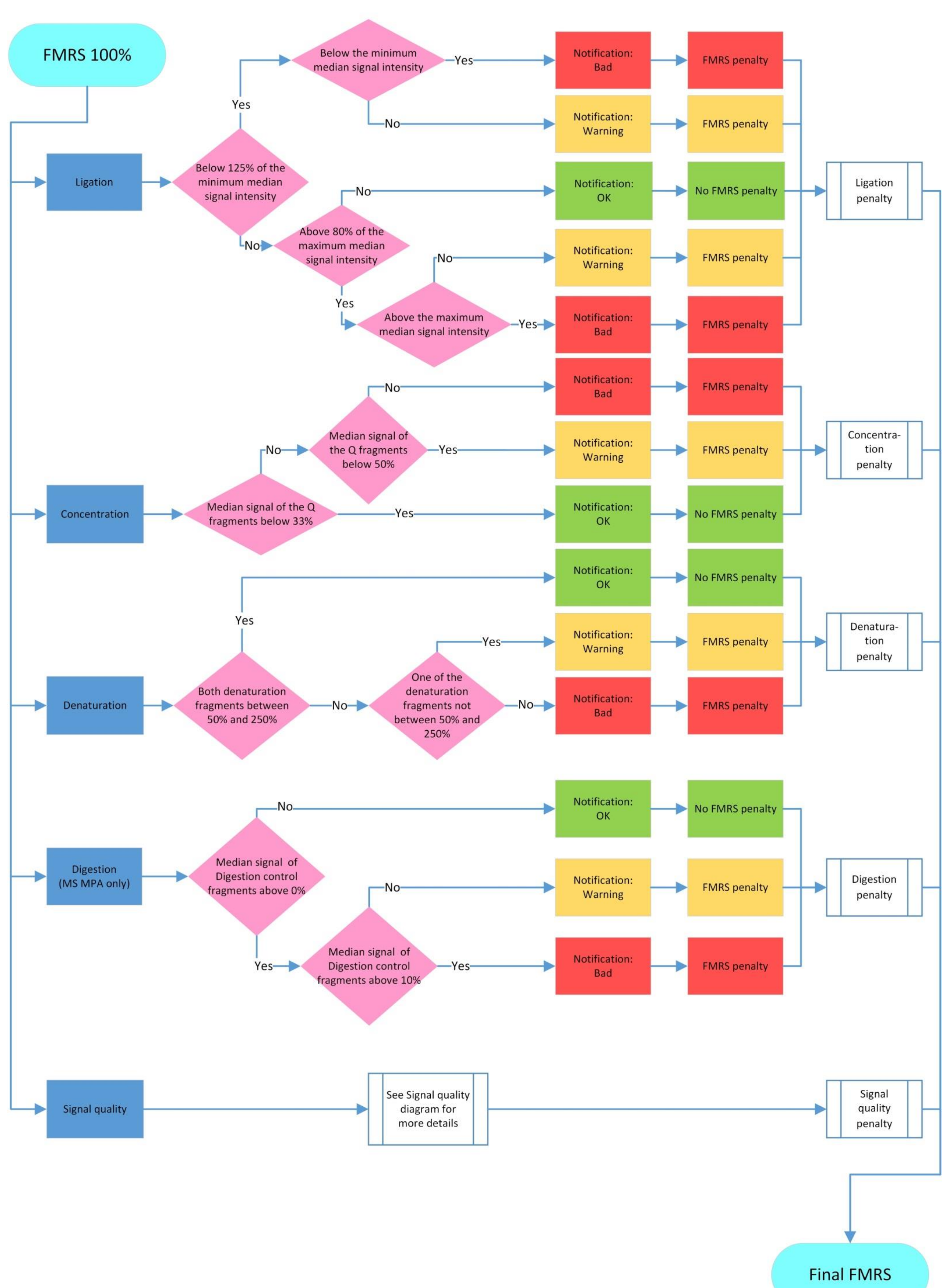


Diagram calculation Fragment MLPA Reaction Score (FMRS)
FMRS 100%
Ligation
Below 125% of the minimum median signal intensity
Yes
Below the minimum median signal intensity
Yes
No
Above 80% of the maximum median signal intensity
No
Yes
Above the maximum median signal intensity
No
Yes
Notification: Bad
Notification: Warning
Notification: OK
Notification: Warning
Notification: Bad
FMRS penalty
FMRS penalty
No FMRS penalty
FMRS penalty
FMRS penalty
Ligation penalty
Concentration
Median signal of the Q fragments below 33%
No
Median signal of the Q fragments below 50%
No
Yes
Yes
Notification: Bad
Notification: Warning
Notification: OK
FMRS penalty
FMRS penalty
No FMRS penalty
Concentra-tion penalty
Denaturation
Both denaturation fragments between 50% and 250%
Yes
No
One of the denaturation fragments not between 50% and 250%
Yes
No
Notification: OK
Notification: Warning
Notification: Bad
No FMRS penalty
FMRS penalty
FMRS penalty
Denatura-tion penalty
Digestion (MS MPA only)
Median signal of Digestion control fragments above 0%
No
Yes
Median signal of Digestion control fragments above 10%
No
Yes
Notification: OK
Notification: Warning
Notification: Bad
No FMRS penalty
FMRS penalty
FMRS penalty
Digestion penalty
Signal quality
See Signal quality diagram for more details
Signal quality penalty
Final FMRS

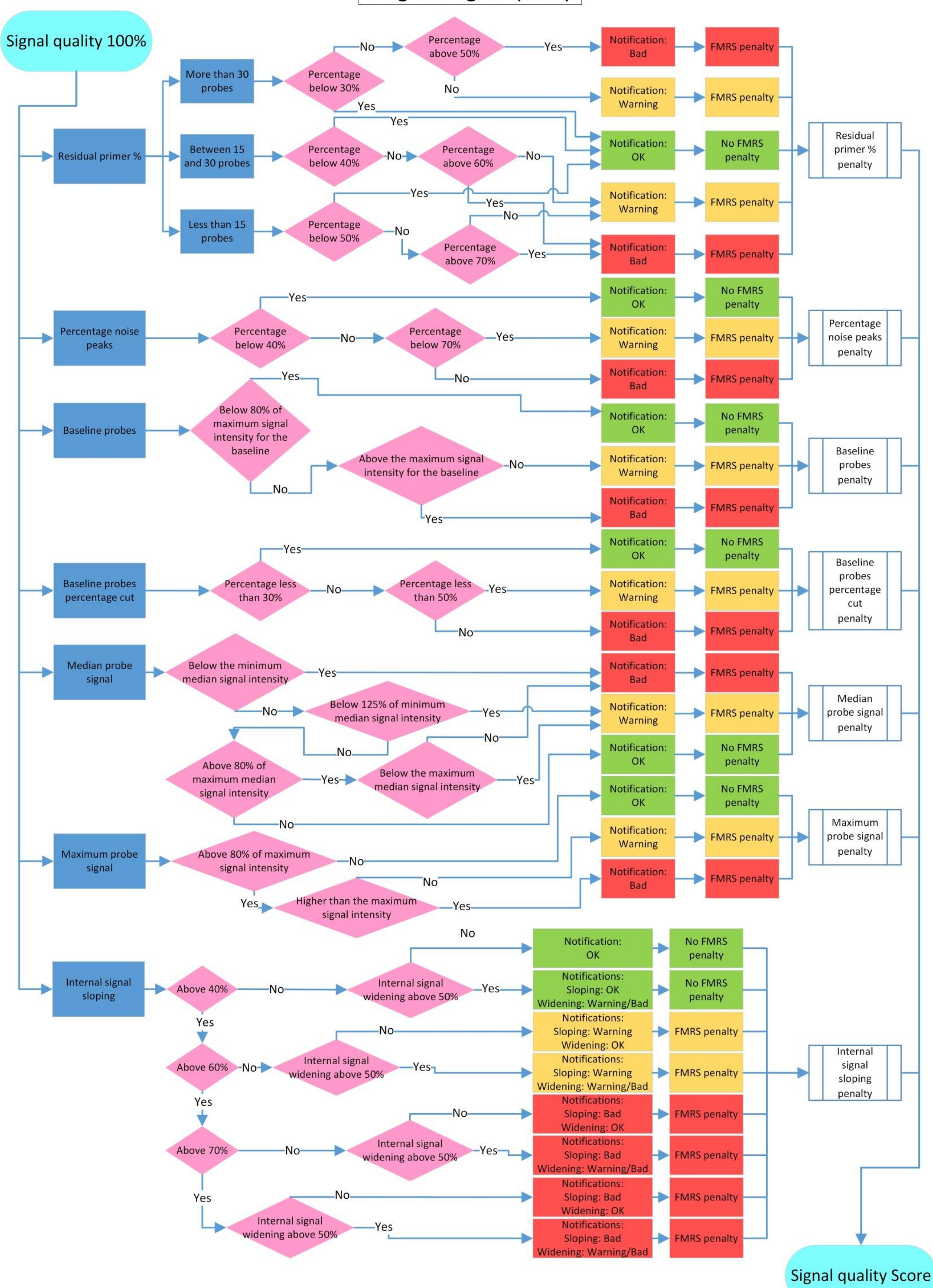


Diagram Signal quality
Signal quality 100%
Residual primer %
More than 30 probes
Between 15 and 30 probes
Less than 15 probes
Percentage below 30%
Percentage above 50%
Percentage below 40%
Percentage above 60%
Percentage below 50%
Percentage above 70%
Percentage noise peaks
Percentage below 40%
Percentage below 70%
Baseline probes
Below 80% of maximum signal intensity for the baseline
Above the maximum signal intensity for the baseline
Baseline probes percentage cut
Percentage less than 30%
Percentage less than 50%
Median probe signal
Below the minimum median signal intensity
Below 125% of minimum median signal intensity
Above 80% of maximum median signal intensity
Below the maximum median signal intensity
Maximum probe signal
Above 80% of maximum signal intensity
Higher than the maximum signal intensity
Internal signal sloping
Above 40%
Above 60%
Above 70%
Internal signal widening above 50%
Yes
No
Notification: Bad
Notification: Warning
Notification: OK
FMRS penalty
No FMRS penalty
Notifications: Sloping: OK Widening: Warning/Bad
Notifications: Sloping: Warning Widening: OK
Notifications: Sloping: Warning Widening: Warning/Bad
Notifications: Sloping: Bad Widening: OK
Notifications: Sloping: Bad Widening: Warning/Bad
Residual primer % penalty
Percentage noise peaks penalty
Baseline probes penalty
Baseline probes percentage cut penalty
Median probe signal penalty
Maximum probe signal penalty
Internal signal sloping penalty
Signal quality Score

FMRS evaluations

Ligation

Background:

This indicates whether the benchmark fragment at 92 nt is not too high or too low. This fragment is used as a standard to which other control fragments are compared (please note that another fragment acts as this standard in some probemixes).

Conditions:

		Notification	Penalty
✓	Above 125% of the minimum median signal and below 80% of the maximum median signal intensity specified for the Capillary Electrophoresis instrument (see **Table 5**)	Ok	No penalty
!	Between 100% and 125% of the minimum median signal specified for the Capillary Electrophoresis instrument (see **Table 5**)	Warning	No penalty
!	Between 80% and 100% of the maximum signal specified for the Capillary Electrophoresis instrument (see **Table 5**)	Warning	No penalty
✗	Below 100% of the minimum median signal specified for the Capillary Electrophoresis instrument (see **Table 5**)	Bad	No penalty
✗	Above 100% of the maximum signal of the size marker specified for the Capillary Electrophoresis instrument (see **Table 5**)	Bad	No penalty

Related issues and solutions:

When this fragment is too low or too high, it is usually accompanied by other phenomena affecting the quality of the raw data. When all other criteria are met and only this criterion fails, no points are subtracted from the FMRS score.

Concentration

Background:

This is a measure for the amount of sample DNA in the MLPA reaction and the activity of the ligase enzyme. The ligase activity and amount of DNA should be sufficient as they are both critical for reliable data analysis and result interpretation. To determine these, Coffalyser.Net compares the four Q-fragments at 64, 70, 76 and 82 nt to the benchmark fragment at 92 nt.

Conditions:

		Notification	Penalty
✓	Median signal of the Q-fragments below 33% of the signal of the benchmark fragment at 92 nt	Ok	No penalty
!	Median signal of the Q-fragments between 33% and 50% of the signal of the benchmark fragment at 92 nt	Warning	15 points
✗	Median signal of the Q-fragments above 50% of the signal of the benchmark fragment at 92 nt	Bad	60 points

Related issues and solutions:

The Q-fragments are DNA and ligase independent and they show increased signal intensities in case of less or no DNA, or diminished ligase activity. A notification might indicate that insufficient DNA has been used. The recommended amount of DNA for MLPA ranges from 50 to 100 ng. Please note that measuring devices might overestimate the DNA concentration.

It is important not to vortex the ligase enzyme as this will destroy the enzyme.

Denaturation

Background:

This is a measure for predominantly DNA denaturation. Incomplete denaturation of sample DNA affects the hybridisation efficiency of probes to their target sequences. As a consequence, these sequences are not completely covered by their probes leading to aberrant and unreliable results, in particular for GC-rich target sequences. Coffalyser.Net assesses the denaturation by comparing the D-fragments (D1 at 88 nt and D2 at 96 nt) to the benchmark fragment at 92 nt.

Conditions:

		Notification	Penalty
✓	Signals of both D-fragments between 50% and 250% of the signal of the benchmark fragment at 92 nt	Ok	No penalty
!	Signals of both D-fragments above 250% of the signal of the benchmark fragment at 92 nt	Warning	15 points
!	Signals of one D-fragment above 250% and the signal of the other between 50% and 250% of the signal of the benchmark fragment at 92 nt	Warning	15 points
!	Signal of one D-fragment above 250% and the signal of the other below 50% of the signal of the benchmark fragment at 92 nt	Warning	15 points
!	Signals of one D-fragment below 50% and the signal of the other between 50% and 250% of the signal of the benchmark fragment at 92 nt	Warning	15 points
✗	Signal of both D-fragments below 50% of the signal of the benchmark fragment at 92 nt	Bad	60 points

Related issues and solutions:.

Low signals of both D-fragments indicate that the DNA denaturation was incomplete. Some contaminants are known to impair DNA denaturation. It might therefore help to dilute the DNA sample. Contaminants that are present in the sample are diluted as well, thereby reducing their effect. Next to this, an extra purification step possibly helps to improve the quality of the samples, which might lead to better results.

Other results of the D-fragments might be an indication of other problems in the MLPA reaction. Contact the Technical Support department of MRC-Holland for further assistance.

Digestion (only DNA/MS-MLPA)

Background:

This indicates whether the digestion by the restriction enzyme HhaI is complete. Most MS-MLPA probemixes contain one or more Digestion control probes. These probes contain a HhaI restriction site that is never methylated and should therefore always be digested. Consequently, no signal for these probes should be present in the raw data of the digested reaction.

Conditions:

		Notification	Penalty
✓	No signal(s) of the Digestion control probe(s) in the digested reaction	Ok	No penalty
!	Median signal of the Digestion control probe(s) in the digested reaction between 0% and 10% of the signal of the benchmark fragment at 92 nt	Warning	20 points
✗	Median signal of the Digestion control probe(s) in the digested reaction above 10% of the signal of the benchmark fragment at 92 nt	Bad	60 points

Related issues and solutions:

A signal of the Digestion control probes in samples to which the HhaI enzyme has been added indicates reduced activity of this enzyme. Ensure that the ligation-digestion reaction is performed at 48° C (in contrast to the 54° C in 'normal' MLPA experiments). It is important not to vortex the HhaI enzyme as this will destroy the enzyme.

