Coffalyser.Net™ Reference Manual

For Coffalyser.Net versions
220513.1739, 240129.1959 and 250317.1029

Intended purpose

Coffalyser.Net is an in vitro diagnostic (IVD)[1] or research use only (RUO) software designed for the analysis of (MS-)MLPA data generated using a SALSA® MLPA® probemix[2] as described in the (MS-)MLPA General Protocol.

The results obtained with Coffalyser.Net analysis software should be interpreted by a clinical molecular geneticist or equivalent.

[1]Coffalyser.Net analysis software is registered for in vitro diagnostic use (IVD) in countries specified at the end of this manual. In all other countries, Coffalyser.Net analysis software is for research use only (RUO).

[2]To be used in combination with a SALSA MLPA Reagent Kit, SALSA HhaI, and SD0XX as specified in the product description of the application-specific probemix.

INTENDED PURPOSE **2**
1. INTRODUCTION **5**
2. LOG IN TO COFFALYSER.NET **6**
3. SHEET LIBRARY & COFFALYSER SHEETS **7**
4. CREATE A CAPILLARY ELECTROPHORESIS (CE) DEVICE **9**
5. CREATE A PROJECT **10**
6.A SET UP A COPY NUMBER ANALYSIS EXPERIMENT AND ANALYSE DATA **11**
6.B SET UP A METHYLATION STATUS ANALYSIS EXPERIMENT AND ANALYSE DATA **15**
7. VIEW ANALYSIS RESULTS **20**
8. EXPORT RESULTS (OPTIONAL) **21**
9. OPEN EXISTING EXPERIMENTS **22**
10. QUALITY SCORES **23**
APPENDIX I – NORMALISATION AND RESULT INTERPRETATION **24**
NORMALISATION 24
Copy number analysis *24*
Methylation-specific MLPA analysis *25*
RESULTS AND INTERPRETATION 26
Copy number analysis *26*
Methylation-specific MLPA analysis *30*
FINAL RATIO VS. INTRA RATIO PERCENTAGE 34
Mutation-specific probes *34*
APPENDIX II - QUALITY SCORES FRAGMENT ANALYSIS **36**
FRAGMENT RUN SEPARATION SCORE (FRSS) 38
..... *39*
FRSS evaluations *40*
FRAGMENT MLPA REACTION SCORE (FMRS) 44
FMRS evaluations *47*
PROBE COUNTER 55
MS-MLPA probemixes *56*
Probemix with mutation-specific probes *56*
Probe ID colours *57*
APPENDIX III - SHEET LIBRARY **58**

UPDATE OF THE SHEET LIBRARY 58
MANAGE THE SHEET LIBRARY 61
Add Coffalyser sheets to the sheet library *62*
Delete Coffalyser sheets from the sheet library *65*
DISPLAY OR HIDE COLUMNS IN THE SHEET LIBRARY 66
APPENDIX IV - COFFALYSER SHEETS 67
DISPLAY OR HIDE COLUMNS IN A COFFALYSER SHEET 69
EDIT COFFALYSER SHEETS 72
Add and delete probes in a Coffalyser sheet *72*
Probe report levels *74*
APPENDIX V - CE DEVICES 76
ADD AND DELETE A CE DEVICE 78
EDIT A CE DEVICE 80
APPENDIX VI - BIN SET 82
INSPECT THE BIN SET 82
CREATE OR ADJUST A MANUAL BIN SET 85
APPENDIX VII - USER ACCOUNTS 87
USER ROLES 87
USER ACCOUNT INFORMATION 90
CREATE AND DELETE USER ACCOUNTS 92
EDIT USER ACCOUNTS 94
EDIT USER PROFILE 96
CONTACT INFORMATION 98
Implemented Changes – compared to the previous version(s). *99*

1. Introduction

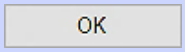
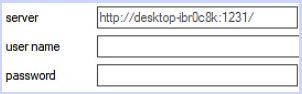
The purpose of this document is to provide a step-by-step guide for the analysis of MLPA data with Coffalyser.Net™. The procedures for login, software set up, data analysis and result export are presented in a chronological order. Instructions for installation of Coffalyser.Net™ can be found in the installation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

IMPORTANT NOTES:

- Coffalyser.Net is registered for in vitro diagnostic (IVD) use in countries specified at the end of this manual. 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 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 after the comparative analysis. 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.mrcholland.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 (MS-)MLPA General Protocol before use. These are available online at www.mrcholland.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. Serious incidents that have occurred in relation to the device shall be reported to MRC Holland and the competent authority of the Member State in which the user and/or the patient is established.
- The Summary of Safety and Performance will be available in the European database on medical devices (Eudamed), https://ec.europa.eu/tools/eudamed.
- Basic UDI-DI: 872021148Coffalyser.NetVM

2. Log in to Coffalyser.Net

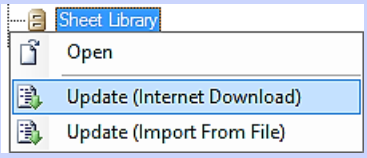
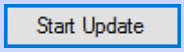
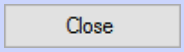
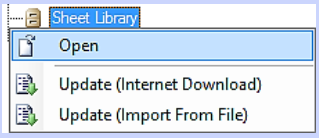
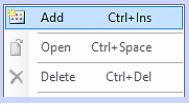
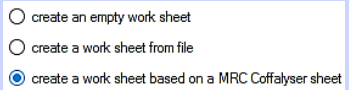
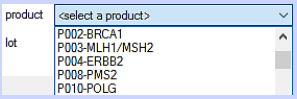
For storage of data (e.g. raw data, experiments, analysis results), Coffalyser.Net uses a database that is hosted on an SQL server. Connections to this database go via a Coffalyser.Net Server Service. All server services that are found in your network are displayed in the Server Selection dialog that is displayed when Coffalyser.Net is started. In most cases, only one server service will be shown. When more server services are present, make sure to select the correct one.

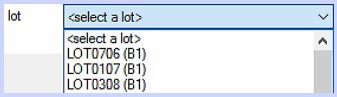
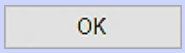
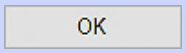
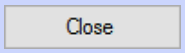
1.	Start Coffalyser.Net	
2.	In the Server Selection window select the server	
3.	Click **OK**	
	The Login window opens	
4.	Enter your user name and password in the designated fields	
5.	Click **Login**	

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 the correct 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 58 and **Appendix IV - Coffalyser sheets** on page 67.

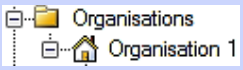
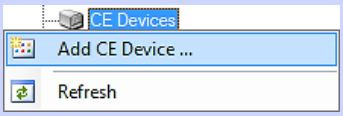
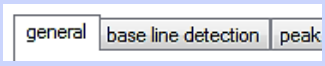
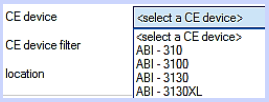
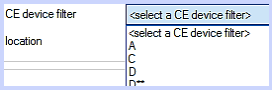
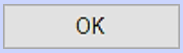
Before you can analyse your data, you need to retrieve the latest information from our servers and create a Coffalyser sheet for your probemix of interest.

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** to start the update	
5.	Once the update is finished, 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	

11.	Select the appropriate lot number from the lot drop-down menu	
12.	Click **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	
15.	Repeat steps 8 to 14 to add more Coffalyser sheets if required	
16.	Click **Close** to close the Manage Coffalyser Work Sheets window	

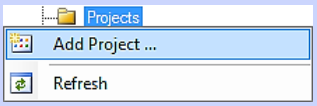
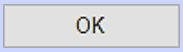
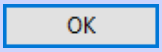
4. Create a Capillary Electrophoresis (CE) device

A CE device in Coffalyser.Net contains parameters for size-calling and peak recognition. Before data can be analysed, a CE device must 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 76.

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	

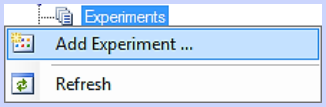
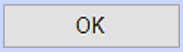
6.a Set up a copy number analysis experiment and analyse data

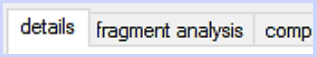
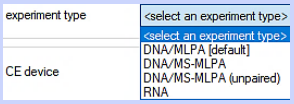
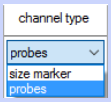
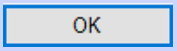
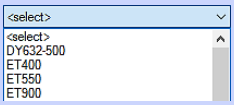
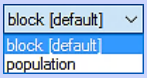
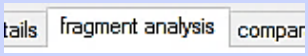
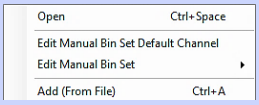
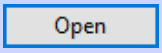
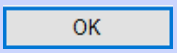
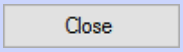
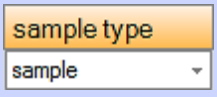
The deepest layer in the data storage structure is formed by experiments. Experiments hold the actual raw MLPA data as well as the analysis results. Per project an unlimited number of experiments can be created.

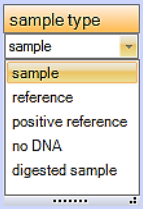
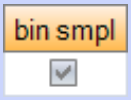
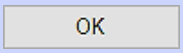
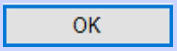
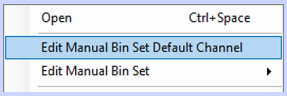
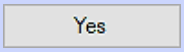
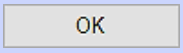
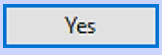
In this chapter the procedure for copy number analysis is described. The combined analysis of copy number and methylation status is described in the next chapter.

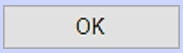
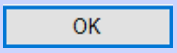
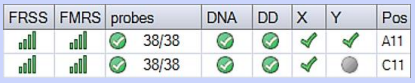
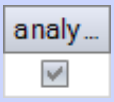
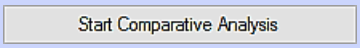
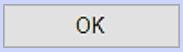
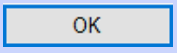
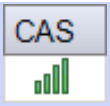
IMPORTANT NOTES:

- The use of a manual bin set is mandatory. This can be done by setting the probe recognition method to *manual* in the Fragment Analysis Settings window. This requires a manual bin set to be present in the Coffalyser sheet.
- All other parameters for fragment analysis and comparative analysis must 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 after the comparative analysis.

1.	Expand the project by clicking the **+** sign	
2.	Right click on the folder *Experiments*	
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/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	
20.	Click **Close** in the Import Files window	
21.	In the column SAMPLE TYPE, click on the cell of a sample	

22.	Click on the arrowhead to expand the list with sample types	
23.	Select a sample type from the list	
24.	Repeat steps 21 to 23 for the other samples	
	If a manual bin set has *not* been created before and is *not* present in the Coffalyser sheet, proceed with step 25. If a manual bin set has been created before and is present in the Coffalyser sheet, continue with step 35.	
25.	In case SALSA binning DNA has been included in the experiment, click the checkbox of this sample in the column BIN SMPL.	
26.	Click **Start Fragment Analysis**	
27.	Leave all settings on default in the Fragment Analysis Settings window and click **OK**	
28.	Click **OK** to close the fragment analysis confirmation message	
29.	Right click in the window and select *Edit Manual Bin Set Default Channel*	
30.	Click **Yes**	
	The Coffalyser Work Sheet Editor – Manual Bin Set window opens	
31.	Check if probes fall inside their bin. For more information see the section **Inspect the bin set** in **Appendix VI - Bin set** on page 82	
32.	When probe signals fall outside their bin, adjust the bin set according to the procedure as described in the section **Create or adjust a manual bin set** in **Appendix VI - Bin set** on page 85	
33.	Click **OK** to close the Coffalyser Work Sheet Editor – Manual Bin Set window	
	A dialog box opens	
34.	Click **Yes** to set the probe recognition method to manual	
35.	Click **Start Fragment Analysis**	

36.	Confirm in the Fragment Analysis Settings window that the probe recognition method is set to *manual*, otherwise select it from the drop-down menu	
37.	Leave all other settings on default and click **OK**	
38.	Click **OK** to close the fragment analysis confirmation message	
	When not all probes have been found as indicated by the probe counter, inspect the bin set (**Inspect the bin set** in **Appendix VI - Bin set** on page 82) and adjust it when necessary (**Create or adjust a manual bin set in Appendix VI - Bin set** on page 85). In case the bin set has been adjusted, follow the procedure from step 35 onwards.	
39.	Check for every sample in the experiment if the FRSS and FMRS [1] scores show 4 green bars, and if the DNA and DD columns show a green icon.	
	Only samples that show 4 green bars for the FRSS and FMRS, and green icons in the DNA and DD columns 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 this (right click on a sample and select Open).	
40.	Navigate to the tab COMPARATIVE ANALYSIS by clicking **Next >>**	
41.	Select samples that show 4 green bars for the FMRS to be included in the comparative analysis by clicking the checkbox in the column ANALYSIS	
42.	Click **Start Comparative analysis**	
43.	Leave all settings on default in the Comparative Analysis Settings window and click **OK**	
44.	Click **OK** to close the comparative analysis confirmation message	
45.	Check if the CAS, FRSS and FMRS [2] scores show 4 green bars for each sample that has been included in the comparative analysis	
46.	Check the PSLP, FSLP, RSQ and RPQ [2] of samples that do not show 4 green bars for troubleshooting by hovering the cursor over these quality checks	
	Only the results of samples that show 4 green bars for the CAS, FRSS and FMRS can reliably be interpreted.	

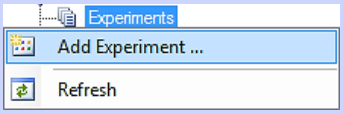
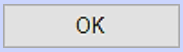
[1] See chapter **10. Quality scores** for a description.
[2] See chapter **10. Quality scores** for a description.

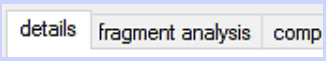
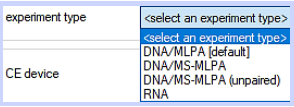
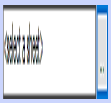
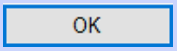
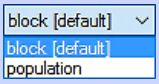
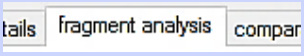
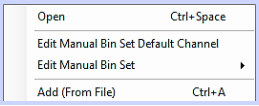
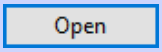
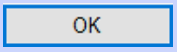
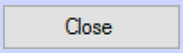
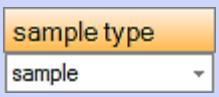
6.b Set up a methylation status analysis experiment and analyse data

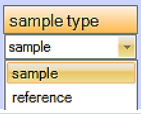
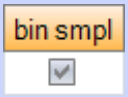
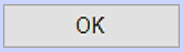
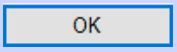
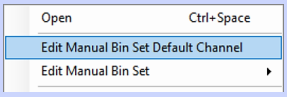
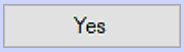
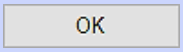
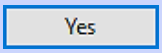
Two types of experiments exist in Coffalyser.Net: one for copy number analysis and one for the combined analysis of copy number and methylation status. In this chapter the combined analysis of copy number and methylation status is described. The procedure for copy number analysis is described in the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

IMPORTANT NOTES:

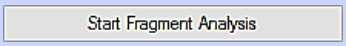
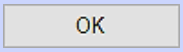
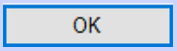
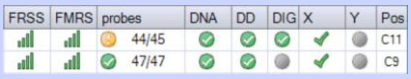
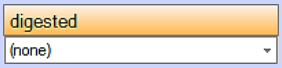
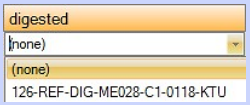
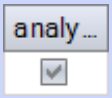
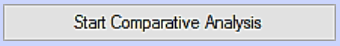
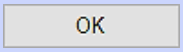
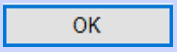
- The use of a manual bin set is mandatory. This can be done by setting the probe recognition method to *manual* in the Fragment Analysis Settings window. This requires a manual bin set to be present in the Coffalyser sheet.
- All other parameters for fragment analysis and comparative analysis must be 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 after the comparative analysis.

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

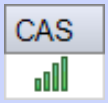
7.	Navigate to the tab DETAILS in the window that opens	
8.	Select *DNA/MS-MLPA* as experiment type from the drop-down menu	
9.	Check for each dye channel if the type of fragments is set correctly. If not, select the appropriate type from the drop-down menu	
10.	Open the sheet library via the button 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	
20.	Click **Close** in the Import Files window	
21.	In the column SAMPLE TYPE, click on the cell of a sample	
22.	Click on the arrowhead to expand the list with sample types	

23.	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	
24.	Repeat steps 21 to 23 for the other samples	
	If a manual bin set is *not* present in the Coffalyser sheet, proceed with step 25. If a manual bin set is present in the Coffalyser sheet, continue with step 35.	
25.	In case SALSA binning DNA has been included in the experiment, click the checkbox of this sample in the column BIN SMPL	
26.	Click **Start Fragment Analysis**	
27.	Leave all settings on default in the Fragment Analysis Settings window and click **OK**	
28.	Click **OK** to close the fragment analysis confirmation message	
29.	Right click in the window and select *Edit Manual Bin Set Default Channel*	
30.	Click **Yes**	
	The Coffalyser Work Sheet Editor – Manual Bin Set window opens	
31.	Check if probes fall inside their bin. For more information see the section **Inspect the bin set** in **Appendix VI - Bin set** on page 82	
32.	When probe signals fall outside their bin, adjust the bin set according to the procedure as described in the section **Create or adjust a manual bin set** in **Appendix VI - Bin set** on page 85	
33.	Click **OK** to close the Coffalyser Work Sheet Editor – Manual Bin Set window	
	A dialog box opens	
34.	Click **Yes** to set the probe recognition method to manual	

[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'.

35.	Click **Start Fragment Analysis**	
36.	Confirm in the Fragment Analysis Settings window that the probe recognition method is set to *manual*, otherwise select it from the drop-down menu	
37.	Leave all other settings on default and click **OK**	
38.	Click **OK** to close the fragment analysis confirmation message	
	When not all probes have been found as indicated by the probe counter, inspect the bin set (**Inspect the bin set** in **Appendix VI - Bin set** on page 82 and adjust it when necessary (**Create or adjust a manual bin set** in **Appendix VI - Bin set** on page 85). In case the bin set has been adjusted, follow the procedure from step 35 onwards.	
39.	Check for every sample in the experiment if the FRSS and FMRS [4] scores show 4 green bars, and if the DNA and DD columns show a green icon. Digested samples should have a green icon in the column DIG.	
	Only samples that show 4 green bars for the FRSS and FMRS, and green icons in the DNA and DD columns 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 this (right click on a sample and select *Open*)	
40.	Navigate to the tab COMPARATIVE ANALYSIS by clicking **Next >>**	
41.	In the column DIGESTED, click on the cell of a (undigested) sample	
42.	Click on the arrowhead to expand the list with digested samples	
43.	Select the digested sample corresponding to the undigested sample	
44.	Repeat steps 41 to 43 for the other samples	
45.	Select samples to be included in the comparative analysis by clicking the checkbox in the column ANALYSIS	
46.	Click **Start Comparative Analysis**	
47.	Leave all settings on default in the Comparative Analysis Settings window and click **OK**	
48.	Click **OK** to close the comparative analysis confirmation message	

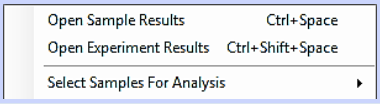
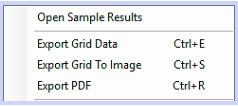
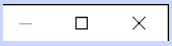
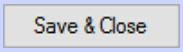
[4] See chapter **10. Quality scores** for a description.

49.	Check if the CAS, FRSS and FMRS [5] scores show 4 green bars for each sample that has been included in the comparative analysis	
50.	Check the PSLP, FSLP, RSQ and RPQ [5] of samples that do not show 4 green bars for troubleshooting by hovering the cursor over these quality checks	
⚠	Only the results of samples that show 4 green bars for the CAS, FRSS and FMRS can reliably be interpreted.	