Signal quality – Residual primer %

Background:

This is a measure for the amount of unused primer and therefore for the efficiency of the PCR reaction. In a successful MLPA reaction the great majority of available primer is incorporated into the MLPA probes. Coffalyser.Net compares the amount of fluorescence of the primer peak (which consists of unused primer) to the total fluorescence of the MLPA probe peaks to calculate whether sufficient primer is incorporated.

Conditions:

Probemixes with more than 30 probes:

		Notification	Penalty
✓	Residual primer percentage below 30%	Ok	No penalty
!	Residual primer percentage between 30% and 50%	Warning	15 points
✗	Residual primer percentage above 50%	Bad	40 points

Probemixes with 15-30 probes:

		Notification	Penalty
✓	Residual primer percentage below 40%	Ok	No penalty
!	Residual primer percentage between 40% and 60%	Warning	15 points
✗	Residual primer percentage above 60%	Bad	40 points

Probemixes with less than 15 probes:

		Notification	Penalty
✓	Residual primer percentage below 50%	Ok	No penalty
!	Residual primer percentage between 50% and 70%	Warning	15 points
✗	Residual primer percentage above 70%	Bad	40 points

Related issues and solutions:

A high percentage of residual primer, which is often visible as a high primer peak in the shorter length region of the electropherogram, indicates that the PCR was suboptimal. This might be accompanied by a low overall peak pattern of the MLPA probes.

Some contaminants are known to have a negative effect on the PCR by affecting the polymerase enzyme. In case of contamination, it might help to dilute the DNA sample. Contaminants that are present in the sample are diluted as well, thereby reducing their effect. Next to this, an extra purification step possibly helps to improve the quality of the samples, which might lead to better results.

Signal quality – Percentage noise peaks

Background:

This is indicates the number of peaks that are detected but not recognised as MLPA probes as percentage of the number of detected MLPA probes.

Conditions:

		Notification	Penalty
✓	Percentage noise peaks below 40%	Ok	No penalty
!	Percentage noise peaks between 40% and 70%	Warning	10 points
✗	Percentage noise peaks above 70%	Bad	20 points

Related issues and solutions:

Large amounts of noise peaks may disturb the quantification of fluorescence of other probe related peaks. Noise peaks can have several causes including a high DNA concentration, too much polymerase, contamination of the DNA sample, and overload of the capillary electrophoresis device.

Problems related to noise peaks might be resolved by diluting the DNA sample. Ensure to add the right amount of polymerase to the PCR mixture. Ensure that the amount of PCR product in the injection mixture does not exceed 10% of the total volume. Diluting the PCR product first before adding it to the injection mixture for electrophoresis might also help to improve results.

Signal quality – Baseline probes

Background:

This is a measure for the average signal intensity in the probe channel when no peaks are passing the detector (the baseline). In a calibrated system this value should usually be close to 0.

Conditions:

		Notification	Penalty
✓	Below 80% of the Probes maximum signal of the baseline specified for the Capillary Electrophoresis instrument (see **Table 5**)	Ok	No penalty
!	Between 80% and 100% of the Probes maximum signal of the baseline specified for the Capillary Electrophoresis instrument (see **Table 5**)	Warning	10 points
✗	Above 100% of the Probes maximum signal of the baseline specified for the Capillary Electrophoresis instrument (see **Table 5**)	Bad	15 points

Related issues and solutions:

An elevated baseline can lead to erroneous size-calling of peaks. A high baseline also decreases the dynamic range of the channel. The CE device performs optimally when the baselines for all channels are lower than 5% of the maximum intensity of the device.

In case of an elevated baseline with an ABI device, remove the capillary array at the manifold end and clean the detection cell by applying a little bit of ethanol. Remove the ethanol by holding a lint-free lab wipe on the side of the detection and blow it dry with compressed air. Ensure that no air bubbles are present in the capillary array and tubing after reinstalling the array.

Signal quality – Baseline probes percentage cut

Background:

This is a measure for the amount of fluorescence below the MLPA probe peaks. In other words, it indicates if the probe peaks fully return to the baseline.

Conditions:

		Notification	Penalty
✓	Baseline probes percentage cut below 30%	Ok	No penalty
!	Baseline probes percentage cut between 30% and 50%	Warning	15 points
✗	Baseline probes percentage cut above 50%	Bad	40 points

Related issues and solutions:

An high Baseline probes percentage cut may be caused by an overload of the capillary electrophoresis device with PCR product. Ensure that the amount of PCR product in the injection mixture does not exceed 10% of the total volume. Diluting the PCR product first before adding it to the injection mixture for electrophoresis might also help to improve results. Next to this, lowering the injection settings (when possible) might help to reduce the Baseline probes percentage cut.

Signal quality – Median probe signal

Background:

This indicates the median height of the peaks of the MLPA probes. For accurate measurement, signal intensities of the MLPA probes should not be too high or too low. The median signal intensity should be at least 3x the signal of the baseline.

Conditions:

		Notification	Penalty
✓	Above 125% of the Probes minimum median signal specified for the Capillary Electrophoresis instrument (see **Table 5**)	Ok	No penalty
✓	Below 80% of the Probes maximum median signal specified for the Capillary Electrophoresis instrument (see **Table 5**)	Ok	No penalty
!	Between 100% and 125% of the Probes minimum median signal specified for the Capillary Electrophoresis instrument (see **Table 5**)	Warning	10 points
!	Between 80% and 100% of the Probes maximum median signal specified for the Capillary Electrophoresis instrument (see **Table 5**)	Warning	10 points
✕	Below 100% of the Probes minimum median signal specified for the Capillary Electrophoresis instrument (see **Table 5**)	Bad	20 points
✕	Above 100% of the Probes maximum median signal specified for the Capillary Electrophoresis instrument (see **Table 5**)	Bad	20 points

Related issues and solutions:

The signals of the MLPA probes can be increased or decreased by adjusting the injection settings. Optimisation of signal intensities should be done using the reference samples as these are expected to have no copy number changes of the probe target sequences.

Signal quality – Maximum probe signal

Background:

This indicates the highest peak in the pattern of the MLPA probes.

Conditions:

		Notification	Penalty
✓	Below 80% of the Probes maximum signal specified for the Capillary Electrophoresis instrument (see **Table 5**)	Ok	No penalty
!	Between 80% and 100% of the Probes maximum signal specified for the Capillary Electrophoresis instrument (see **Table 5**)	Warning	10 points
✕	Above 100% of the Probes maximum signal specified for the Capillary Electrophoresis instrument (see **Table 5**)	Bad	15 points

Related issues and solutions:

When signal intensities are close to the maximum detection limit of the CE device, measurement of these signals is less accurate. The signals of the MLPA probes can be decreased by lowering the injection settings. Optimisation of signal intensities should be done using the reference samples as these are expected to have no copy number changes of the probe target sequences.

Signal quality – Internal signal sloping

Background:

This is a measure of the drop in signal intensity of the fragments in the peak pattern of the MLPA probes that is proportional to the length (also known as signal to size drop or, simply, sloping).

Conditions:

		Notification	Penalty
✓	Sloping lower than 40%	Ok	No penalty
!	Sloping between 40% and 60%	Warning	5 points
✗	Sloping between 60% and 70%	Bad	15 points
✗	Sloping above 70%	Bad	30 points

Related issues and solutions:

Sloping that is only visible in the peak pattern of the MLA probes (and not in the peak pattern of the size marker) is introduced in the MLPA experiment itself. It is usually a result of a decreased amplification efficiency of the polymerase for the longer MLPA probes. This reduced efficiency can be caused by things such as contaminants in the sample, evaporation during the overnight hybridisation step or evaporation when the ligase enzyme is added.

Depending on the exact cause, several solutions are available for this problem:

- Reduce the time that the wells are uncovered by using multichannel pipets, especially during the addition of the ligase.
- Ensure that the tubes are closed properly. Some plastics deform due to the heat. In that case, switching to a different brand of tubes might be worthwhile.
- Use a thermocycler with heated lid and ensure that it works properly.
- It might help to dilute the DNA sample. Contaminants that are present in the sample are diluted as well, thereby reducing their effect. Next to this, an extra purification step possibly helps to improve the quality of the samples, which might lead to better results.

Signal quality – Internal signal widening

Background:

Like signal widening of the size marker, signal widening of MLPA probes is introduced during capillary electrophoresis. It is therefore seen in the peak patterns of the size marker and the MLPA probes. The phenomenon of signal widening appears in the electropherogram as peaks being broader at their base and less sharp than usual.

Conditions:

		Notification	Penalty
✓	Signal widening lower than 50%, but no sloping	Ok	No penalty
!	Signal widening above 50%, but no sloping	Warning	No penalty
!	Signal widening above 50%, and sloping between 40% and 60%	Warning	15 points
!	Signal widening above 50% *, and sloping between 60% and 70%	Warning	35 points
!	Signal widening above 50% *, and sloping above 70%	Warning	60 points

*When signal widening is more than 80%, the notification will be 'Bad'. However, the penalty will not change

Related issues and solutions:

Sometimes signal widening occurs randomly and by rerunning the samples this problem may be solved. In case it persists and/or when it is accompanied by signal sloping, check the capillary electrophoresis instrument for flaws and check the age of the capillary array and polymer. These may have to be replaced.

Appendix III - Sheet library

For reliable analysis and result interpretation, it is important that peaks in raw run data are properly recognised as signals coming from MLPA probes and fragments. Coffalyser.Net uses so-called Coffalyser sheets for this process. A Coffalyser sheet contains all necessary information that is specific for one lot of a probemix. Coffalyser sheets are stored in the sheet library.

The sheet library in Coffalyser.Net consists of two sections: a hidden and an active one. After updating the library, all available Coffalyser sheets are stored in the hidden section. Before you can analyse your data, you have to add the Coffalyser sheet to the active section. The reason for this setup is that more than 400 probemixes are available with numerous lots. As the active section only holds the Coffalyser sheets that you have added, it is easier to find the correct sheet for your experiment. In addition, it is possible to make adjustments to the sheets in the active section (e.g. adding synthetic probes to the sheet), which are not saved in the original Coffalyser sheet in the hidden section. It is therefore possible to add the original Coffalyser sheet, as provided by MRC-Holland, to the active section of the library again.

Update of the sheet library

When a new lot of a probemix or a completely new probemix is released, its corresponding Coffalyser sheet is made available for downloading. Coffalyser sheets are also sometimes updated, for example to add product notifications. In order to get these new or modified Coffalyser sheets, you'll need to update the sheet library.

In Coffalyser.Net a sheet library update function is incorporated, which downloads all available Coffalyser sheets from the MRC-Holland server. See the section **Procedure: Update sheet library (Internet download**) for instructions. It is also possible to import the sheet library file into the software manually. This is predominantly useful when Coffalyser.Net is installed on a computer that is not connected to the internet. The section **Procedure: Update sheet library (Import from file**) describes how to do this. It is important to update the sheet library regularly. The software will notify you when the sheet library has not been updated for more than 7 days.

NOTE: The update of the sheet library will only update the Coffalyser sheets in the hidden section of the sheet library. The sheets in the active section remain unaffected.

NOTE: The software gives a notification when the update of the sheet library has successfully been completed. This notification includes the number of updated items. In case it states that 0 items were updated, this indicates that the library was already up to date.

PROCEDURE: UPDATE SHEET LIBRARY (INTERNET DOWNLOAD)

1.	Right click on *Sheet Library*	Organisations / MRC-Holland / Users / Sheet Library — Open / Update (Internet Download) / Update (Import From File)
2.	Select Update (Internet Download)	
	The Download Updates (MRC-Holland) window opens	
3.	Click **Start Update**	Start Update
4.	In the Internet Permission window click **Yes** or **Always**	Yes / Always

	Wait for the update process to complete	
5.	Click **Close** to close the Download Updates (MRC-Holland) window	Close

Procedure: Update sheet library (Import from file)

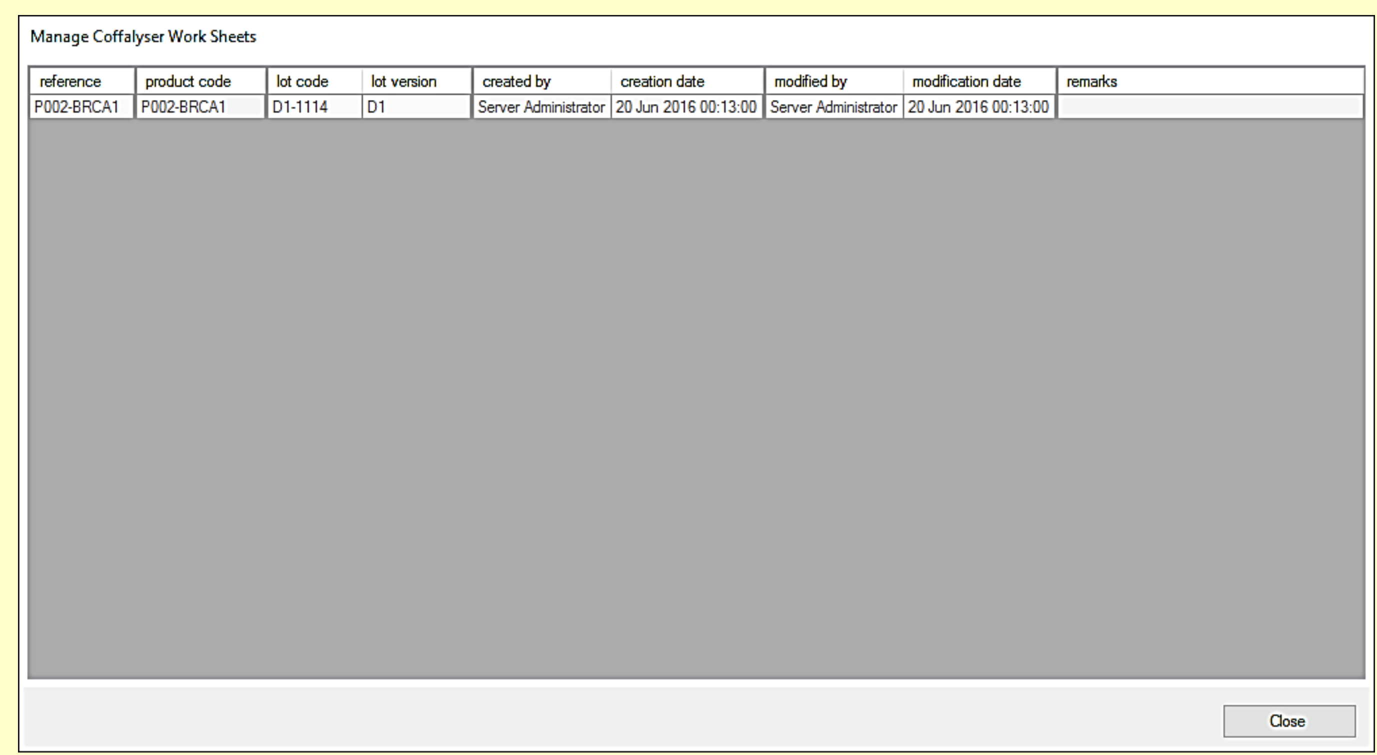
1.	Log in to your MyMLPA account on www.mlpa.com	My MLPA - Login
2.	Navigate to the section MY COFFALYSER.NET	
3.	Click on Coffalyser.Net – sheet library update and save the file	Coffalyser.Net - sheet library update
4.	Copy the sheet library file onto a USB drive	
5.	Take the USB drive and plug it into the computer on which Coffalyser.Net is installed	
6.	In Coffalyser.Net right click on *Sheet Library*	
7.	Select Update (Import From File)	Update (Import From File)
	The Download Updates (MRC-Holland) window opens	
8.	Click **Start Update**	Start Update
	A dialog box opens	
9.	Navigate to the location on the USB drive where the sheet library file is saved	
10.	Select the file and click **Open**	Open

11.	Click **Close** to close the Download Updates (MRC-Holland) window	Close

Manage the sheet library

Coffalyser sheets can be managed in the active section of the sheet library: they can be added, deleted and edited. This allows users to modify the sheet library and its contents to their needs. Coffalyser.Net also keeps track of the creation and modification dates of Coffalyser sheets and by which user this was done, which is especially useful in a multiuser environment.

Box 1: Manage Coffalyser Work Sheet window



Reference column
The reference name of the probemix is displayed here.

Product code column
The product code of the probemix is displayed here.

Lot code column
The lot number of the probemix is displayed here.

Lot version column
The version number of the probemix is displayed here.

Created by column
The name of the user who created the Coffalyser sheet is displayed here.

Creation date column
The date and time the Coffalyser sheet was created is displayed here.

Modified by column
The name of the user who last modified the Coffalyser sheet is displayed here.

Modification date column
The date and time the Coffalyser sheet was last modified is displayed here.

Remarks column
Remarks made in the Coffalyser sheet are displayed here.

Close button
Closes the Manage Coffalyser Work Sheets window.

Add Coffalyser sheets to the sheet library

There are two sources from which Coffalyser sheets can be added to the active section of the sheet library.

1. The hidden section of the sheet library. This is the most common and recommended option. It requires that the sheet library is up to date.

2. A Coffalyser sheet file. This is useful when you want to share a (modified) Coffalyser sheet between two computers that are not in the same network.

Coffalyser.Net also allows you to add empty Coffalyser sheets (or blanc templates) to the active section of the sheet library. This is useful when you have a custom probemix that is not based on a probemix from MRC-Holland. All probe-related information has to be added manually to the Coffalyser sheet before you can analyse data.

PROCEDURE: ADD A COFFALYSER SHEET FROM THE HIDDEN SECTION (CREATE A WORK SHEET BASED ON A MRC COFFALYSER SHEET)

Step	Action	Screenshot
1.	Right click on *Sheet Library*	Organisations / MRC-Holland / Users / Sheet Library
2.	Select *Open*	Open / Update (Internet Download) / Update (Import From File)
	The Manage Coffalyser Work Sheets window opens	
3.	Right click in the window and select *Add*	Add Ctrl+Ins / Open Ctrl+Space / Delete Ctrl+Del
	The Add Coffalyser Work Sheet form appears	
4.	Select create a work sheet based on a MRC Coffalyser sheet	create an empty work sheet / create a work sheet from file / create a work sheet based on a MRC Coffalyser sheet
5.	Select the appropriate probemix from the product drop-down menu	product P028-FHL; lot; P026-Sotos / P027-Uveal melanoma / P028-FHL / P029-WBS / P030-RYR1
6.	Select the appropriate lot number from the lot drop-down menu	product P028-FHL; lot <select a lot>; <select a lot> / LOT0611 (A1) / B1-050215d (B1) / B1-1215 (B1)
7.	Click **OK**	OK

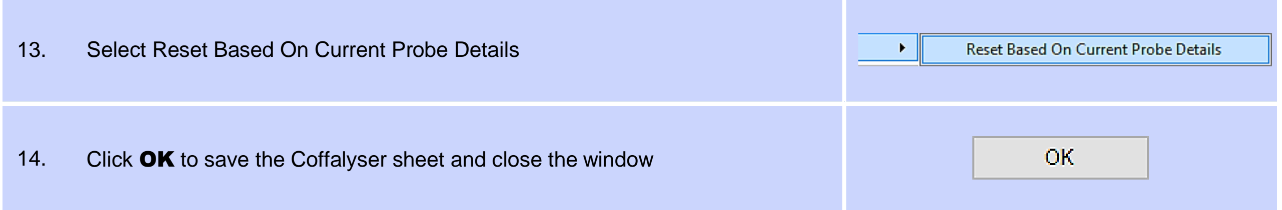
	The Coffalyser Work Sheet Editor window opens	
8.	Click **OK** to save the Coffalyser sheet and close the Coffalyser Work Sheet Editor window	OK

Procedure: Import a Coffalyser sheet file (create a work sheet from file)

1.	Right click on *Sheet Library*	Organisations MRC-Holland Users Sheet Library Open Update (Internet Download) Update (Import From File)
2.	Select *Open*	
	The Manage Coffalyser Work Sheets window opens	
3.	Right click in the window and select *Add*	Add Ctrl+Ins Open Ctrl+Space Delete Ctrl+Del
	The Add Coffalyser Work Sheet form appears	
4.	Select create a work sheet from file	create an empty work sheet create a work sheet from file create a work sheet based on a MRC Coffalyser sheet
5.	Click **OK**	OK
	A dialog box opens	
6.	Navigate to the location where the Coffalyser sheet file is stored	
7.	Select the Coffalyser sheet file and click **Open**	Open
	The Coffalyser Work Sheet Editor window opens	
8.	Click **OK** to save the Coffalyser sheet and close the Coffalyser Work Sheet Editor window	OK

PROCEDURE: ADD AN EMPTY COFFALYSER SHEET

1.	Right click on *Sheet Library*	Organisations MRC-Holland Users Sheet Library Open Update (Internet Download) Update (Import From File)
2.	Select *Open*	
	The Manage Coffalyser Work Sheets window opens	
3.	Right click in the window and select *Add*	Add Ctrl+Ins Open Ctrl+Space Delete Ctrl+Del
4.	Select create an empty work sheet	create an empty work sheet create a work sheet from file create a work sheet based on a MRC Coffalyser sheet
5.	Click **OK**	OK
	The Coffalyser Work Sheet Editor window opens	
6.	Navigate to the tab DETAILS	details | probes | MRC-Holland product notifications
7.	Enter the required information in the designated fields	
8.	Navigate to the tab PROBES	details | probes | MRC-Holland product notifications
9.	Right click in the window and select *Add*	Add Delete Ctrl+Del Manual Bin Set Bounds Replace Control Fragments With Probe Report Level Probe Order 1 Probe Ctrl+Ins 3 Probes 5 Probes 10 Probes 25 Probes 40 Probes
10.	Select the desired number of probes you want to add	
11.	Enter the required information in the applicable fields (see Appendix IV - Coffalyser sheets)	
12.	Right click in the window and select *Probe Order*	Probe Report Level Probe Order Copy To Clipboard



13.	Select Reset Based On Current Probe Details	Reset Based On Current Probe Details
14.	Click **OK** to save the Coffalyser sheet and close the window	OK

Delete Coffalyser sheets from the sheet library

Coffalyser sheets can be deleted from the sheet library via the designated function. However, this is only possible for Coffalyser sheets that are not linked to an experiment. If you want to delete a Coffalyser sheet that is linked to an experiment, it is necessary to delete the experiment first.

PROCEDURE: DELETE A COFFALYSER SHEET

1.	Right click on *Sheet Library*	Organisations MRC-Holland Users Sheet Library Open Update (Internet Download) Update (Import From File)
2.	Select *Open*	
	The Manage Coffalyser Work Sheets window opens	
3.	Right click on the Coffalyser sheet you wish to delete and select *Delete*	Add Ctrl+Ins Open Ctrl+Space Delete Ctrl+Del
4.	Click **Yes** to confirm you want to delete the Coffalyser sheet	Yes

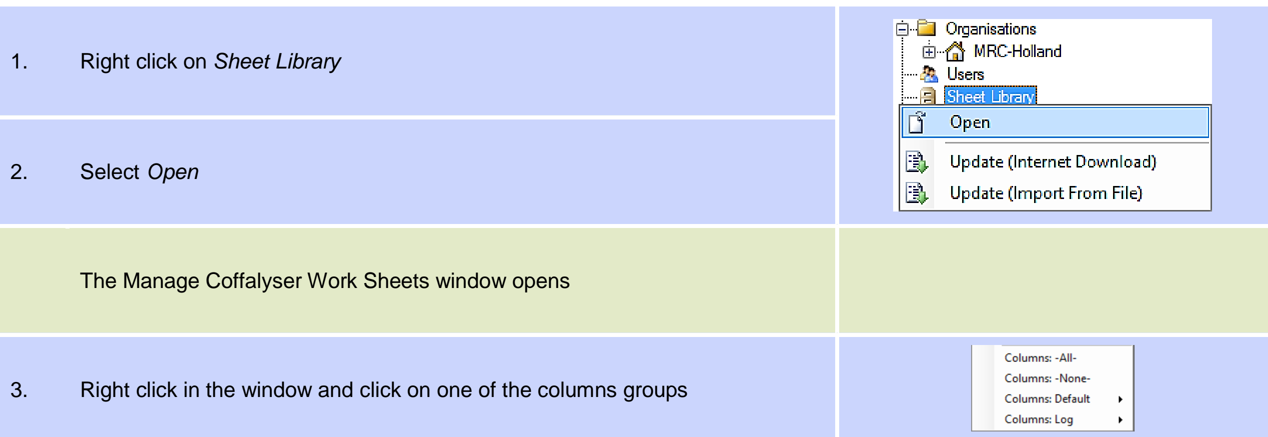
Display or hide columns in the sheet library

Coffalyser.Net allows you to select which columns are displayed in the sheet library. It is possible to display or hide multiple columns at once by selecting a group or select individual columns from these groups.

Four groups exist:

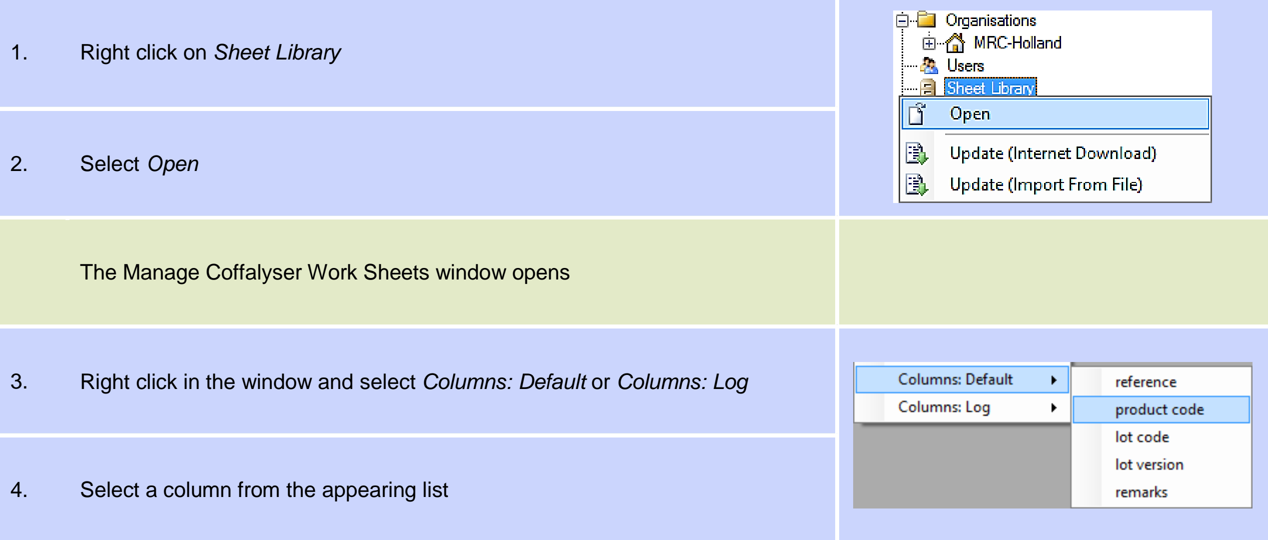
- **Columns: -All-**: Displays all columns.
- **Columns: -None-**: Hides all columns.
- **Columns: Default**: Displays the default columns reference, product code, lot code, lot version and remarks.
- **Columns: Log**: Displays the log columns created by, creation date, modified by and modification date.

PROCEDURE: DISPLAY OR HIDE MULTIPLE COLUMNS AT ONCE



Step	Action
1.	Right click on *Sheet Library*
2.	Select *Open*
	The Manage Coffalyser Work Sheets window opens
3.	Right click in the window and click on one of the columns groups

PROCEDURE: DISPLAY OR HIDE A SINGLE COLUMN

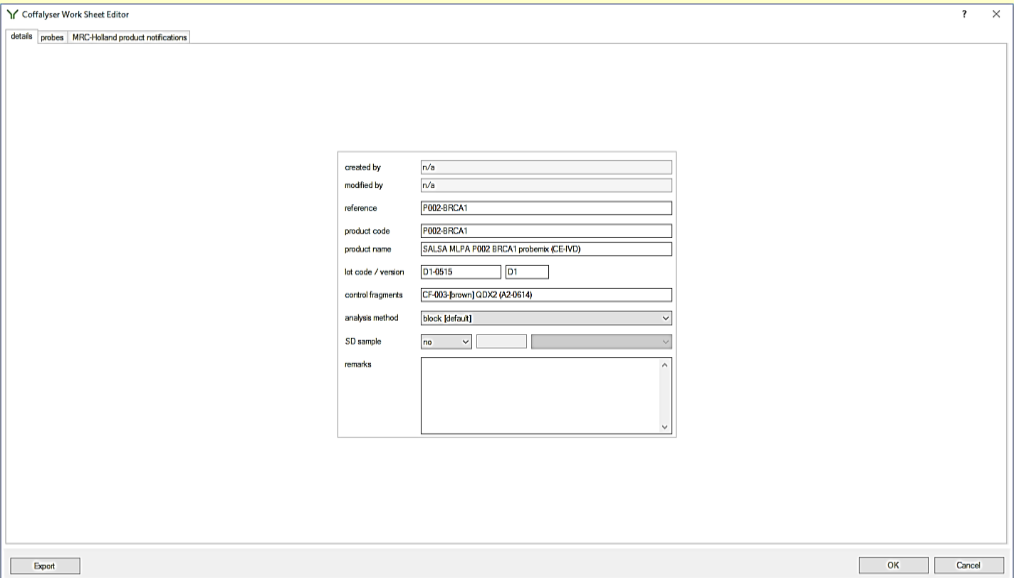


Step	Action
1.	Right click on *Sheet Library*
2.	Select *Open*
	The Manage Coffalyser Work Sheets window opens
3.	Right click in the window and select *Columns: Default* or *Columns: Log*
4.	Select a column from the appearing list

Appendix IV - Coffalyser sheets

A Coffalyser sheet contains information of all probes that have been included in the corresponding probemix. All relevant and necessary information of a probemix is stored in the three tabs of a Coffalyser sheet.

Box 2: Coffalyser Work Sheet Editor window – details tab



Created by
The creation date + time and the name of the user who created the Coffalyser sheet are displayed here.

Modified by
The modification date + time and the name of the user who last modified the Coffalyser sheet are displayed here.

Reference
The reference name of the probemix is displayed here.

Product code
The product code of the probemix is displayed here.

Lot code / version
The lot and version number of the probemix are displayed here.

Control fragments
The set of control fragments included in the probemix is displayed here.

Analysis method
The analysis method is displayed here.

SD sample
It is indicated whether or not a SD sample is available for this probemix. If so, the number of this SD sample is displayed in the adjacent text field and the intended purpose (Binning only or Binning & reference sample) is displayed in the drop-down menu.