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

7. View analysis results

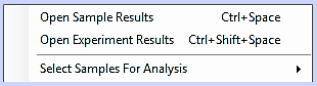
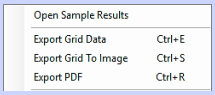
Coffalyser.Net offers the possibility to view the analysis results of all samples at once, but results of individual samples can also be viewed. It is recommended to first view the results of the complete experiment. In this way samples of interest can easily be spotted and subsequently be assessed in more detail by opening the results of these samples. Consult the most recent product description and (MS-)MLPA General Protocol for interpretation of the results. 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 COMPARATIVE ANALYSIS tab and select *Open Experiment Results* to open the results of the complete experiment	
	The Comparative analysis experiment explorer opens	
2.	Right click on a sample of interest and select *Open Sample Results* to open the sample results	
	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	

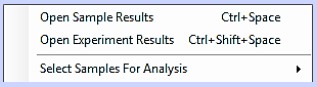
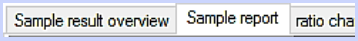
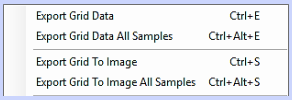
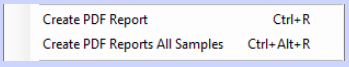
8. Export results (optional)

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**).

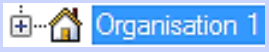
EXPORT RESULTS OF A COMPLETE EXPERIMENT

1.	Navigate to the tab COMPARATIVE ANALYSIS	
2.	Right click in the window and select *Open Experiment Results* to open the results of the complete experiment	
	The Comparative analysis experiment explorer opens	
3.	Right click in the window and select the desired method to export the results	

EXPORT RESULTS OF AN INDIVIDUAL SAMPLE

1.	Navigate to the tab COMPARATIVE ANALYSIS	
2.	Right click on a sample and select *Open Sample Results* to access the sample results	
	The Comparative Analysis Sample Results Explorer opens	
3.	Navigate to the tab SAMPLE REPORT	
4.	Right click in the window and select the desired method to export the results	
5.	Right click in the window and select *Create PDF Report* to export the results to a pdf file when desired	

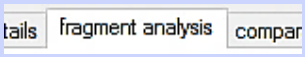
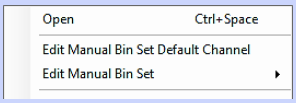
9. Open existing experiments

1.	Expand the organisation that holds the experiment by clicking the **+** sign	
2.	Expand the project that holds the experiment by clicking the **+** sign	
3.	Expand the folder Experiments by clicking the **+** sign	
4.	Right click on the experiment you want to open	
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 36 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 36 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	
2.	Right click on a sample and select *Open*	
	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, require normalisation before they can 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 done for every **reference probe** included in the probemix. 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 in the final ratio of target probe 1 in sample 1.

This procedure is repeated for all probes (except mutation-specific probes and probes with a very low signal, see section **Final ratio vs. intra ratio percentage** on page 34).

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.

More information

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: https://doi.org/10.5772/21898).

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.

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

This procedure is repeated for all probes (except mutation-specific probes and probes with a very low signal, see section **Final ratio vs. intra ratio percentage** on page 34).

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).

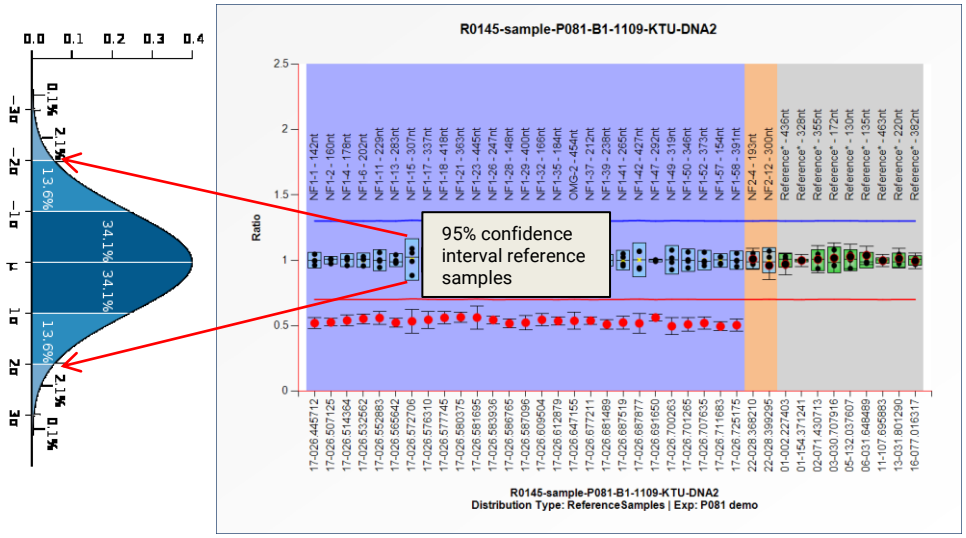


Figure 1. 95% confidence intervals over the reference samples.

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).

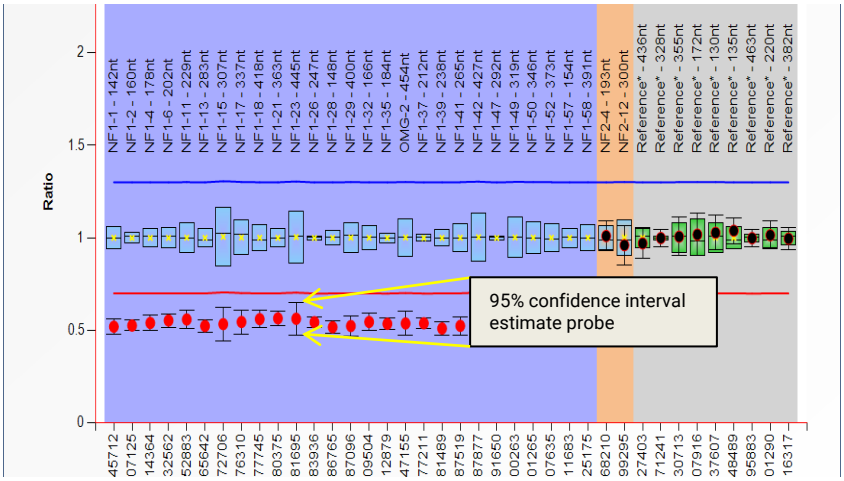


Figure 2. 95% confidence interval estimates of probes in a sample.

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, this 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.

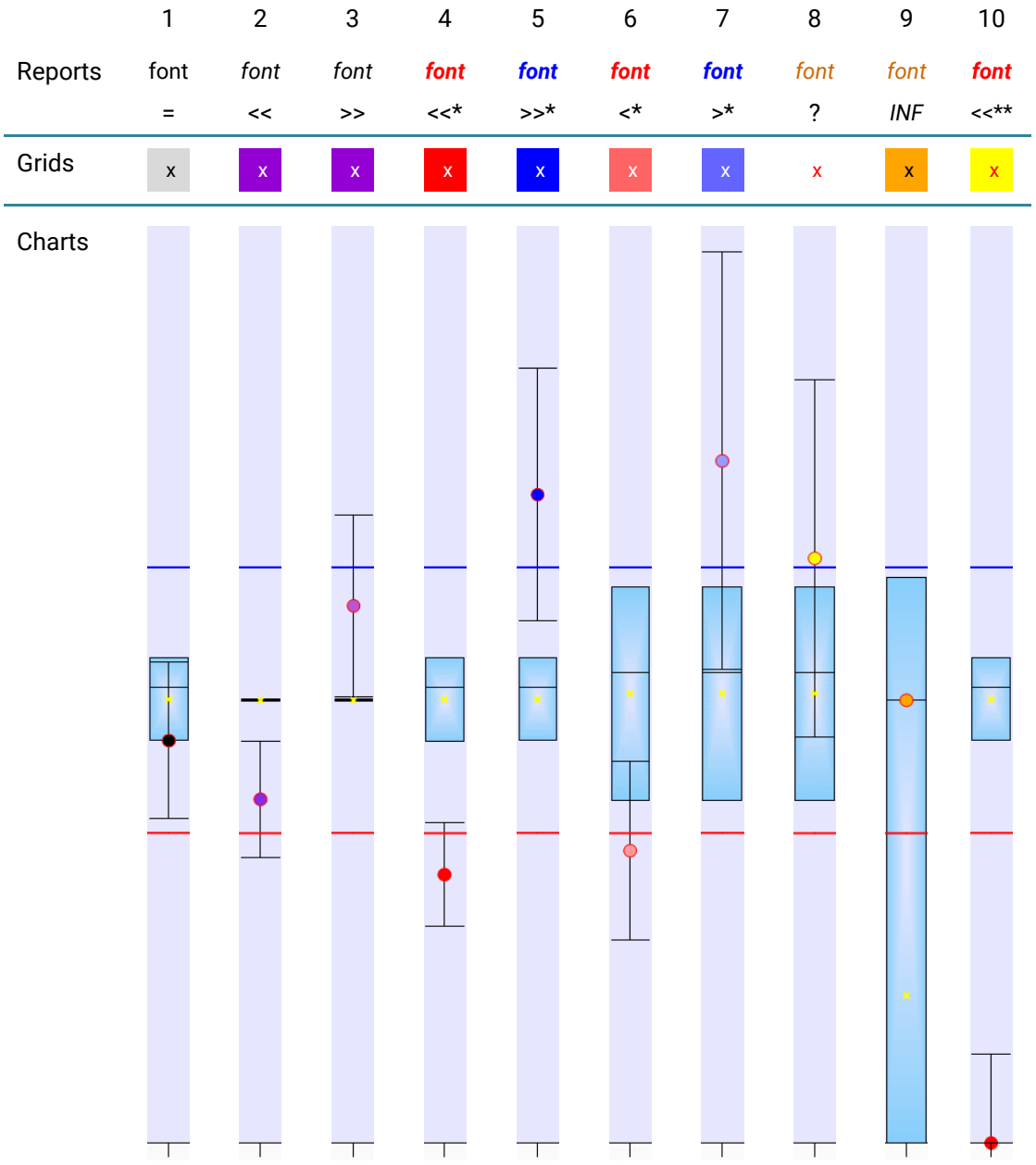


Figure 3 Probe results in Coffalyser.Net

Situation 1: Probe results do not indicate a copy number change: in pdf reports the ratio has a black font colour and an equal to (=) sign is shown in the column [REF], and grey or green cells (target probes) and yellow cells (reference probes) are shown in the grids. In the ratio charts the 95% confidence interval estimates of the probes overlap with the 95% confidence intervals of the same probes over the reference samples (the blue boxes).

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 the ratio is in italics and has a black font colour. In the column [REF] this is indicated by two less-than brackets (<<). In the grids, cells are coloured purple to indicate that the lower arbitrary border has not been crossed. 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 the ratio is in italics and has a black font colour. In the column [REF] this is indicated by two greater-than brackets (>>). In the grids, cells are coloured purple to indicate that the upper arbitrary border has not been crossed. 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 has been crossed. In pdf reports the ratio is in bold and italics, and has a red font colour. In the column [REF] 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 (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 has been crossed. In pdf reports the ratio is in bold and italics, and has a blue font colour. In the column [REF] 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 (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 has been crossed. In pdf reports the ratio is in bold and italics, and has a red font colour. In the column [REF] 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 (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 has been crossed. In pdf reports the ratio is in bold and italics, and has a blue font colour. In the column [REF] 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 (the blue line).

Situation 8: The probe result is inconclusive, although the (in this case lower) arbitrary border has been crossed. In pdf reports the ratio is in italics, and has a brown font colour. In the column [REF] this is indicated by a question mark (?). In grids, cells are white. In ratio charts the probe ratio dot is coloured yellow.

Situation 9: The probe result indicates a strange, uncommon situation in which the majority of the reference samples does not have a signal for this probe. A ratio is calculated based on the reference samples that do have a signal, which is dangerous. This situation is most often encountered in special probemixes, when no dedicated reference samples are used, and/or when incorrect reference samples have been selected. In pdf reports the ratio is in italics, and has a brown font colour. In the column [REF] this is indicated by the term *INF*. In grids, cells are orange. In ratio charts the probe ratio dot is coloured orange.

Situation 10: The probe result indicates that no signal has been found. In pdf reports the ratio is in bold and italics, and has a red font colour. In the column [REF] this is indicated by two less-than brackets with two asterisks (<<**). In grids, cells are yellow. In ratio charts the probe ratio dot is coloured red.

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 ratio is calculated for every probe, only the ratios 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 [HhaI] in their names.

The calculated methylation ratio of a methylation-specific probe indicates how many copies 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 Examples of MS-MLPA results

Copy number analysis		Methylation status analysis	
Final ratio	Number of copies	Methylation result	Number of methylated copies
1.0	2	0.5	1
1.0	2	1.0	2
2.0	4	0.5	2
0.5	1	1.0	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), 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

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).

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 the 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).

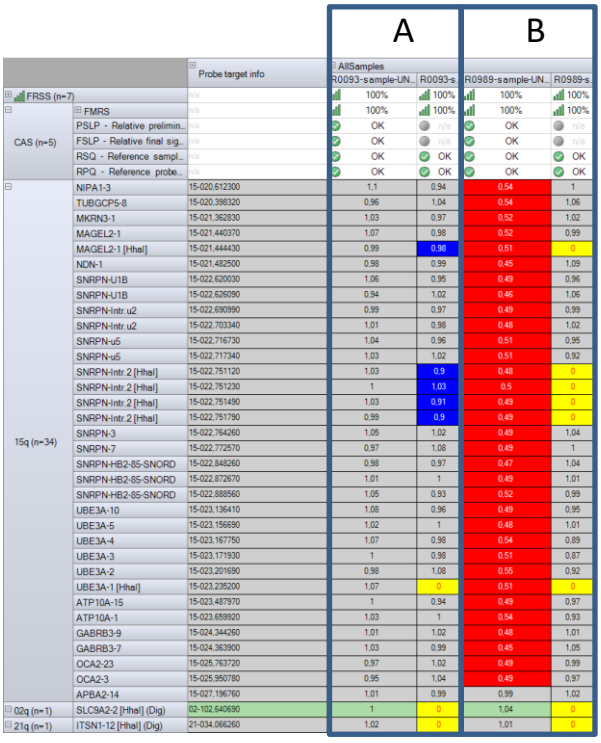


Figure 4 Ratio overview of the Comparative Analysis Experiment Explorer in which the results of the copy number analysis and the methylation status analysis are grouped per sample. Probes containing an HhaI site have the label [HhaI] in their names. **A:** Sample R0093 **B:** Sample R0400.

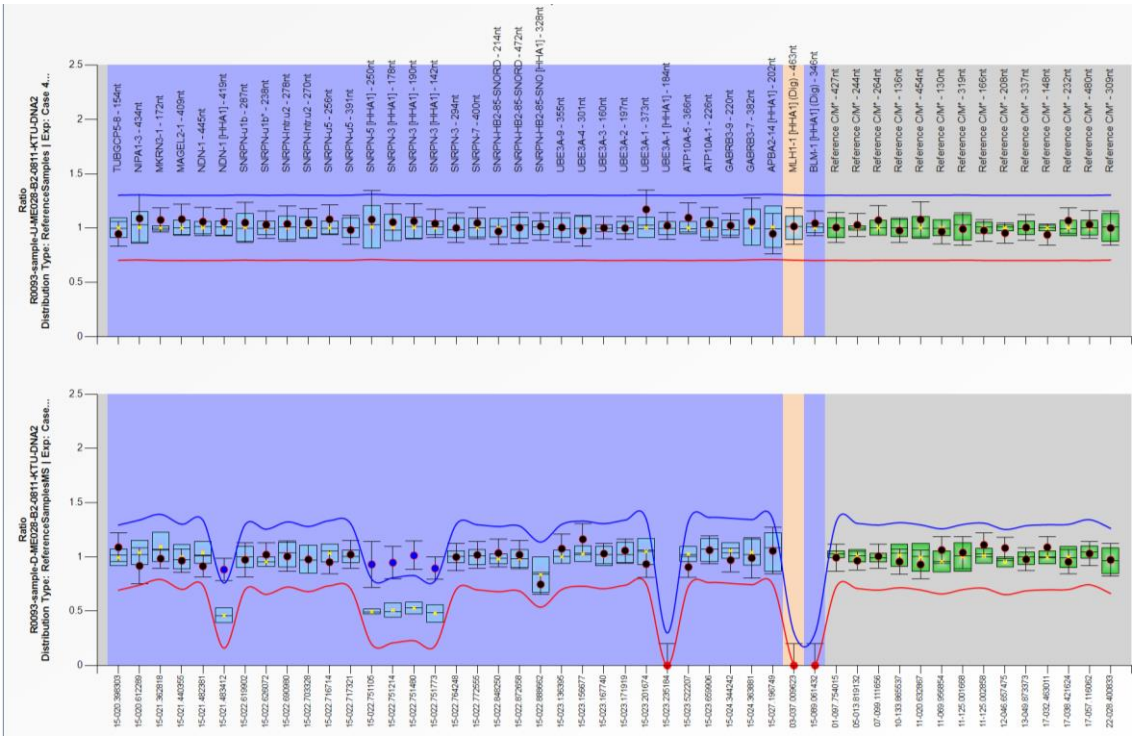
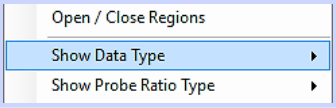
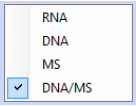


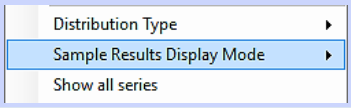
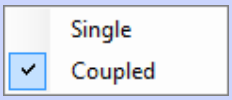
Figure 5 Ratio charts of a sample in the Comparative Analysis Sample Results Explorer. The upper chart displays the results of the undigested counterpart / the copy number analysis, and the lower chart displays the results of the digested counterpart / the methylation status analysis.

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*	
2.	From the appearing list select *DNA* or *MS* to only display the results of the undigested samples or digested samples, respectively	
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*	
2.	From the appearing list select *Single*	
3.	To see the combined results again, follow steps 1 and 2 and select *Coupled*	

Final ratio vs. intra ratio percentage

As described above, the normalisation is done in two steps: against the reference probes within the sample and against the reference samples. This results in a final ratio. This applies to the great majority of probes. For some probes, however, it is not possible or not meaningful to calculate a final ratio. In such cases, Coffalyser.Net calculates an intra ratio percentage, which is the result of the normalisation against the reference *probes* within the same sample. The reference *samples* are *not* taken into account at all. This is used in the following situations:

- For all mutation-specific probes, as these typically have no signal in the reference samples which means that no final ratio can be calculated.
- For signals ≤ 10% of the median signal of the reference probes in the sample. These signals are considered to be unexpectedly low, and may represent background signals or noise, so an intra ratio percentage is displayed as warning.
- For probes with a signal in the digested reaction of an MS-MLPA probemix but with no signal in the undigested reaction, in which case no final ratio can be calculated. This is an unexpected result but can occur due to noise signals or sample swaps.

Note that the intra ratio percentage cannot be used do draw conclusions about copy numbers, but only about whether a probe signal is present or absent. An intra ratio percentage only represents how high a probe signal was as compared to the reference probes in that reaction.

Normalisation

Coffalyser.Net only calculates an intra-normalised ratio for probes in the beforementioned situations. It does that by dividing the signal of the 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.

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

To distinguish this from the final ratios of the other probes, Coffalyser.Net displays intra-normalised ratios as percentages. In the ratio chart the intra-normalised ratio is presented as an orange box.

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, a mutation-specific probe only produces a signal in samples with the relevant mutation.

Binning DNA (SD)

Because signals of mutation-specific probes are usually absent in normal samples, the automatically determined bins for these probes might not be correct. Consequently, they might 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 Binning 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 useful for creating a manual bin set. See section **6.a Set up a copy number analysis experiment and analyse data** (step 25) and section **6.b Set up a methylation status analysis experiment and analyse data** (step 25) for instruction on the use of an SD sample in Coffalyser.Net. **Appendix VI - Bin set** on page 82 contains instructions for creating a manual bin set.