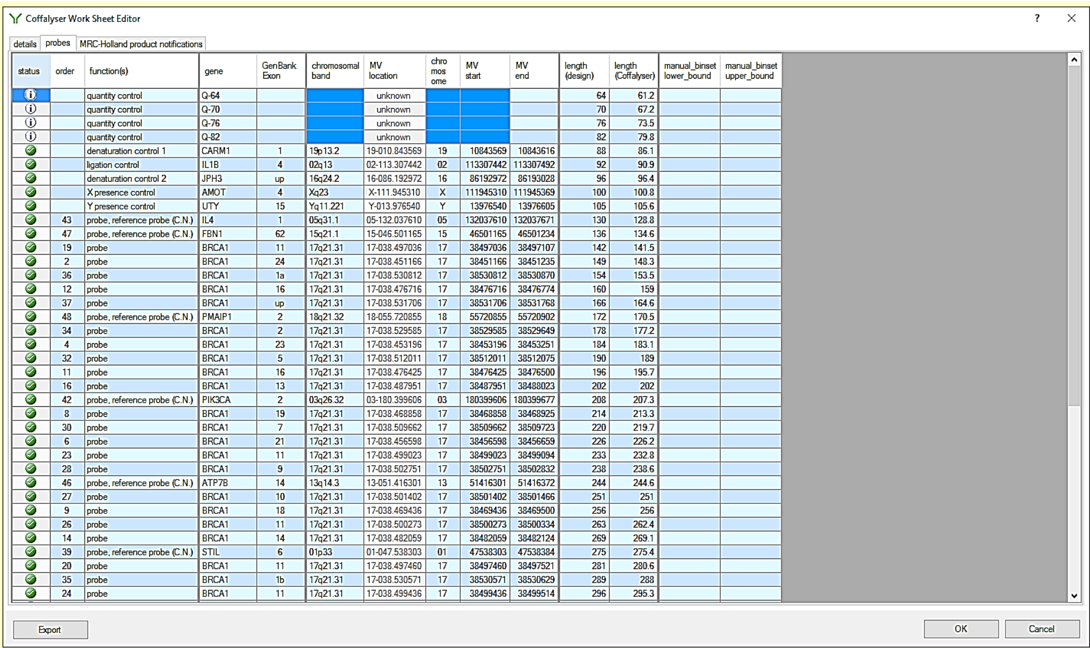
Remarks
Remarks about this worksheet or probemix can be entered in the text field.

Export button
Exports the Coffalyser sheet as .bin file.

OK button
Saves (changes in) the worksheet and closes the Coffalyser Work Sheet editor.

Cancel button
Closes the Coffalyser Work Sheet editor without saving changes.

Box 3: Coffalyser Work Sheet Editor window – probes tab



status	order	function(s)	gene	GenBank Exon	chromosomal band	MV location	chromosome	MV start	MV end	length (design)	length (Coffalyser)	manual_binset lower_bound	manual_binset upper_bound
		quantity control	Q-64			unknown				64	61.2		
		quantity control	Q-70			unknown				70	67.2		
		quantity control	Q-76			unknown				76	73.5		
		quantity control	Q-82			unknown				82	79.8		
		denaturation control 1	CARM1	1	19p13.2	19-010.843569	19	10843569	10843616	88	86.1		
		ligation control	IL1B	4	02q13	02-113.307442	02	113307442	113307492	92	90.9		
		denaturation control 2	JPH3	up	16q24.2	16-086.192972	16	86192972	86193028	96	96.4		
		X presence control	AMOT	4	Xq23	X-111.945310	X	111945310	111945369	100	100.8		
		Y presence control	UTY	15	Yq11.221	Y-013.976540	Y	13976540	13976605	105	105.6		
	43	probe, reference probe (C.N.)	IL4	1	05q31.1	05-132.037610	05	132037610	132037671	130	128.8		
	47	probe, reference probe (C.N.)	FBN1	62	15q21.1	15-046.501165	15	46501165	46501234	136	134.6		
	19	probe	BRCA1	11	17q21.31	17-038.497036	17	38497036	38497107	142	141.5		
	2	probe	BRCA1	24	17q21.31	17-038.451166	17	38451166	38451235	149	148.3		
	36	probe	BRCA1	1a	17q21.31	17-038.530812	17	38530812	38530870	154	153.5		
	12	probe	BRCA1	16	17q21.31	17-038.476716	17	38476716	38476774	160	159		
	37	probe	BRCA1	up	17q21.31	17-038.531706	17	38531706	38531768	166	164.6		
	48	probe, reference probe (C.N.)	PMAIP1	2	18q21.32	18-055.720855	18	55720855	55720902	172	170.5		
	34	probe	BRCA1	2	17q21.31	17-038.529585	17	38529585	38529649	178	177.2		
	4	probe	BRCA1	23	17q21.31	17-038.453196	17	38453196	38453251	184	183.1		
	32	probe	BRCA1	5	17q21.31	17-038.512011	17	38512011	38512075	190	189		
	11	probe	BRCA1	16	17q21.31	17-038.476425	17	38476425	38476500	196	195.7		
	16	probe	BRCA1	13	17q21.31	17-038.487951	17	38487951	38488023	202	202		
	42	probe, reference probe (C.N.)	PIK3CA	2	03q26.32	03-180.399606	03	180399606	180399677	208	207.3		
	8	probe	BRCA1	19	17q21.31	17-038.468858	17	38468858	38468925	214	213.3		
	30	probe	BRCA1	7	17q21.31	17-038.509662	17	38509662	38509723	220	219.7		
	6	probe	BRCA1	21	17q21.31	17-038.456598	17	38456598	38456659	226	226.2		
	23	probe	BRCA1	11	17q21.31	17-038.499023	17	38499023	38499094	233	232.8		
	28	probe	BRCA1	9	17q21.31	17-038.502751	17	38502751	38502832	238	238.6		
	46	probe, reference probe (C.N.)	ATP7B	14	13q14.3	13-051.416301	13	51416301	51416372	244	244.6		
	27	probe	BRCA1	10	17q21.31	17-038.501402	17	38501402	38501466	251	251		
	9	probe	BRCA1	18	17q21.31	17-038.469436	17	38469436	38469500	256	256		
	26	probe	BRCA1	11	17q21.31	17-038.500273	17	38500273	38500334	263	262.4		
	14	probe	BRCA1	14	17q21.31	17-038.482059	17	38482059	38482124	269	269.1		
	39	probe, reference probe (C.N.)	STIL	6	01p33	01-047.538303	01	47538303	47538384	275	275.4		
	20	probe	BRCA1	11	17q21.31	17-038.497460	17	38497460	38497521	281	280.6		
	35	probe	BRCA1	1b	17q21.31	17-038.530571	17	38530571	38530629	289	288		
	24	probe	BRCA1	11	17q21.31	17-038.499436	17	38499436	38499514	296	295.3		

Status
Displays if all relevant probe information is entered correctly.

Order
Displays the number on which probes are ordered in screens and reports.

Function(s)
Displays the function(s) of a probe in the probemix.

Gene
Displays the gene to which the probe is targeted.

GenBank Exon
Displays the exon of the gene to which the probe is targeted.

Chromosomal band
Displays the chromosomal band of the target sequence of the probe.

MV location
Displays the location of the target sequence of the probe based on NCBI Map Viewer HG18.

Chromosome
Displays the chromosome on which the target sequence of the probe is located.

MV start
Displays the start location of the target sequence of the probe based on NCBI Map Viewer HG18.

MV end
Displays the end location of the target sequence of the probe based on NCBI Map Viewer HG18.

Length (design)
Displays the length on which the probe has been designed.

Length (Coffalyser)
Displays the length of the probe as found by MRC-Holland.

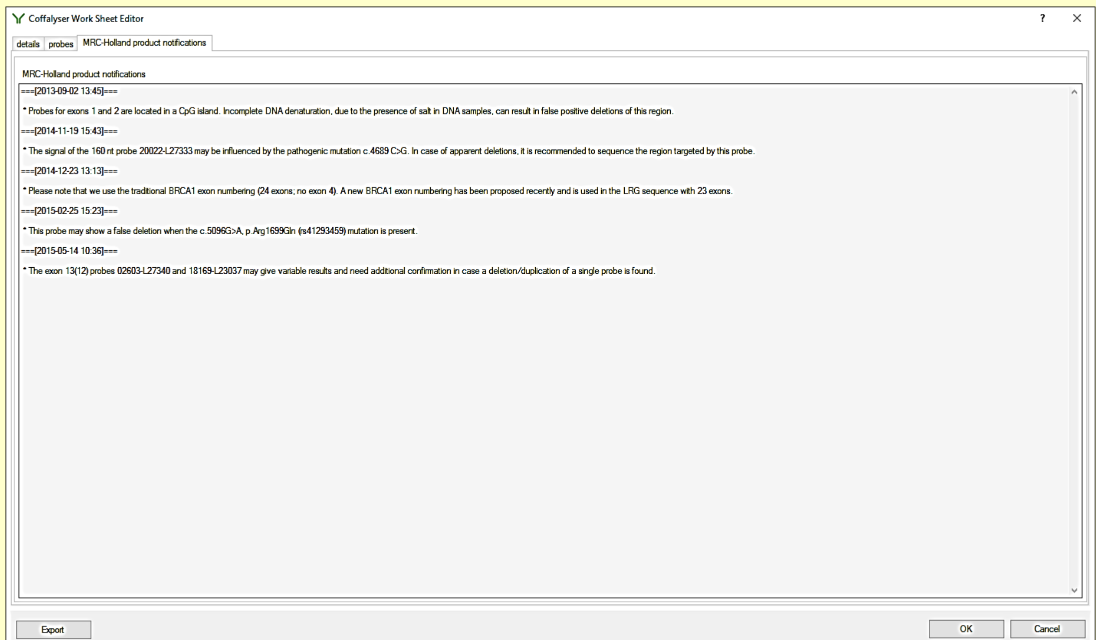
Manual_binset_lower_bound
Displays the lower boundary of the probe's bin.

Manual_binset_upper_bound
Displays the upper boundary of the probe's bin.

OK button
Saves (changes in) the Coffalyser sheet and closes the Coffalyser Work Sheet editor.

Cancel button
Closes the Coffalyser Work Sheet editor without saving changes.

Box 4: Coffalyser Work Sheet Editor window – MRC-Holland product notifications tab



MRC-Holland product notifications
All notifications about the product and/or probes are displayed here.

Export button
Exports the Coffalyser sheet as .bin file.

OK button
Saves (changes in) the Coffalyser sheet and closes the Coffalyser Work Sheet editor.

Cancel button
Closes the Coffalyser Work Sheet editor without saving changes.

Display or hide columns in a Coffalyser sheet

When a Coffalyser sheet is opened, Coffalyser.Net only shows a subset of columns. However, it allows you to select other columns to be displayed as well. It is possible to display multiple columns at once by selecting a group or by selecting individual columns from these groups. Columns can be hidden in a similar fashion. In Table 6 to Table 9 all columns are presented per group, together with a description. In addition, it is noted whether information in a column is mandatory.

Table 6. Columns: default

Name	Description	Mandatory: Yes / No
Status	Displays if all relevant probe information is entered correctly.	N/A – Is automatically displayed
Order	Displays the number on which probes are ordered in screens and reports.	Yes
Function(s)	Displays the function(s) of a probe in the probemix.	Yes
Gene	Displays the gene to which the probe is targeted.	Yes
GenBank Exon	Displays the exon of the gene to which the probe is targeted.	No
Chromosomal band	Displays the chromosomal band of the target sequence of the probe.	Yes
MV location	Displays the location of the target sequence of the probe based on NCBI Map Viewer HG18.	Yes
Chromosome	Displays the chromosome on which the target sequence of the probe is located.	Yes
MV start	Displays the start location of the target sequence of the probe based on NCBI Map Viewer HG18.	Yes
MV end	Displays the end location of the target sequence of the probe based on NCBI Map Viewer HG18.	Yes
Length (design)	Displays the length on which the probe has been designed.	Yes
Length (Coffalyser)	Displays the length of the probe as found by MRC-Holland.	Yes
Manual_binset_lower_bound	Displays the lower boundary of the probe’s bin.	Yes
Manual_binset_upper_bound	Displays the upper boundary of the probe’s bin.	Yes

Table 7. Columns: Advanced

Name	Description	Mandatory: Yes / No
Report	Displays the manner how probes are labelled in screens and reports.	Yes
Copy number variable	Indicates whether copy number variants exist in healthy individuals.	No
Copy number (normal)	Displays the number of copies in healthy individuals.	No
Copy number (SD_sample)	Displays the number of copies in the SD sample (when applicable).	No
Probe_weight (target)	Displays the weight of the probe in the slope correction procedure.	No – Not functional yet
Probe_weight (copy_number)	Displays the weight of the probe in the normalisation procedure.	No – Not functional yet

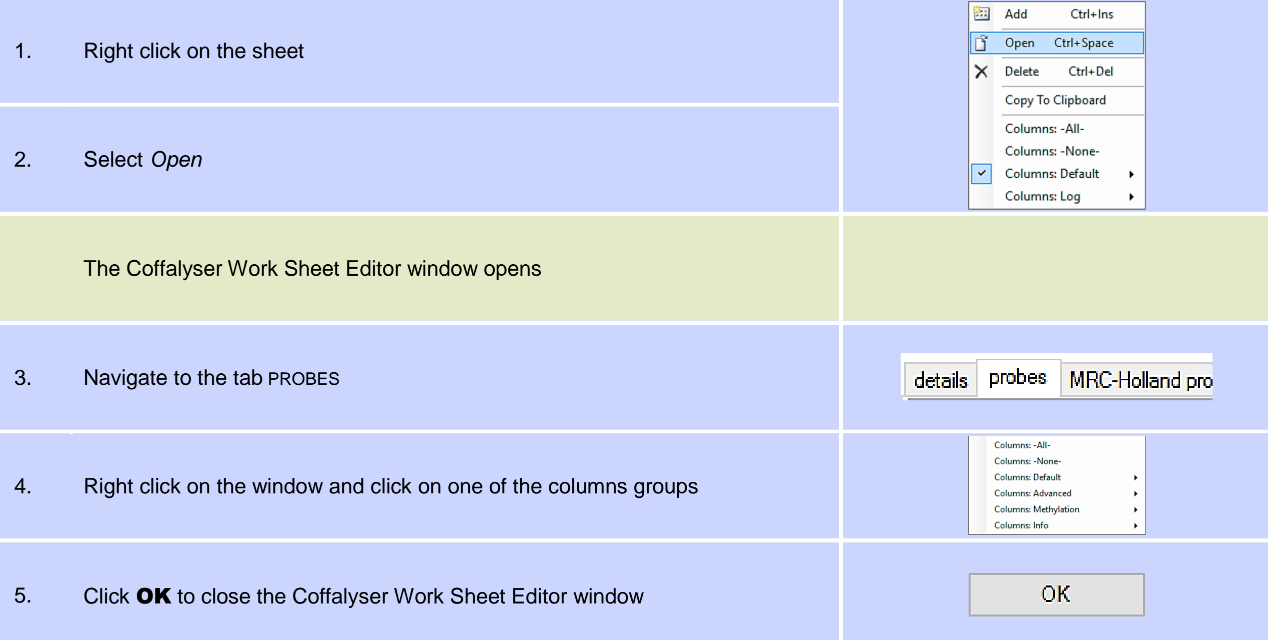
Table 8. Columns: Methylation

Name	Description	Mandatory: Yes / No
Normal methylation % (male)	Displays the methylation percentage of the probe in healthy males.	No – Only mandatory when a value is present in the Normal Methylation % (female) field
Normal methylation % (female)	Displays the methylation percentage of the probe in healthy females.	No – Only mandatory when a value is present in the Normal Methylation % (male) field
HHA1	Indicates the presence of one or more HhaI restriction sites in the hybridizing sequence of the probe.	No
HPA2	Indicates the presence of one or more HpaII restriction sites in the hybridizing sequence of the probe.	No