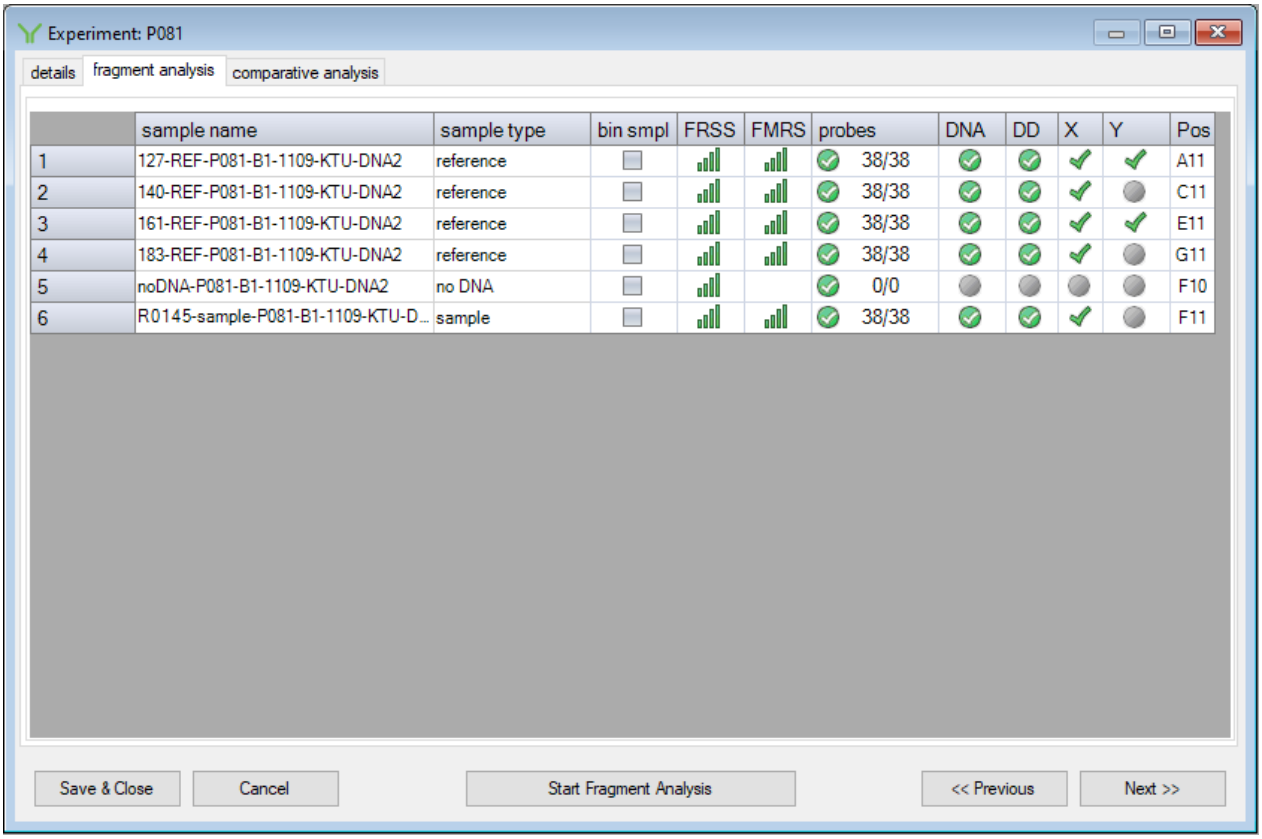
IMPORTANT NOTES:

- The intra ratio percentage for a mutation-specific probe does not indicate the percentage of cells carrying the mutation!
- 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 can even block the analysis completely.

After fragment analysis Coffalyser.Net displays an overview in the tab *fragment analysis* (**Figure 6**).



	sample name	sample type	bin smpl	FRSS	FMRS	probes	DNA	DD	X	Y	Pos
1	127-REF-P081-B1-1109-KTU-DNA2	reference				38/38					A11
2	140-REF-P081-B1-1109-KTU-DNA2	reference				38/38					C11
3	161-REF-P081-B1-1109-KTU-DNA2	reference				38/38					E11
4	183-REF-P081-B1-1109-KTU-DNA2	reference				38/38					G11
5	noDNA-P081-B1-1109-KTU-DNA2	no DNA				0/0					F10
6	R0145-sample-P081-B1-1109-KTU-D...	sample				38/38					F11

Figure 6. Experiment – fragment analysis tab.

The following quality scores are shown:

- **FRSS**: Main score of the quality assessment for the fragment separation (see section **Fragment Run Separation Score (FRSS)**).
- **FMRS**: Main score of the quality assessment for the MLPA reaction (see section **Fragment MLPA Reaction Score (FMRS)**).
- **Probes**: Probe counter.
- **DNA**: Check for DNA concentration (also part of the FMRS).
- **DD**: Check for DNA denaturation (also part of the FMRS).
- **DIG**: Check for digestion. Only visible in experiments in which the experiment type is set to DNS/MS-MLPA (also part of the FMRS).
- **X**: Indicates the presence of a X chromosome control fragment.
- **Y**: Indicates the presence of a Y chromosome control fragment.

This enables you to quickly distinguish samples with high quality raw data from those with low quality raw data.

Only samples that show 4 green bars for the FRSS and FMRS, and green icons in the DNA and DD columns, should be used in the rest of the analysis. Start troubleshooting for those samples that do not show this.

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

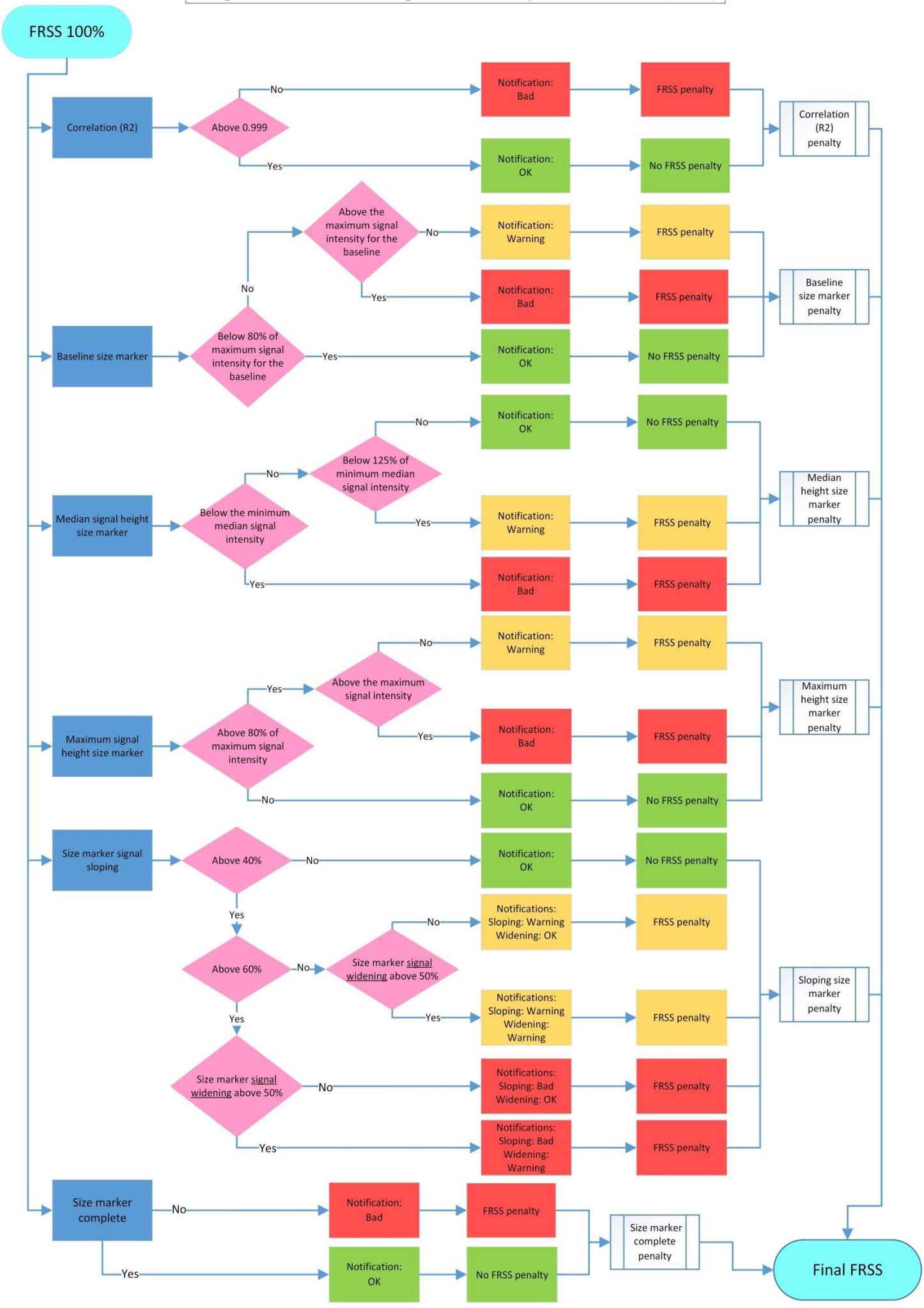
FRSS	Points
	≥ 90
	≥ 75 and < 90
	≥ 45 and < 75
	> 25 and < 45
	≤ 25

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 peak amplitude (in RFU)	Size marker minimum median signal (in RFU)	Size marker maximum signal (in RFU)
ABI 310(0)	700	200	100	7,000
ABI 3130(xl)	700	200	100	7,000
ABI 3700	1,000	300	100	30,000
ABI 3730(xl)	1,000	300	100	30,000
ABI 3500(xl)	1,000	300	100	30,000
ABI SeqStudio (Flex)	1,000	300	100	30,000
Beckman CEQ 2000	12,000	3000	1,000	160,000
Beckman CEQ 8000	12,000	3000	1,000	160,000
Beckman CEQ 8800/GEXP	12,000	3000	1,000	160,000
MegaBACE 1000	1,000	70	100	30,000
Promega Spectrum Compact	1,000	70	100	30,000
Hitachi DS3000	1,000	70	100	30,000

Diagram calculation Fragment Run Separation Score (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 3× the signal of the baseline.

This evaluation is only relevant for those devices for which the *Size marker minimum peak amplitude* is smaller than the *Size marker minimum median signal* (see **Table 3**)

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 for the MLPA fragments. 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 above the set detection threshold.

Conditions:

		Notification	Penalty
	All fragments of the size marker present and above *Size marker minimum peak amplitude* specified for the Capillary Electrophoresis instrument (see **Table 3**)	Ok	No penalty
	Not all fragments of the size marker present and/or above *Size marker minimum peak amplitude* specified for the Capillary Electrophoresis instrument (see **Table 3**)	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 prolonging 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 peak amplitude (in RFU)	Probes minimum median signal (in RFU)	Probes maximum median signal (in RFU)	Probes maximum signal (in RFU)
ABI 310(0)	700	200	300	5,000	7,500
ABI 3130(xl)	700	200	300	5,000	7,500
ABI 3700	1,000	300	300	26,000	31,000
ABI 3730(xl)	1,000	300	300	26,000	31,000
ABI 3500(xl)	1,000	300	300	26,000	31,000
ABI SeqStudio (Flex)	1,000	300	300	26,000	31,000
Beckman CEQ 2000	12,000	3,000	3,000	150,000	170,000
Beckman CEQ 8000	12,000	3,000	3,000	150,000	170,000
Beckman CEQ 8800/GEXP	12,000	3,000	3,000	150,000	170,000
MegaBACE 1000	1,000	300	300	26,000	31,000
Promega Spectrum Compact	1,000	300	300	26,000	31,000
Hitachi DS3000	1,000	300	300	26,000	31,000

Diagram calculation Fragment MLPA Reaction Score (FMRS)

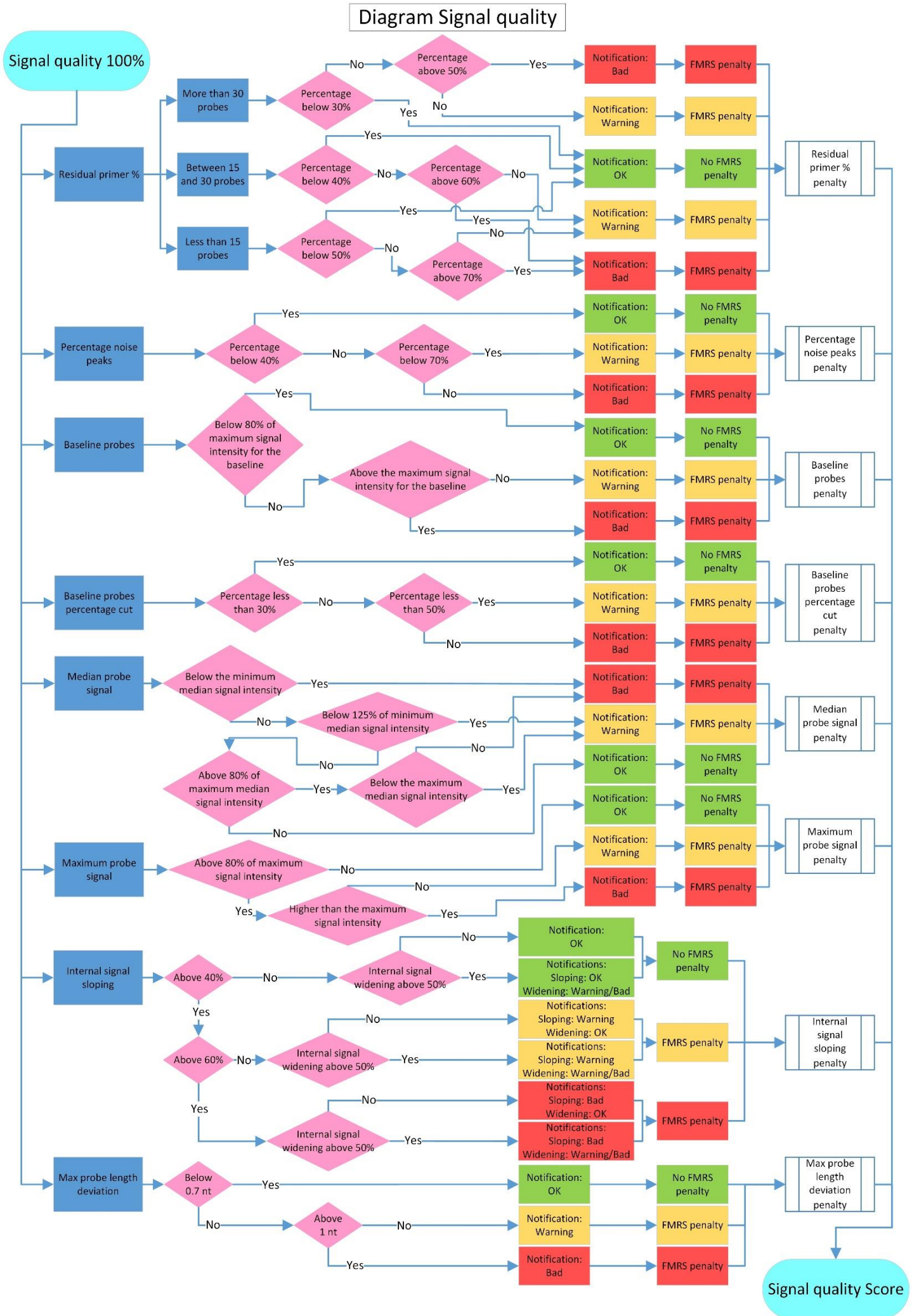


Diagram Signal quality
Signal quality 100%
Residual primer %
More than 30 probes
Between 15 and 30 probes
Less than 15 probes
Percentage below 30%
Percentage above 50%
Percentage below 40%
Percentage above 60%
Percentage below 50%
Percentage above 70%
Percentage noise peaks
Percentage below 40%
Percentage 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%
Internal signal widening above 50%
Above 60%
Internal signal widening above 50%
Max probe length deviation
Below 0.7 nt
Above 1 nt
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
Max probe length deviation penalty
Signal quality Score
Yes
No

FMRS evaluations

Benchmark

Background:

This indicates whether the benchmark fragment, which is usually at 92 nt, is not too high or too low. This fragment is used as a standard to which other control fragments are compared.

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	20 points
	Between 80% and 100% of the *maximum signal* specified for the Capillary Electrophoresis instrument (see **Table 5**)	Warning	20 points
	Below 100% of the *minimum median signal* specified for the Capillary Electrophoresis instrument (see **Table 5**)	Bad	60 points
	Above 100% of the *maximum signal* specified for the Capillary Electrophoresis instrument (see **Table 5**)	Bad	60 points

Related issues and solutions:

When this fragment is too low or too high, this is usually accompanied by other phenomena affecting the quality of the raw data. In case this fragment is present in the probemix, but not detected due to an incorrect bin set, the notification will be 'Bad' and Coffalyser.Net will subtract points from the FMRS. This can be solved by adjusting the bin according to the procedure in **Create or adjust a manual bin set** in **Appendix VI - Bin set** on page 85.

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, which is usually at 92 nt.

Conditions:

		Notification	Penalty
	Median signal of the Q-fragments below 33% of the signal of the benchmark fragment	Ok	No penalty
	Median signal of the Q-fragments between 33% and 50% of the signal of the benchmark fragment	Warning	15 points
	Median signal of the Q-fragments above 50% of the signal of the benchmark fragment	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 usually ranges from 50 to 100 ng (see the protocol and application-specific product description for details). 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 predominantly a measure for 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, usually at 88 nt, and D2, usually at 96 nt) to the benchmark fragment, which is usually at 92 nt.

Conditions:

		Notification	Penalty
	Signals of both D-fragments between 50% and 250% of the signal of the benchmark fragment	Ok	No penalty
	Signals of both D-fragments above 250% of the signal of the benchmark fragment	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	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	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	Warning	15 points
	Signal of both D-fragments below 50% of the signal of the benchmark fragment	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, which is usually 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, which is usually 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 detected MLPA probe peaks in a sample to calculate whether sufficient primer is incorporated.

Conditions:

Samples with more than 30 detected MLPA probe peaks:

		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

Samples with 15-30 detected MLPA probe peaks:

		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

Samples with less than 15 detected MLPA probe peaks:

		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 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:

A 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 3× 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 MLPA 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.

Signal quality – Max probe length deviation

Background:

This indicates if all probe peaks are not too far off the centre of their bins in a sample. For reliable analysis, probe signals should fall within their bin in order to be correctly identified by the software.

Conditions:

		Notification	Penalty
	Max probe length deviation 0.7 nt or smaller	Ok	No penalty
	Max probe length deviation between 0.7 nt and 1.0 nt	Warning	30 points
	Max probe length deviation 1.0 nt or larger	Bad	60 points

Related issues and solutions

When peaks of the same probe deviate too much from the centre of the bin in several samples, the bin set might not be correct. This can happen when changes have been made to the capillary electrophoresis device or electrophoresis conditions, such as using a different polymer type, size standard or another capillary array. Adjusting the bin set may be necessary in such a case.

When over time probes deviate further from the centre of the bin, this may be indicative of problems with the capillary electrophoresis device. It is then recommended to check the capillary electrophoresis instrument for flaws and check the age of the capillary array and polymer. These may have to be replaced.

Probe counter

IMPORTANT NOTES:

- Peaks will only be recognised as probe signals when they are higher than the *Probes minimum peak amplitude (in RFU)* specified for each instrument type (see Table 5) AND when they are higher than 10% of the median signal intensity over the reference probes.
- In rare cases, slope correction may cause low signals to cross the beforementioned thresholds, which may result in the probe counter changing after comparative analysis.
- When probes, for which the value in the column COPY NUMBER (NORMAL) in the Coffalyser sheet is >0, are not detected, the probe counter will show a lower number of detected probes. Reference samples will get a lower FMRS score in case one or more target or reference probes are not detected. Patient/test samples will only get a reduced FMRS score when reference probes are not detected.

The probe counter in the fragment analysis tab shows the number of probes found / the number of probes expected for each sample.

The number of expected probes indicates the number of probes that is expected to generate a signal in healthy individuals. For most probemixes this is equal to the number of probes in the probemix. See

Figure 7 for an example. If less probes are found than expected, it may be due to a problem in the experiment/analysis, but it can also be a real result (e.g. homozygous deletion). In these situations the peak patterns should be inspected.

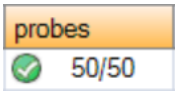


Figure 7. Example of the probe counter of a probemix. 50 probes have been found and 50 probes are expected.

MS-MLPA probemixes

For most MS-MLPA probemixes, the number of expected probes in digested samples is lower than in undigested samples. The reason is that most MS-MLPA probemixes contain digestion control probes that should be completely digested by the restriction enzyme. Next to this, some probes with a restriction site are expected to be completely digested in samples of healthy individuals. These probes are therefore expected not to generate a signal.