Table 9. Columns: Info

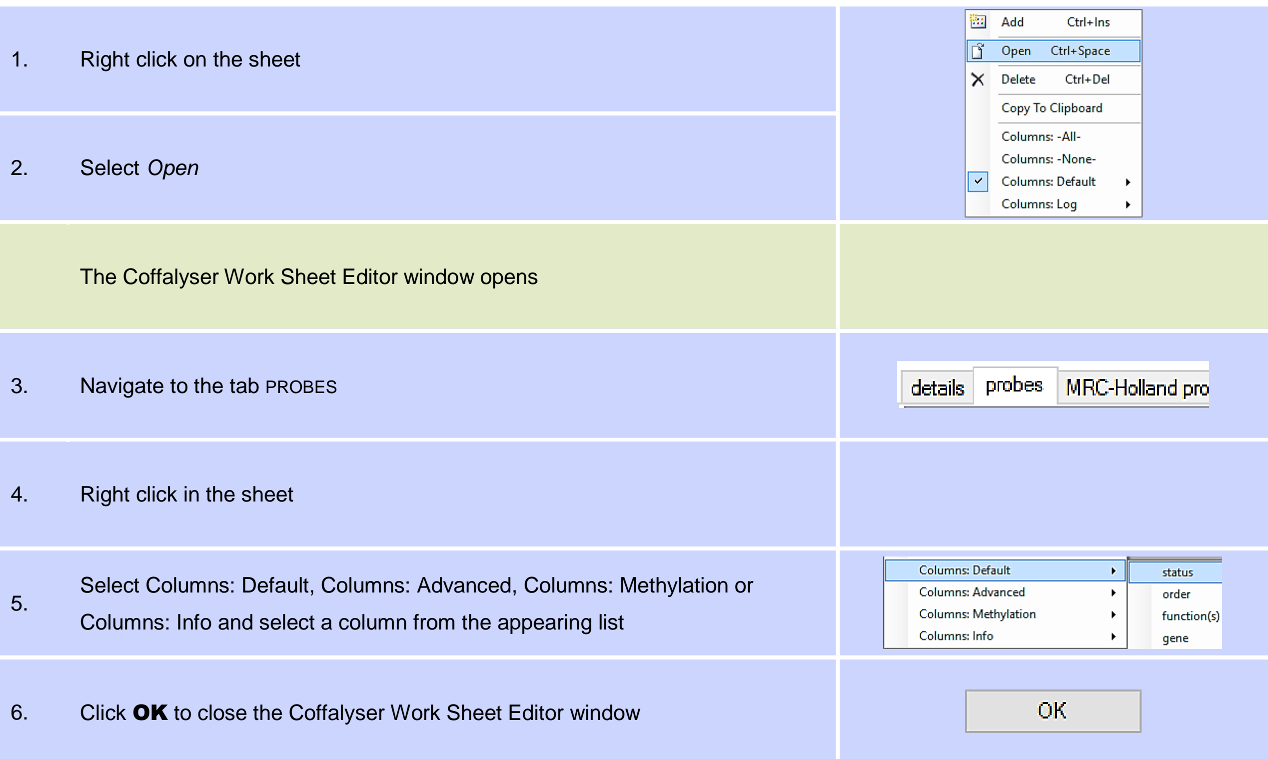
Name	Description	Mandatory: Yes / No
Probe number	Displays the probe number.	N/A – This is the MRC-Holland's probe number
GenBank info	Displays the Genbank accession number of the sequence used for probe design. It is usually followed by the location of the ligation site in this sequence.	No
Position	Displays whether the target sequence of the probe is located in the exon, intron or both.	No
Direction	Displays whether the probe targets the leading or lagging strand	No
Mutation details	Displays the details of the mutation targeted by a mutation specific probe.	Yes – Only for mutation specific probes

PROCEDURE: DISPLAY OR HIDE MULTIPLE COLUMNS AT ONCE



1.	Right click on the sheet
2.	Select *Open*
	The Coffalyser Work Sheet Editor window opens
3.	Navigate to the tab PROBES
4.	Right click on the window and click on one of the columns groups
5.	Click **OK** to close the Coffalyser Work Sheet Editor window

PROCEDURE: DISPLAY OR HIDE A SINGLE COLUMN



1.	Right click on the sheet
2.	Select *Open*
	The Coffalyser Work Sheet Editor window opens
3.	Navigate to the tab PROBES
4.	Right click in the sheet
5.	Select Columns: Default, Columns: Advanced, Columns: Methylation or Columns: Info and select a column from the appearing list
6.	Click **OK** to close the Coffalyser Work Sheet Editor window

Edit Coffalyser sheets

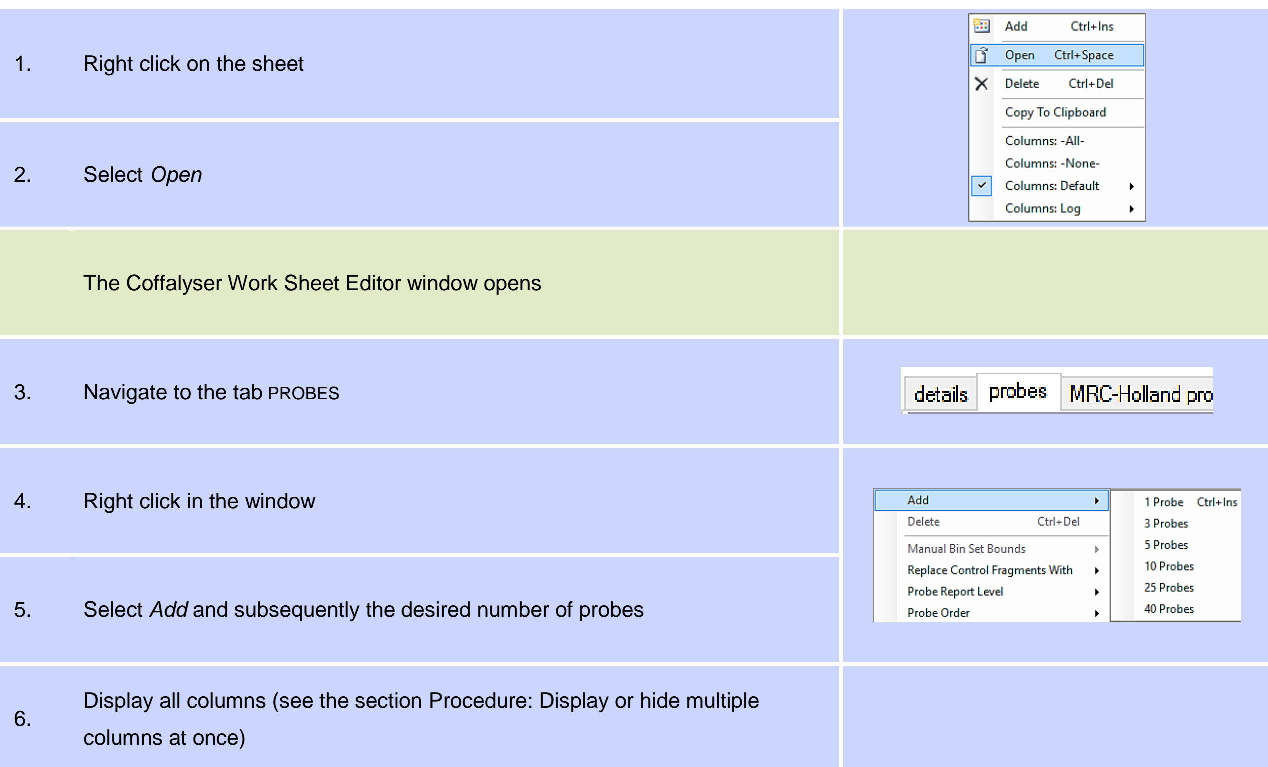
Coffalyser sheets can be modified to a great extent. It is for instance possible to add extra information to probes and to change the way probes are displayed in the screens and reports. The paragraphs in this section deal with all functions that can be used to adjust Coffalyser sheets.

> IMPORTANT NOTE:
>
> Coffalyser sheets should not be edited in a diagnostic setting. Changes made to a Coffalyser sheet will have an effect on the analysis results!

Add and delete probes in a worksheet

Coffalyser.Net allows you to add and remove probes from a Coffalyser sheet. These functions are predominantly useful when you have a customised MLPA probemix.

PROCEDURE: ADD PROBES TO A COFFALYSER SHEET



Step	Action
1.	Right click on the sheet
2.	Select *Open*
	The Coffalyser Work Sheet Editor window opens
3.	Navigate to the tab PROBES
4.	Right click in the window
5.	Select *Add* and subsequently the desired number of probes
6.	Display all columns (see the section Procedure: Display or hide multiple columns at once)

7.	Enter the relevant information in the designated fields	
8.	Right click in the window	
9.	Select Probe Order and subsequently Reset Based On Current Probe Details	Probe Order ▸ Reset Based On Current Probe Details
10.	Click **OK** to close the Coffalyser Work Sheet Editor window	OK

PROCEDURE: DELETE A PROBE FROM A COFFALYSER SHEET

1.	Right click on the sheet	Add Ctrl+Ins Open Ctrl+Space Delete Ctrl+Del Copy To Clipboard Columns: -All- Columns: -None- Columns: Default Columns: Log
2.	Select *Open*	
	The Coffalyser Work Sheet Editor window opens	
3.	Navigate to the tab PROBES	details probes MRC-Holland pro
4.	Right click on the probe you wish to delete and select *Delete*	Add Delete Ctrl+Del Manual Bin Set Bounds Replace Control Fragments With
5.	Click **Yes** to confirm you want to delete the probe	Yes
6.	Right click in the window	
7.	Select Probe Order and subsequently Reset Based On Current Probe Details	Probe Order ▸ Reset Based On Current Probe Details
8.	Click **OK** to close the Coffalyser Work Sheet Editor window	OK

Control fragments in a Coffalyser sheet

It is possible to replace the set of control fragments in a Coffalyser sheet by another set. This function is not intended to be used with probemixes sold by MRC-Holland, but only with self-designed probemixes.

Procedure: Change control fragment set in a Coffalyser sheet

1.	Right click on the sheet	Add Ctrl+Ins / Open Ctrl+Space / Delete Ctrl+Del / Copy To Clipboard / Columns: -All- / Columns: -None- / Columns: Default / Columns: Log
2.	Select *Open*	
	The Coffalyser Work Sheet Editor window opens	
3.	Navigate to the tab PROBES	details / probes / MRC-Holland pro
4.	Right click in the window and select *Replace Control Fragments with*	Manual Bin Set Bounds / Replace Control Fragments With / Probe Report Level
5.	Select the appropriate set of control fragments and lot number	CF001-[orange] Q92 (Q92) / CF002-[pink] QD old (QD) / CF003-[brown] QDX2 / CF004-(pink) QD2 (new) / CF005-[purple] DNA n-h (QD-NH) / CF006-[white] RNA human (QR); A1-0612 / A2-0113 / A2-0513 / A2-0614
6.	Click **Yes** to confirm you want to replace the control fragment set	Yes
7.	Right click in the window	Probe Order / Reset Based On Current Probe Details
8.	Select Probe Order and subsequently Reset Based On Current Probe Details	Probe Order / Reset Based On Current Probe Details
9.	Click **OK** to close the Coffalyser Work Sheet Editor window	OK

Probe report levels

It is possible to change the probe identities in screens and reports. For instance, the name of the gene targeted by a probe or the chromosomal position can be displayed. This is done via the Probe report level functionality in the Coffalyser sheet. Table 10 lists all possible probe report levels together with a description of each one of them.

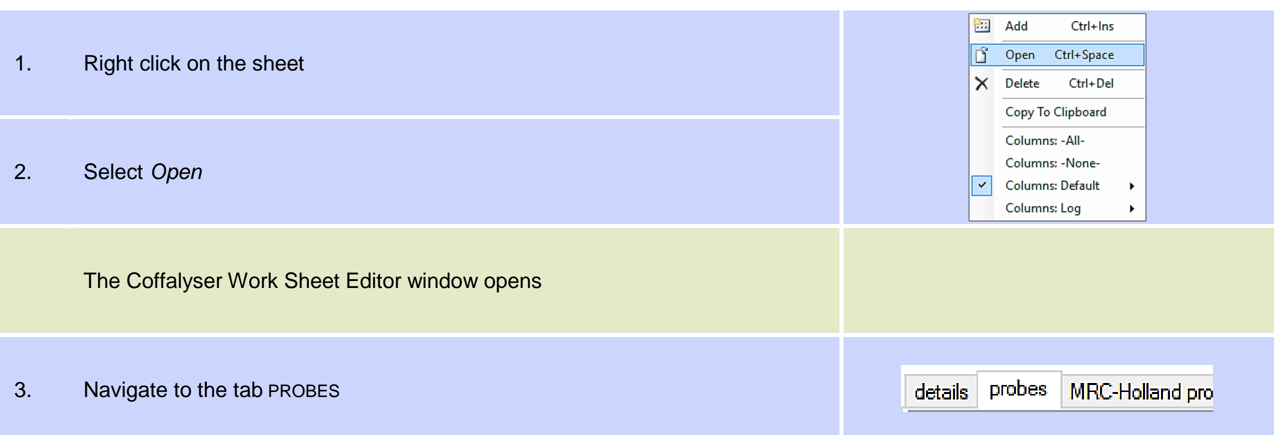
Table 10. Probe report level

Probe report level	Result in screens and reports
Exclude from report	Probes are not visible*.
Anonymous	The role of the probe is displayed (i.e. Reference or Target).
Chromosomal position	The chromosomal position of the target location of the probe is displayed.
Gene name	The name of the gene that is targeted by the probe is displayed.
Gene name + Exon	The name of the gene and the exon number that is targeted by the probe is displayed.

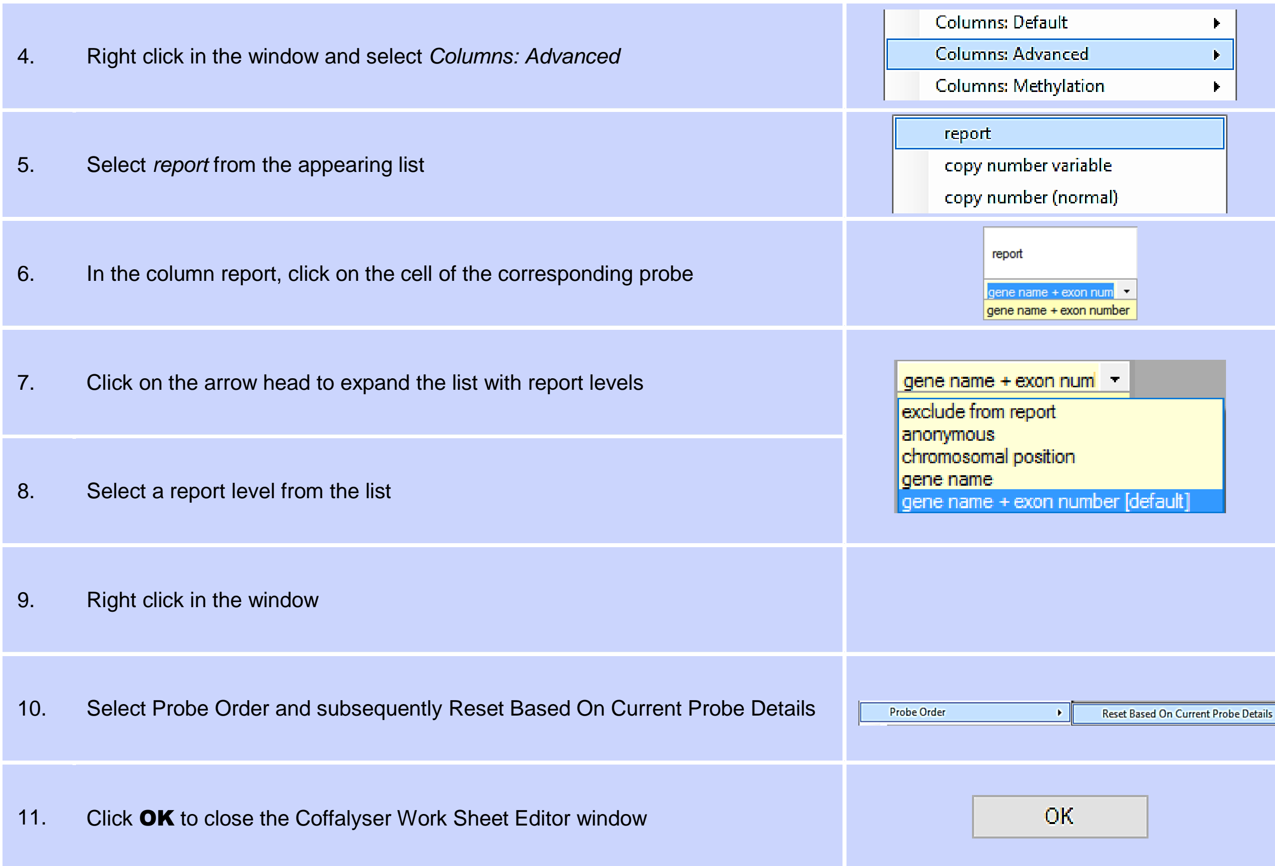
*Probes that are excluded from the report are not visible, but they are included in the analysis.