Incomplete digestion and/or methylation of the target sequence (which can be a real result) may lead to signals of probes that are expected to be absent in a digested sample. This results in an increase in the number of unexpected probes in the probe counter. The probe counter will then display the number of found probes / number of expected probes | number of unexpected probes. Figure 8 shows an example of the probe counter for a digested sample in which the digestion was not complete. 6 probes that were expected to be fully digested showed a signal, resulting in the probe counter showing 6 unexpected probes.

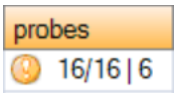


Figure 8. Example of the probe counter of a digested sample in which the digestion was not complete.

Note that contamination with another probemix or PCR product may also result in the probe counter showing unexpected probes.

Probemix with mutation-specific probes

Several probemixes contain one or more mutation-specific probes. In case a mutation-specific probe is included in the probemix, which only generates a signal when the mutation is present in a sample, the number of expected probes is 1 less than the total number of probes in the probemix. Signals of mutation-specific probes are unexpected because they are unexpected in healthy individuals.

For example, when a probemix contains 51 probes of which one is a mutation-specific probe, 50 probes will be found and 50 probes will be expected in a sample of a healthy individual. The probe counter will appear as in Figure 7.

When the mutation, for which a mutation-specific probe is designed, is present in a sample, the mutation-specific probe will generate a signal. The probe counter will then display the number of

found probes / number of expected probes | number of unexpected probes. For example, when a probemix containing 50 probes + 1 mutation-specific probe is used on a sample that harbours the mutation, 50 probes will be found, 50 probes are expected and there will be 1 unexpected signal. The probe counter will appear as in Figure 9.

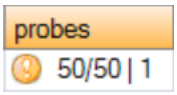


Figure 9. Example of the probe counter of a sample tested with a probemix containing a mutation-specific probe. 50 probes have been found, 50 probes are expected and 1 probe is unexpected, which is the mutation-specific probe.

In case the mutation, for which a mutation-specific probe is designed, is present in a sample and this sample has a homozygous deletion, the probe counter may look like 45/50 | 1.

Probe ID colours

In the tabs *genomic profile* of the (fragment analysis) Sample Results Explorer and *Electropherograms* of the Comparative Analysis Sample Results Explorer Coffalyser.Net visualises which probe signals are expected, unexpected and below or above 10% of the signals of the reference probes in a sample by highlighting the probe IDs.

Table 6. Overview of the colours of the probe ID

	Colour probe ID	**Probe signal expected / unexpected**	**Probe signal below or above 10% of the signals of the reference probes**
1	No colour	Expected	Above
2	Orange	Expected	Below
3	Yellow	Unexpected	Above
4 *	Lavender	Unexpected	Below
5	Pale Violet Red	Only when the median signal of the reference probes is 0 / cannot be calculated	

* Probes in this category are not counted in the probe counter, but they are included in the comparative analysis

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 need 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. creating a manual bin set, 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

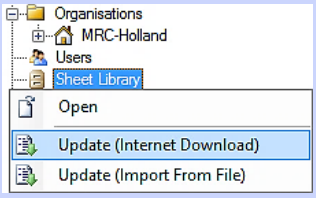
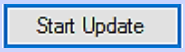
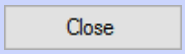
When a new lot of a probemix or a completely new probemix is released, its corresponding Coffalyser sheet is made available for downloading. Coffalyser sheets are also sometimes updated, for example to add product notifications. In order to get these new or modified Coffalyser sheets, you'll need to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

In Coffalyser.Net a sheet library update function is incorporated, which downloads all available Coffalyser sheets from the MRC Holland servers. 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 or that cannot connect to our servers. 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 and any manual bin sets stored within them remain unaffected.

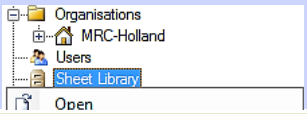
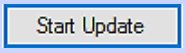
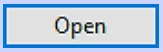
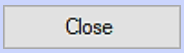
NOTE: The software gives a notification when the update of the sheet library has successfully been completed. This notification includes the number of updated items. In case it states that 0 items were updated, this indicates that the library was already up to date.

PROCEDURE: UPDATE SHEET LIBRARY (INTERNET DOWNLOAD)

1.	Right click on *Sheet Library*	
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**	
	Wait for the update process to complete	
5.	Click **Close** to close the Download Updates (MRC-Holland) window	

PROCEDURE: UPDATE SHEET LIBRARY (IMPORT FROM FILE)

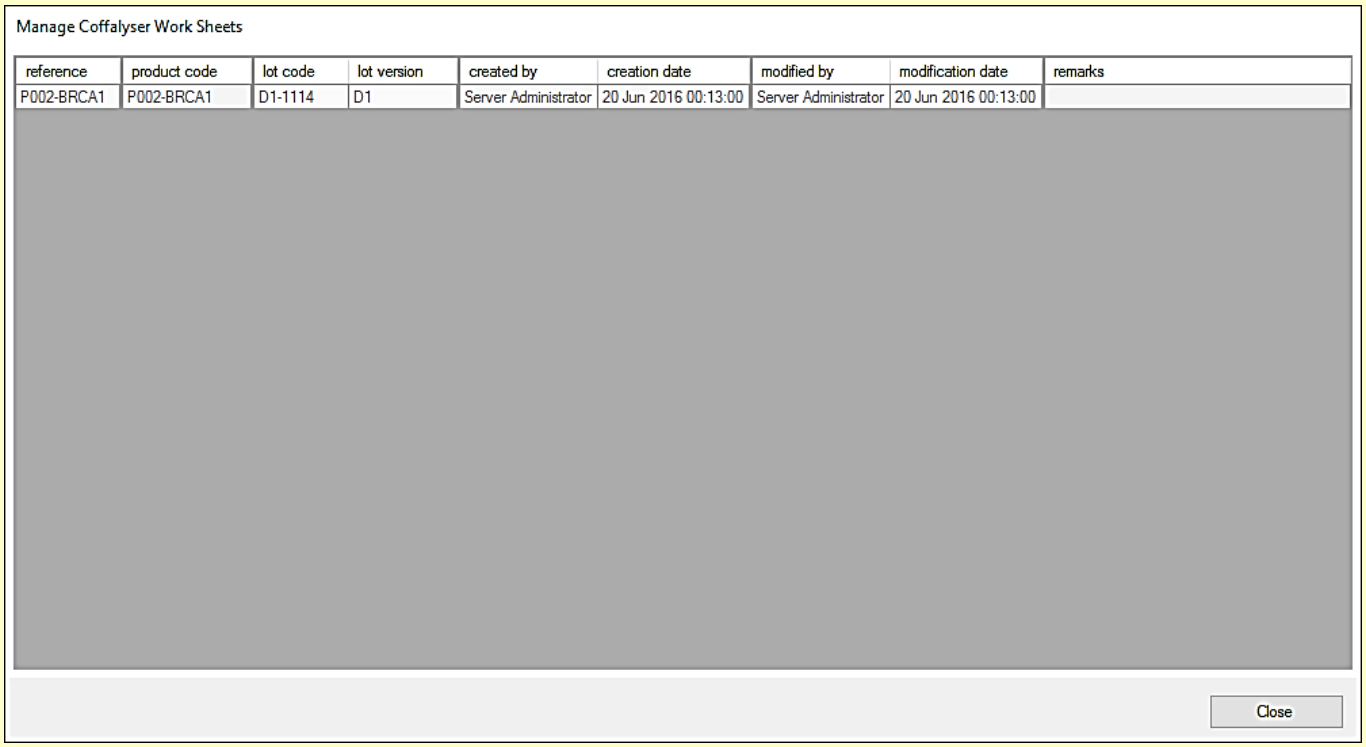
1.	Sign in to your MRC Holland account on www.mrcholland.com	
2.	Click the icon to open the account menu	
3.	Select *Software* from the account menu	
4.	Click on the Coffalyser.Net logo	
5.	Under the header Downloads, click on *Coffalyser.Net – sheet library update* and save the file	Coffalyser.Net – sheet library update
6.	Copy the sheet library file onto a USB drive	
7.	Take the USB drive and plug it into the computer on which Coffalyser.Net is installed	
8.	In Coffalyser.Net right click on *Sheet Library*	

9.	Select *Update (Import From File)*	
	The Download Updates (MRC-Holland) window opens	
10.	Click **Start Update**	
	A dialog box opens	
11.	Navigate to the location on the USB drive where the sheet library file is saved	
12.	Select the file and click **Open**	
13.	Click **Close** to close the Download Updates (MRC-Holland) window	

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.

Product code column
The product code of the probemix.

Lot code column
The lot number of the probemix.

Lot version column
The version number of the probemix.

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

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

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

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

Remarks column
Remarks made in the Coffalyser sheet.

Close button
Closes the Manage Coffalyser Work Sheets window.

Add Coffalyser sheets to the sheet library

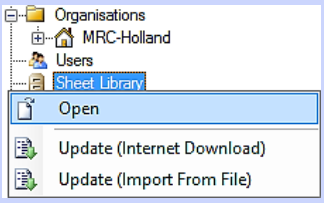
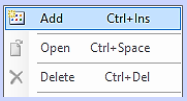
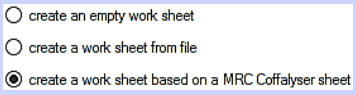
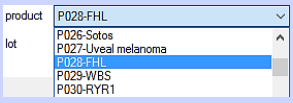
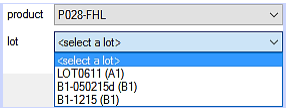
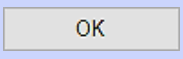
There are two sources from which Coffalyser sheets can be added to the active section of the sheet library.

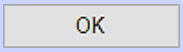
1. The hidden section of the sheet library. This is the most common and recommended option. It requires that the sheet library is up to date.

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.