The probe report levels can be adjusted for each probe separately, but also for a subset of probes. The following subsets exist in Coffalyser.Net: 1. All probes, 2. Control fragments, 3. Non-reference Probes, 4. Reference Probes (Any Type), 5. Reference Probes (Copy Number), 6. Reference Probes (Methylation).

PROCEDURE: CHANGE THE PROBE REPORT LEVEL OF INDIVIDUAL PROBES



1.	Right click on the sheet	
2.	Select *Open*	
	The Coffalyser Work Sheet Editor window opens	
3.	Navigate to the tab PROBES	



4.	Right click in the window and select *Columns: Advanced*
5.	Select *report* from the appearing list
6.	In the column report, click on the cell of the corresponding probe
7.	Click on the arrow head to expand the list with report levels
8.	Select a report level from the list
9.	Right click in the window
10.	Select Probe Order and subsequently Reset Based On Current Probe Details
11.	Click **OK** to close the Coffalyser Work Sheet Editor window

Procedure: Change the probe report level of a subset of probes

1.	Right click on the sheet
2.	Select *Open*
	The Coffalyser Work Sheet Editor window opens
3.	Navigate to the tab PROBES
4.	Right click in the window and select *Probe Report Level*
5.	Select a probe category from the appearing list

6.	Select a report level from the appearing list	exclude from report anonymous chromosomal position gene name gene name + exon number
7.	Right click in the window	
8.	Select Probe Order and subsequently Reset Based On Current Probe Details	Probe Order ▸ Reset Based On Current Probe Details
9.	Click **OK** to close the Coffalyser Work Sheet Editor window	OK

Appendix V - CE devices

Each type of capillary electrophoresis (CE) device that can be used for fragment separation has its own specifications. Based on these specifications, parameters for size calling and peak recognition have been defined. In Coffalyser.Net these parameters are stored in each of the available CE devices.

Supported devices

In Coffalyser.Net the most common capillary electrophoresis devices are supported. Table 11 presents a list with all supported instruments and formats of the raw data files.

Table 11.

Instrument	Format raw data files
ABI Genetic Analyzer devices	.fsa
Beckman Coulter CEQ devices	.esd
MegaBACE 1000 devices	.rsd

A correctly configured CE device in Coffalyser.Net is a prerequisite for reliable MLPA data analysis. It should therefore be assured that:

- The type of CE device in Coffalyser.Net resembles the instrument that is used in the lab.
- The filter set with which the instrument has been calibrated, is selected in the software.

Filter set

A filter set defines which fluorescent dyes are recognised in each dye channel. Table 12 contains an overview of common filter sets and their corresponding dyes for the MLPA probes and size marker.

Table 12.

<table>
<tr><td>Instrument</td><td>Filter set</td><td>Dye MLPA probes</td><td>Dye size marker</td></tr>
<tr><td rowspan="3">ABI Genetic Analyzers</td><td>C</td><td>6-FAM™</td><td>TAMRA</td></tr>
<tr><td>D</td><td>6-FAM™</td><td>ROX</td></tr>
<tr><td>G5</td><td>6-FAM™</td><td>LIZ™</td></tr>
<tr><td>Beckman Coulter</td><td>Cy3-Cy5</td><td>Cy5</td><td>Cy3</td></tr>
<tr><td rowspan="2">MegaBACE 1000</td><td>FilterSet 1</td><td>6-FAM™</td><td>ET-ROX</td></tr>
<tr><td>FilterSet 2</td><td>6-FAM™</td><td>ET-ROX</td></tr>
</table>

Box 5: CE Device Properties window – general tab

Created by
The creation date + time and the name of the user who created the CE device are displayed here.

Modified by
The modification date + time and the name of the user who modified the properties of the CE device are displayed here.

CE device*
The type of the CE device is displayed here.

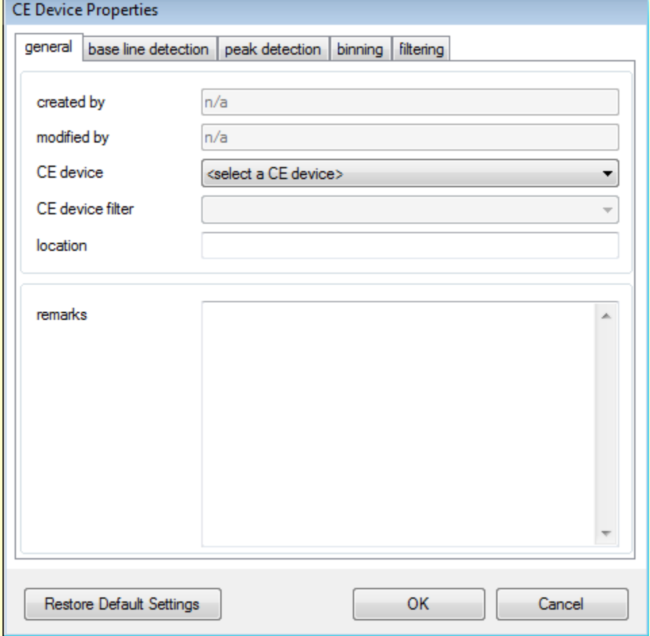
CE device filter*
The filter set of the CE device is displayed here.

Location
The location or other identification of the CE device is displayed here.

Remarks
Remarks about the CE device are displayed here.

Restore default settings button
Returns all settings to their default value.

* Mandatory



OK button
Saves (changes in) the CE device and closes the CE Device Properties window.

Cancel button
Closes the CE Device Properties window without saving changes.

Add and delete a CE device

To be able to analyse MLPA data, a CE device has to be created in an organisation. An organisation can hold multiple CE devices. CE devices can only be created and deleted by Organisation administrators and Server administrators. Note that a CE device can only be deleted when it is not selected in an experiment.

PROCEDURE: CREATE CE DEVICE

1.	Right click on the folder *CE Devices*	CE Devices Add CE Device ... Refresh
2.	Select *Add CE Device …*	
	The CE Device Properties window opens	
3.	Navigate to the tab GENERAL	general | base line detection | peak
4.	Select the CE device type used for electrophoresis from the CE device drop-down menu	CE device: <select a CE device> CE device filter location <select a CE device> ABI - 310 ABI - 3100 ABI - 3130 ABI - 3130XL
5.	Select the filter set used during electrophoresis from the CE device filter drop-down menu [8]	CE device filter: <select a CE device filter> location <select a CE device filter> A C D
6.	Fill in the Location text field when desired	
7.	Fill in the Remarks text field when desired	
8.	Click **OK** to save the CE device and close the window	OK

[8] The available filter sets depend on the chosen CE device type

PROCEDURE: DELETE CE DEVICE

1.	Expand the folder CE Devices by clicking the **+** sign next to this folder	Projects CE Devices Settings Users
2.	Right click on the CE device you want to remove and select *Delete*	Refresh Delete Properties Properties (Read Only)
3.	Click **Yes** to confirm you want to delete the selected CE device	Yes

Edit a CE device

It is possible to adjust the properties of any created CE device. This functionality is reserved for Organisation administrators and Server administrators. The parameters for size calling and peak recognition can easily be reset to their default values, in case these have been changed.

IMPORTANT NOTES:

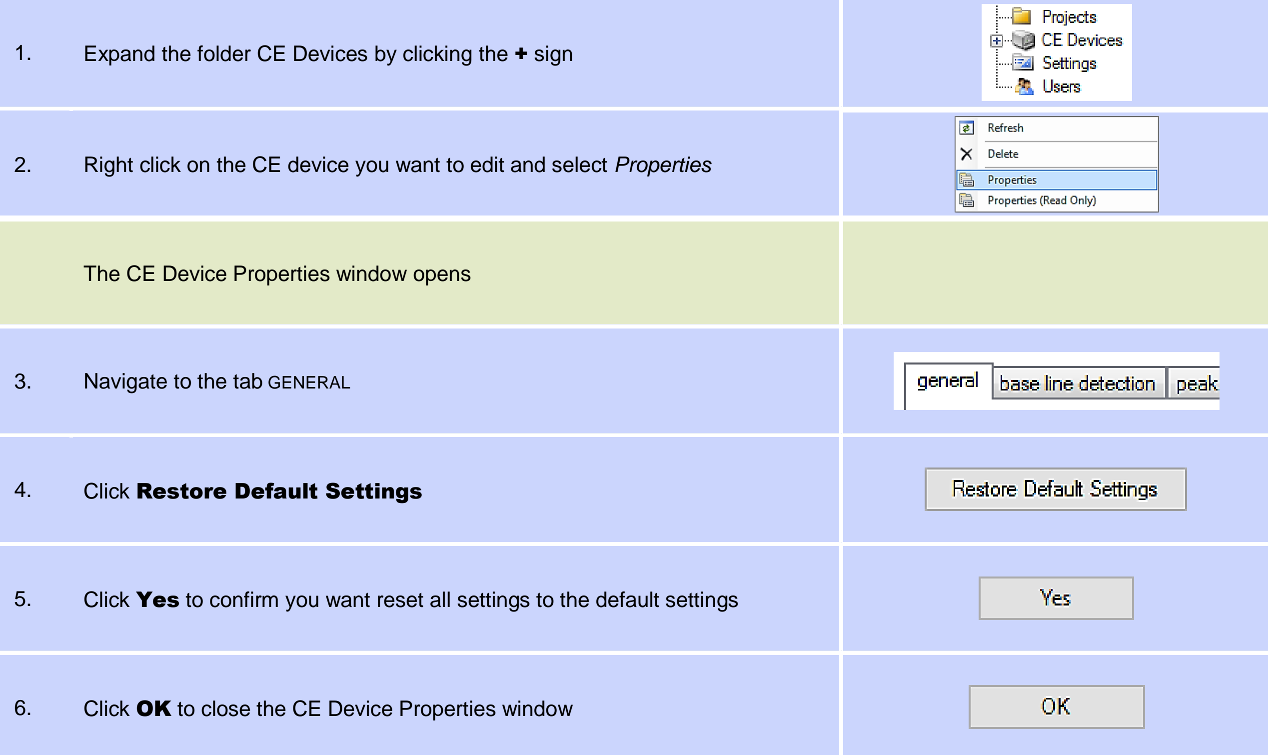
- Only the properties in the tab General of a CE device should be edited.
- The other tabs contain the parameters for size calling and peak recognition. These parameters have been set after extensive testing and should not be changed in a diagnostic setting.

Changing the settings of a CE device will influence size calling and peak recognition. This will have an effect on the analysis results!

PROCEDURE: EDIT CE DEVICE PROPERTIES

Step	Action	
1.	Expand the folder CE Devices by clicking the **+** sign	Projects CE Devices Settings Users
2.	Right click on the CE device you want to edit and select *Properties*	Refresh Delete Properties Properties (Read Only)
	The CE Device Properties window opens	
3.	Navigate to the tab GENERAL	general | base line detection | peak
4.	Edit the information in the relevant fields	
5.	Click **OK** to save the changes and close the window	OK

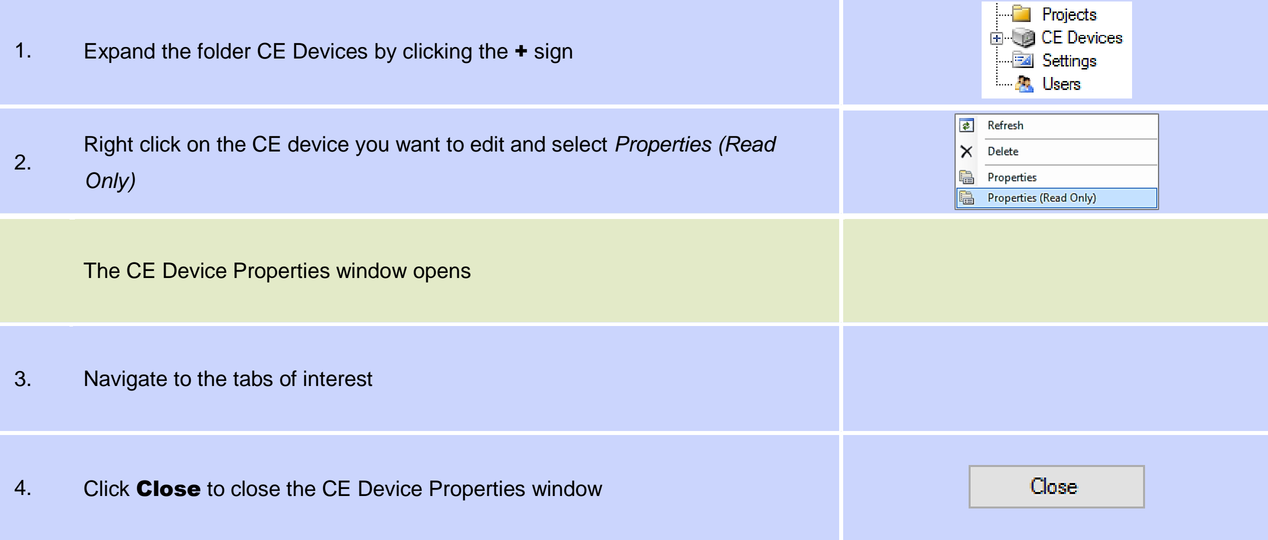
PROCEDURE: RESTORE DEFAULT SETTINGS



Step	Action	Screen
1.	Expand the folder CE Devices by clicking the **+** sign	Projects CE Devices Settings Users
2.	Right click on the CE device you want to edit and select *Properties*	Refresh Delete Properties Properties (Read Only)
	The CE Device Properties window opens	
3.	Navigate to the tab GENERAL	general | base line detection | peak
4.	Click **Restore Default Settings**	Restore Default Settings
5.	Click **Yes** to confirm you want reset all settings to the default settings	Yes
6.	Click **OK** to close the CE Device Properties window	OK

PROCEDURE: VIEW CE DEVICE PROPERTIES

The procedure below describes how any user in an organisation can view the properties of a CE device. It is not possible to alter settings.



Step	Action	Screen
1.	Expand the folder CE Devices by clicking the **+** sign	Projects CE Devices Settings Users
2.	Right click on the CE device you want to edit and select *Properties (Read Only)*	Refresh Delete Properties Properties (Read Only)
	The CE Device Properties window opens	
3.	Navigate to the tabs of interest	
4.	Click **Close** to close the CE Device Properties window	Close

Appendix VI - Bin set

For reliable analysis and result interpretation, it is important that peaks in raw run data are properly recognised as signals coming from MLPA probes and fragments. The lengths of these probes and fragments slightly differ between samples in an experiment. For instance in one sample a fragment has a length of 140.25 nt, whereas in another sample it is 140.45.

To link these signals to the same probe, Coffalyser.Net uses a collection of bins, a so-called bin set. A bin is a range of base pairs (by default 4) in which Coffalyser.Net looks for a signal in all samples included in an experiment. For each probe and fragment a bin is present in the bin set.

Inspect the bin set

In Coffalyser.Net, each bin is displayed as a vertical bar and each signal as a black dot with a size label. When a signal has been found in a bin, this bin will be green. An example of a correct bin set is presented in Figure 8: in all bins a signal has been found, resulting in the bins to be green.

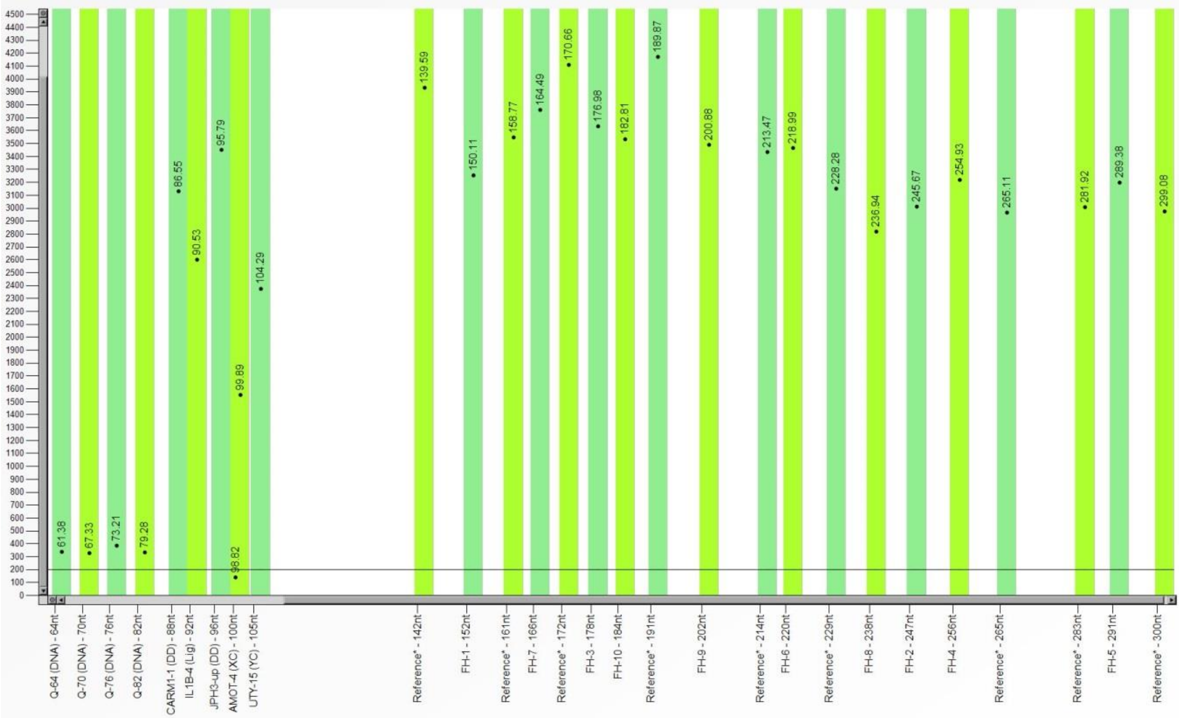


Figure 8. Correct bin set in Coffalyser.Net

When no signal has been found, the bin will be red. See Figure 9 for an example of an incorrect bin set. In this case no signal has been detected in one bin in this sample. This bin has turned red and the probe signal lies just outside of it.

Please note that in samples with a homozygous deletion, not all probes will generate a signal. In that case the related bins will also be red and no signal is present outside the bins!

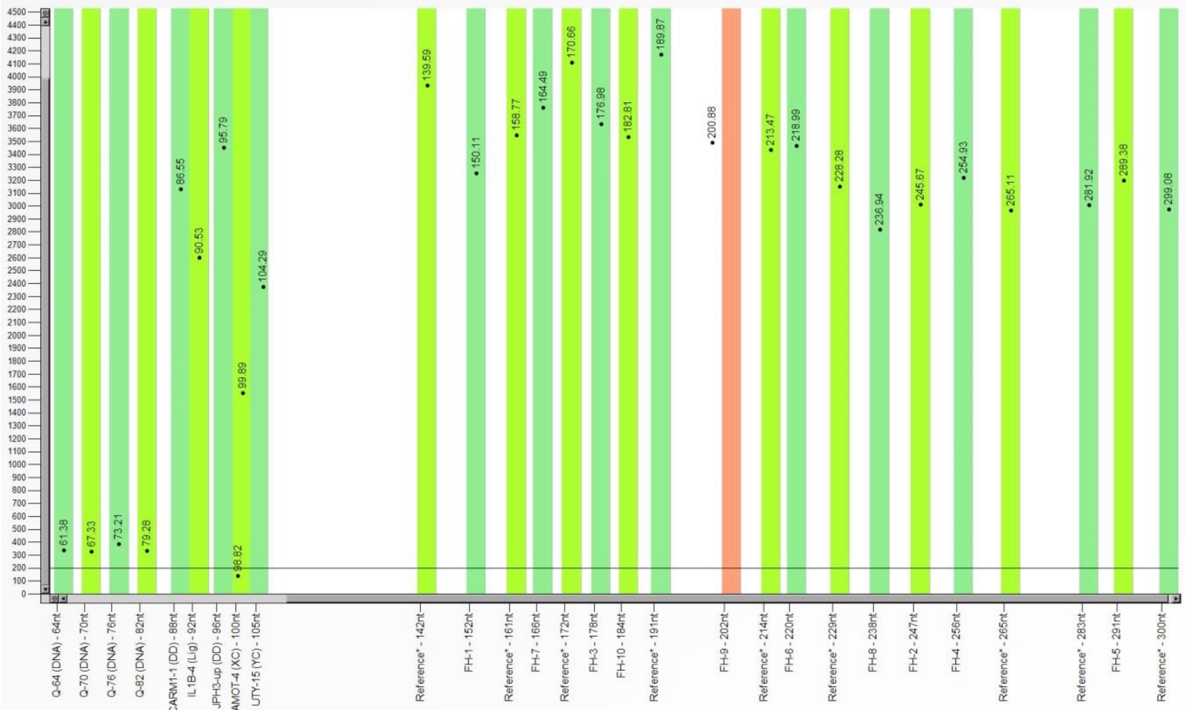
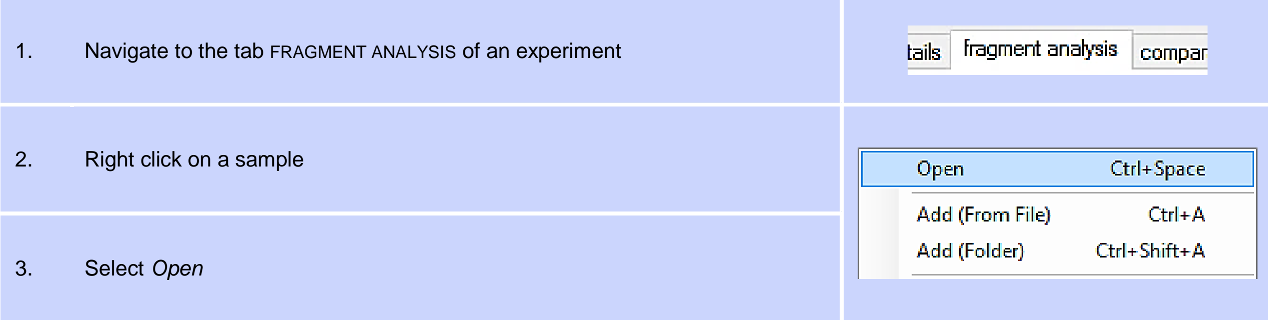


Figure 9. Incorrect bin set in Coffalyser.Net. The signal of the FH exon 9 probe falls outside its bin.

A bin set can be checked at two places: in the Sample Results Explorer and in the Edit Manual Bin Set window.

PROCEDURE: INSPECT BIN SET IN THE SAMPLE RESULTS EXPLORER



1.	Navigate to the tab FRAGMENT ANALYSIS of an experiment	tails / fragment analysis / compar
2.	Right click on a sample	Open Ctrl+Space Add (From File) Ctrl+A Add (Folder) Ctrl+Shift+A
3.	Select *Open*	

	The Sample Results Explorer window opens	
4.	Navigate to the tab BINNING PROFILE	steps binning profile fragment
5.	Check the graph for red bins where a signal is located just outside the bin	
6.	Inspect other samples by selecting them from the list on the left side of the window when desired	

PROCEDURE: INSPECT BIN SET IN THE EDIT MANUAL BIN SET WINDOW

1.	Navigate to the tab FRAGMENT ANALYSIS of an experiment	tails fragment analysis compar
2.	Right click in the window and select *Edit Manual Bin Set*	Include In Comparative Analysis Edit Manual Bin Set Export Data
3.	Select the channel that contains the MLPA probes	
	The Coffalyser Work Sheet Editor – Manual Bin Set window opens	
4.	Check the graph for red bins where a signal is located just outside the bin	
5.	Inspect other samples by selecting them from the list on the left side of the window when desired	

Adjust a bin set

Sometimes the actual lengths of probes obtained with a capillary electrophoresis instrument differ too much from the expected lengths. Consequently, these probe signals fall outside the bin set that Coffalyser.Net has created. As a result, the software won't detect these signals and issues a warning for missing probes. To solve this problem, the bin set can be adjusted thereby creating a manual bin set.

PROCEDURE: ADJUST A BIN SET

1.	Navigate to the tab FRAGMENT ANALYSIS of an experiment	tails / fragment analysis / compar
2.	Right click in the window and select *Edit Manual Bin Set*	Include In Comparative Analysis Edit Manual Bin Set Export Data
3.	Select the channel that contains the MLPA probes	
	The Coffalyser Work Sheet Editor – Manual Bin Set window opens	
4.	Right click in the grid and select *Manual Bin Set Bounds*	Delete Ctrl+Del Manual Bin Set Bounds Replace Control Fragments With
5.	Select Set Based On Current Experiment Results (All Rows)	Set Based On Current Sample Results (All Rows) Set Based On Current Experiment Results (All Rows) Resolve Bin Set Overlap
6.	Select a sample in which the bin set is incorrect	
7.	Look up in the chart the actual length of the fragment that falls outside its bin	
8.	In the column MANUAL BINSET LOWER BOUND change the value to a value below the actual length of the fragment	manual_binset lower_bound
9.	In the column MANUAL BINSET UPPER BOUND change the value to a value above the actual length of the fragment	manual_binset upper_bound
	Do not make the bin larger than 4 nt and ensure that it does not overlap with other bins	

10.	When necessary, repeat steps 6 to 9 to adjust the bin for other fragments	
11.	Click **OK** to close the Coffalyser Work Sheet Editor – Manual Bin Set window	OK
	A dialog box opens	
12.	Click **Yes** to set the probe recognition method to manual	Yes

Appendix VII - User accounts

Coffalyser.Net offers the possibility to share its database with multiple users. For privacy, security and organisational purposes, different levels of access exist, which can be assigned to user accounts. These levels are linked to user roles in the software. Five user roles can be distinguished: Server administrator, Organisation administrator, Organisation user, Project administrator and Project user. A complete overview of all functionalities/rights specified per user role is provided in Table 13.

User roles

Project user

A Project user is the lowest user role with the least amount of rights. An Organisation user can be made Project user by the Project administrator, Organisation administrator and Server administrator. The following functions are available to Project users:

- View the properties (Read only) of an organisation
- View the properties (Read only) of a CE device
- View the properties (Read only) of a project
- View the contents of a project
- Create an experiment in projects of which he is Project user
- Open an experiment in projects of which he is Project user
- Adjust the properties of an experiment in projects of which he is Project user
- Modify an experiment (e.g. add/remove samples, change analysis method) in projects of which he is Project user
- View the properties (Read only) of an experiment

Project administrator

The Project administrator user role is one level higher than Project user. A Project administrator has full rights within his project. An Organisation user can become a Project administrator in two ways. An Organisation user automatically becomes Project administrator of any project he creates. Alternatively, the rights of Project administrator can be given to him by the Server administrator, Organisation administrator or another Project administrator.

Besides the same rights as Project users, Project administrators have the following rights:

- Delete (own) projects of which he is Project administrator

- Adjust the properties of a project of which he is Project administrator. This includes adjusting project user roles
- Delete experiments in projects of which he is Project administrator

Organisation user

The role of Organisation user is an intermediate level between Organisation administrator and Project administrator/user. An Organisation user is part of an organisation, but he has no specific rights. He can become Project administrator or user when these rights are given to him or when he creates a project. Only a Server administrator can assign the role of Organisation user to a user account.

Organisation administrator

The Organisation administrator is the highest user role within an organisation. A Server administrator can assign the role of Organisation administrator to a user account. The main function of the Organisation administrator role is to set up and maintain the structure of his organisation. To fulfil this task, an Organisation administrator has the same rights as a Project administrator, but extra functionalities are available to him:

- Change the organisational role of a user within his organisation
- Adjust the properties of an organisation
- Create a CE device
- Delete a CE device
- Adjust the properties/settings of a CE device

Server administrator

This is the highest level. A Server administrator has the same rights as lower level users, but also rights that are exclusive for this role. These are:

- Create organisations
- Delete organisations
- Open the database folder 'Users'
- Create user accounts
- Assign organisations to user accounts

In addition, a Server administrator has a complete overview of all organisations present in the database.

Table 13. Overview of user role functionalities

	Server administrator	Organisation administrator	Organisation user	Project administrator	Project user
Create a user	●				
Delete a user	●	●[1]			
Change the organisational role of a user	●	●[2]			
Create an organisation	●				
Delete an organisation	●				
Adjust the properties of an organisation	●	●[2]			
View the properties of an organisation (read only)	●	●	●	●	●
Create a CE device	●	●			
Delete a CE device	●	●			
Adjust the properties of a CE device	●	●			
View the properties of a CE device (read only)	●	●	●	●	●
Create a project	●	●	●		
Delete a project	●	●	●[3]	●	
Adjust the properties of a project, including user roles	●	●	●[3]	●	
View contents of a project	●	●	●	●	●
View the properties of a project (read only)	●	●	●	●	●
Create an experiment	●	●	●[4]	●	●
Delete an experiment	●	●	●[3]	●	
Open an experiment	●	●	●[4]	●	●
Adjust the properties of an experiment	●	●	●[4]	●	●

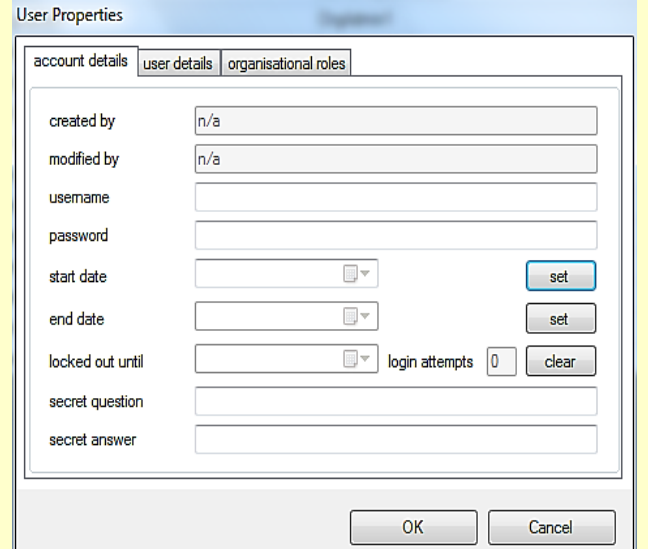
	Server administrator	Organisation administrator	Organisation user	Project administrator	Project user
Modify an experiment (e.g. add/remove samples etc.)	●	●	● [4]	●	●
View the properties of an experiment (read only)	●	●	●	●	●

[1] An Organisation administrator can delete a user account from the software when the organisation is assigned to this user account and when no other organisations are assigned to this user account.
[2] Only possible in the organisation of which the user is Organisation administrator.
[3] Only possible when the Organisation user is also Project administrator of the project.
[4] Only possible when the Organisation user is also Project administrator or Project user of the project.

User account information

All user account information, including user roles, can be accessed in the User Properties form. This form automatically opens when you create a new, or edit an existing, user account. It consists of three tabs where you can enter all mandatory and optional information regarding the user account. User account information is stored in the Coffalyser.Net database.

Box 6: User properties window – account details tab



Username *
Name with which a user logs in on his account.

Password *
Password of the user account.

Start * / End date
When set, the user can only log in during this period. The set/clear buttons activate the date picker in the input fields or clears the date from the input field.

Locked out until / Login attempts
Shows date until which a user is locked out due to invalid login attempts. This number is displayed in the field at the right. The clear button releases the lock on the user account.

Secret question
Input field for a question that will be asked in case of forgotten password.

Secret answer
Input field for the answer to the secret question.

OK button
Saves the changes and closes the User Properties menu.

Cancel button
Closes the User Properties menu without saving changes.

* Mandatory

Box 7: User properties window – user details tab

Surname *
Input field for the surname of the user.

Given name(s)
Input field for the given name(s) of the user.

E-mail address
Input field for the e-mail address of the user.

Department
Input field for the department of the user.

Function
Input field for the function of the user.

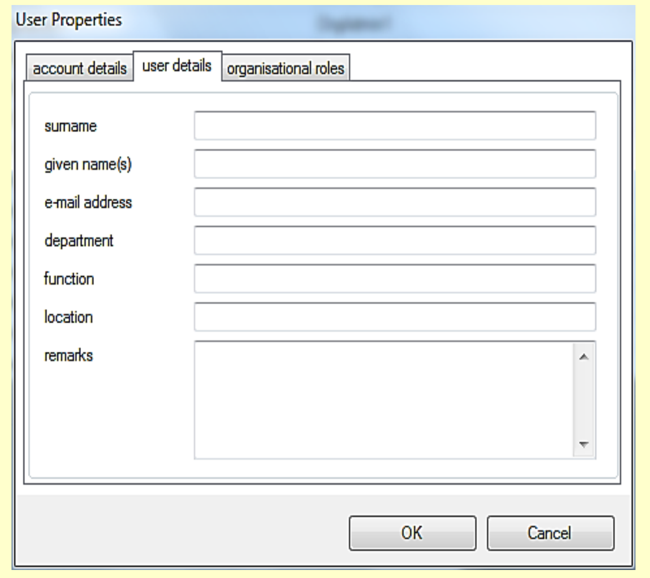
Location
Input field for the location/room of the user.

Remarks
Input field for additional remarks.

OK button
Saves the changes and closes the User Properties menu.

Cancel button
Closes the User Properties menu without saving changes.

* Mandatory



Box 8: User properties window – organisational roles tab

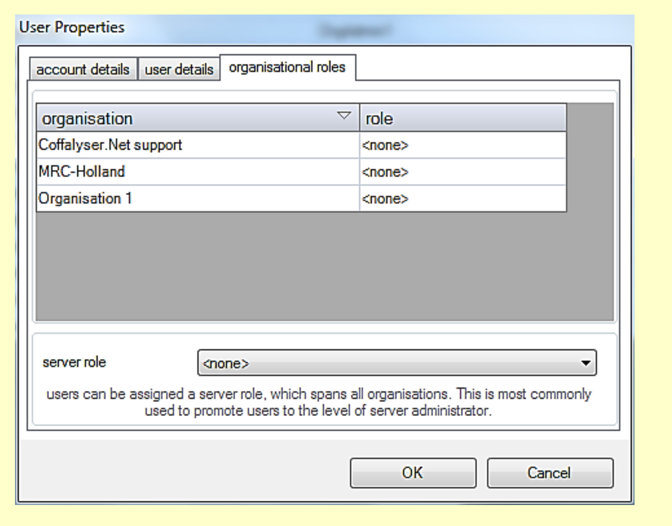
Organisation column
List with Organisations.

Role column
Indicates the current role of a user in an Organisation.

Server role
Indicates whether the user is a Server administrator or not.

OK button
Saves the changes and closes the User Properties menu.

Cancel button
Closes the User Properties menu without saving changes.



Create and delete user accounts

User accounts can be created and deleted by Server administrators only. Both can be done in the *database* folder 'Users'. Once a user account has been created, it is recommended that the Server administrator informs the user to change the password of this account and to enter a secret question + answer. See paragraph **Edit user profile** for instructions.

PROCEDURE: CREATE USER ACCOUNT

1.	Right click on *Users* in the database	
2.	Select *Open*	
	The Users window opens	
3.	Right click and select *Add user*	
	The User Properties form opens	
4.	Navigate to the tab ACCOUNT DETAILS and enter all relevant information	
5.	Navigate to the tab USER DETAILS and enter all relevant information	
6.	Navigate to the tab ORGANISATIONAL ROLES	
7.	In the column role click on the cell next to an organisation the user should have access to In the column role click on the cell next to an organisation the user should have access to	
8.	Click on the arrow head to expand the list with organisational roles	
9.	Select a role from the list	

10.	Repeat steps 7 to 9 for other organisations the user should have access to	
11.	Click **OK** to save the new user account and close the USER PROPERTIES form	OK
12.	Click **Close** to close the Users window	Close

PROCEDURE: DELETE USER ACCOUNT

1.	Right click on *Users* in the database	Organisations Users Open
2.	Select *Open*	
	The Users window opens	
3.	Right click on a user account and select *Delete*	View Edit Add User Delete
4.	Click **Yes** to confirm you want delete the user account	Yes
5.	Click **Close** to close the Users window	Close

Edit user accounts

Server administrators can grant or deny user accounts access to organisations by changing the organisational role. Next to this, they can reset the password of user accounts when the user forgot his password and didn't enter a secret question and answer. These actions can be performed in the *database* folder 'Users'.

Organisation administrators can adjust the organisational role of a user account for their own organisation. This can be done in any of the *organisation's* 'Users' folder.

Before users can access the projects created by other users, they first have to be granted permission to do so. This can be done by Project administrators, Organisation administrators and Server administrators in the Project properties.

PROCEDURE: EDIT USER ACCOUNT

1.	Right click on *Users*	Organisations / Users / Open
2.	Select *Open*	
	The Users window opens	
3.	Right click on a user account and select *Edit*	View / Edit / Add User / Delete
	The User Properties form opens	
4.	Edit the information in the respective tabs as desired	User Properties: account details / user details / organisational roles
5.	Click **OK** to save the changes and close the USER PROPERTIES form	OK
6.	Click **Close** to close the Users window	Close

PROCEDURE: EDIT PROJECT USER ROLE

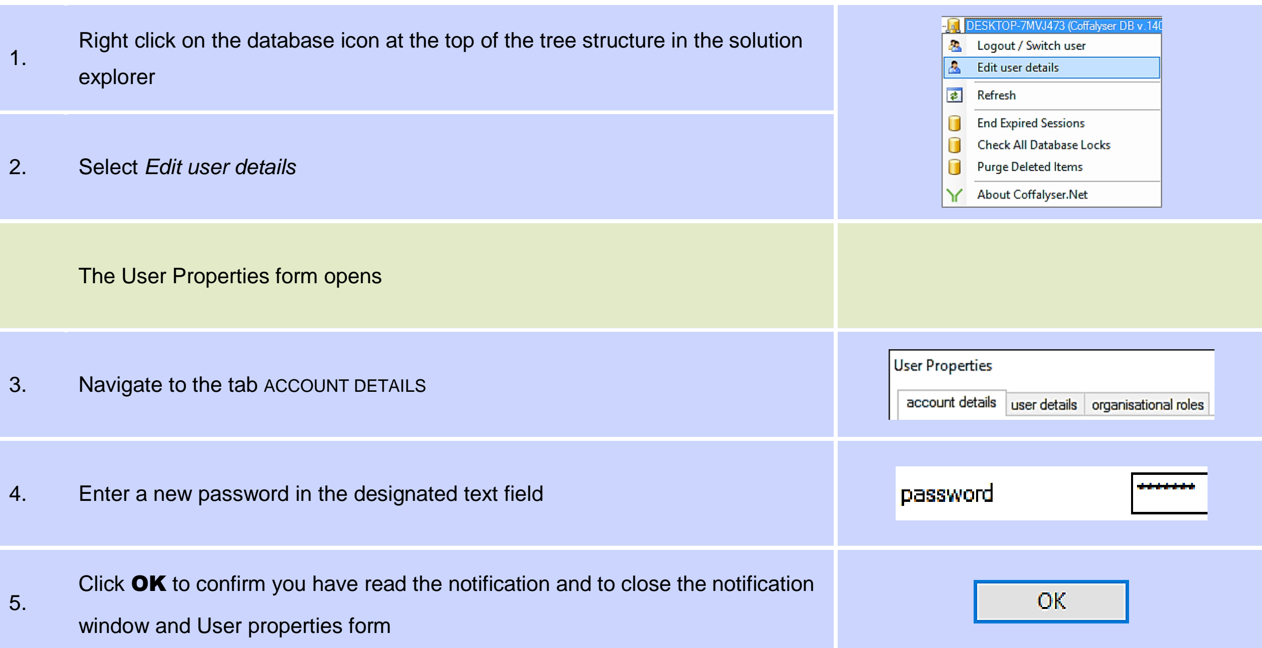
This part describes how access rights to a project can be adjusted for Organisation users. This can be done by Project administrators, Organisation administrators and Server administrators.

1.	Expand the folder *Projects* in an organisation by clicking the **+** sign next to this folder	MRC-Holland Projects CE Devices Settings Users
2.	Right click on a project	Refresh Delete Properties Properties (Read Only)
3.	Select Properties	
	The Project properties window opens	
4.	Navigate to the tab USER ROLES and adjust the role for one or more Organisation users	Project: Example Project details user roles
5.	Click **OK** to save the changes	OK
6.	Click **OK** to confirm you have read the notification and to close the notification window	OK

Edit user profile

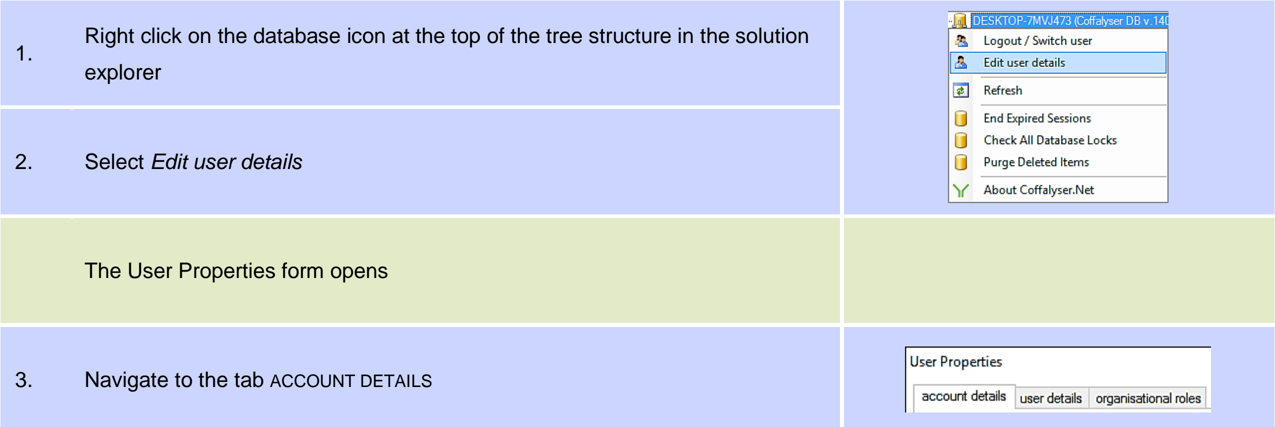
This part describes how users can edit the details of their own user account. A user can only change his password, secret question and answer to this question in the tab account details. Information about the user can be added or adjusted in the tab user details.

PROCEDURE: CHANGE PASSWORD



1.	Right click on the database icon at the top of the tree structure in the solution explorer	DESKTOP-7MVJ473 (Coffalyser DB v 14(Logout / Switch user Edit user details Refresh End Expired Sessions Check All Database Locks Purge Deleted Items About Coffalyser.Net
2.	Select *Edit user details*	
	The User Properties form opens	
3.	Navigate to the tab ACCOUNT DETAILS	User Properties account details | user details | organisational roles
4.	Enter a new password in the designated text field	password *******
5.	Click **OK** to confirm you have read the notification and to close the notification window and User properties form	OK

PROCEDURE: CHANGE SECRET QUESTION + ANSWER



1.	Right click on the database icon at the top of the tree structure in the solution explorer	DESKTOP-7MVJ473 (Coffalyser DB v 14(Logout / Switch user Edit user details Refresh End Expired Sessions Check All Database Locks Purge Deleted Items About Coffalyser.Net
2.	Select *Edit user details*	
	The User Properties form opens	
3.	Navigate to the tab ACCOUNT DETAILS	User Properties account details | user details | organisational roles

4.	Enter a new secret question in the designated text field	secret question
5.	Enter the answer to the secret question in the designated text field	secret answer
6.	Click **OK** to confirm you have read the notification and to close the notification window and User properties form	OK

PROCEDURE: ADD OR CHANGE USER INFORMATION

1.	Right click on the database icon at the top of the tree structure in the solution explorer	DESKTOP-7MVJ473 (Coffalyser DB v.140 Logout / Switch user Edit user details Refresh End Expired Sessions Check All Database Locks Purge Deleted Items About Coffalyser.Net
2.	Select *Edit user details*	
	The User Properties form opens	
3.	Navigate to the tab USER DETAILS	User Properties account details · user details · organisational roles
4.	Enter / change information as desired in the applicable text fields	
5.	Click **OK** to confirm you have read the notification and to close the notification window and User properties form	OK

Contact Information

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Implemented Changes – compared to the previous version(s).

Version 04 – November 2018

- A text has been added about which type of results should be visually confirmed in the electropherogram / raw data
- A warning has been added that a bin should not be made larger than 4 basepairs
- “Every MS-MLPA probemix contains one or more Digestion control probes” has been replaced by “Most MS-MLPA probemixes contain one or more Digestion control probes”
- A note has been added that an update of the sheet library only updates the Coffalyser sheets in the hidden section of the sheet library. The sheets in the active section remain unaffected.
- Filter set A has been removed from Table 12 as ROX labelled primers for MLPA probes are discontinued.
- A warning has been added that a CE device can only be deleted when not linked to an experiment.
- A warning has been added in a footnote that the percentage mentioned for mutation specific probes is NOT the percentage of cells carrying the mutation.
- Adjusted some texts in the Important note boxes 1, 2 and 3
- Some minor textual adjustments have been made.
- Description of the normalization in copy number analysis has been adjusted, so it now describes the actual calculations correctly.
- Description of the normalization for mutation specific probes has been adjusted, so it now describes the actual calculations correctly.
- Descriptions of MS-MLPA normalization and result interpretation have been added. These parts are not included in version 03.
- It is mentioned now that No DNA samples to which HhaI has been added should be defined as ‘NoDNA’

Version 03 – March 2017

- Implemented Changes box added
- In the table ‘Probemixes with 15-30 probes’ on page 46, the percentage of residual primer resulting in the notification ‘Bad’ has been corrected (50% has been changed to 60%)
- In the table with results of mutation specific probes when reference samples have been used on page 31/32, the text has been corrected in the column Patient sample for the situation when reference samples do not have a signal for a mutation specific probe (*Signal of mutation specific probe below 10% of median signal of the reference probes* has been changed to *Signal of mutation specific probe present in the sample*)
- To all procedures in Appendix VI an extra step has been added at the start (Navigate to the tab FRAGMENT ANALYSIS of an experiment)
- In **Figure 3** the symbols as displayed in the reports have been corrected

Version 02 – November 2016

- Coffalyser.Net Reference Manual has completely been rewritten

Version 01

- New document