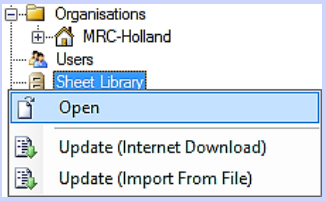
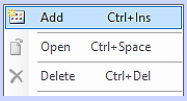
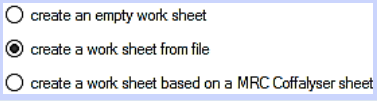
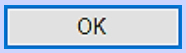
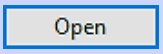
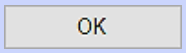
Coffalyser.Net also allows you to add empty Coffalyser sheets (or blank templates) to the active section of the sheet library. This is useful when you have a custom probemix that is not based on a probemix from MRC Holland. All probe-related information has to be added manually to the Coffalyser sheet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

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

1.	Right click on *Sheet Library*	
2.	Select *Open*	
	The Manage Coffalyser Work Sheets window opens	
3.	Right click in the window and select *Add*	
	The Add Coffalyser Work Sheet form appears	
4.	Select *create a work sheet based on a MRC Coffalyser sheet*	
5.	Select the appropriate probemix from the product drop-down menu	
6.	Select the appropriate lot number from the lot drop-down menu	
7.	Click **OK**	
	The Coffalyser Work Sheet Editor window opens	

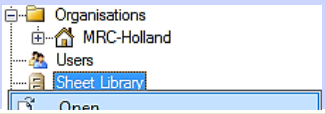
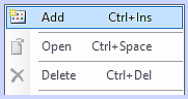
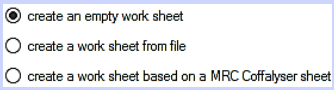
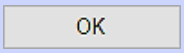
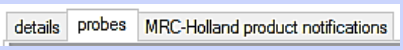
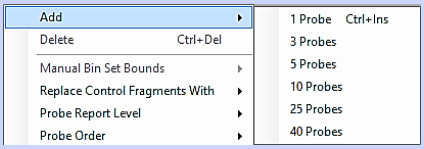
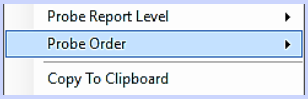
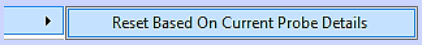
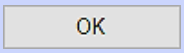
8.	Click **OK** to save the Coffalyser sheet and close the Coffalyser Work Sheet Editor window	

PROCEDURE: IMPORT A COFFALYSER SHEET FILE (CREATE A WORK SHEET FROM FILE)

1.	Right click on *Sheet Library*	
2.	Select *Open*	
	The Manage Coffalyser Work Sheets window opens	
3.	Right click in the window and select *Add*	
	The Add Coffalyser Work Sheet form appears	
4.	Select *create a work sheet from file*	
5.	Click **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**	
	The Coffalyser Work Sheet Editor window opens	
8.	Click **OK** to save the Coffalyser sheet and close the Coffalyser Work Sheet Editor window	

PROCEDURE: ADD AN EMPTY COFFALYSER SHEET

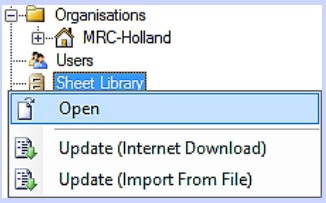
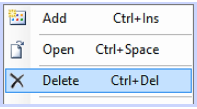
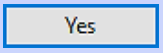
1.	Right click on *Sheet Library*	

2.	Select *Open*	
	The Manage Coffalyser Work Sheets window opens	
3.	Right click in the window and select *Add*	
4.	Select create an empty work sheet	
5.	Click **OK**	
	The Coffalyser Work Sheet Editor window opens	
6.	Navigate to the tab DETAILS	
7.	Enter the required information in the designated fields	
8.	Navigate to the tab PROBES	
9.	Right click in the window and select *Add*	
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*	
13.	Select *Reset Based On Current Probe Details*	
14.	Click **OK** to save the Coffalyser sheet and close the window	

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*	
2.	Select *Open*	
	The Manage Coffalyser Work Sheets window opens	
3.	Right click on the Coffalyser sheet you wish to delete and select *Delete*	
4.	Click **Yes** to confirm you want to delete the Coffalyser sheet	

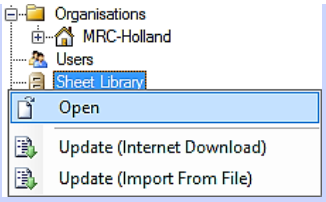
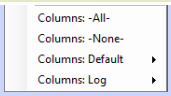
Display or hide columns in the sheet library

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.

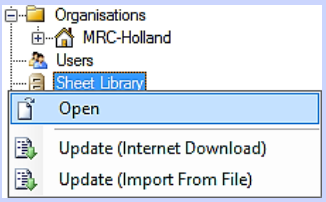
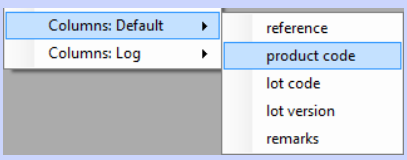
Four groups exist:

- **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

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	

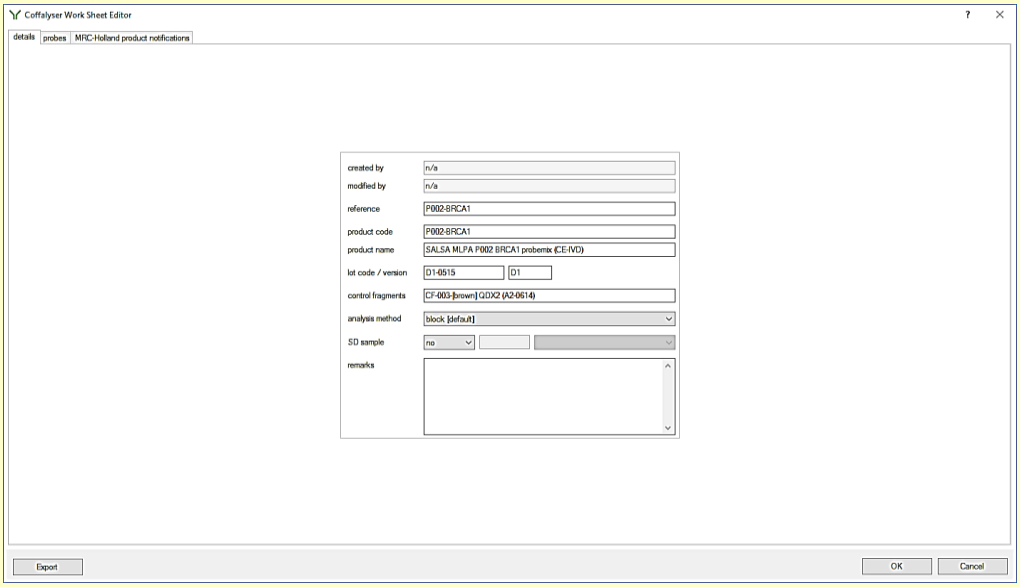
PROCEDURE: DISPLAY OR HIDE A SINGLE COLUMN

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.

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

Reference
The reference name of the probemix.

Product code
The product code of the probemix.

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

Control fragments
The set of control fragments included in the probemix.

Analysis method
The analysis method.

SD sample
It is indicated whether or not an 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.

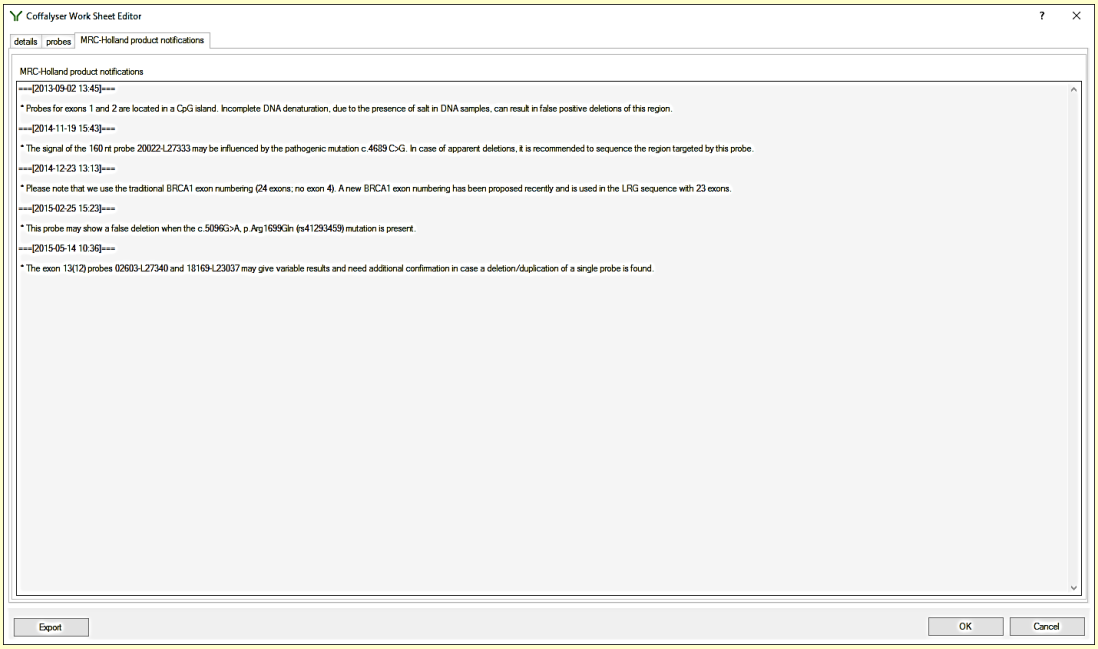
Remarks
Remarks about this worksheet or probemix can be entered in the text field.

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



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

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.

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

Gene
The gene to which the probe is targeted.

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

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

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

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

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

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

Manual_binset_lower_bound
The lower boundary of the probe's bin.

Manual_binset_upper_bound
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.

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 7 to Table 10 all columns are presented per group, together with a description. In addition, it is noted whether information in a column is mandatory. All required information is already available for sheets retrieved from the servers of MRC Holland. You only need to understand the details of a Coffalyser sheet if you work with a custom probemix.

Table 7. Columns: default

Name	Description	Mandatory: Yes / No
Status	Shows if all relevant probe information is entered correctly.	N/A – Is automatically displayed
Order	The number by which probes are ordered in screens and reports.	Yes
Function(s)	The function(s) of a probe in the probemix.	Yes
Gene	The gene to which the probe is targeted.	Yes
GenBank Exon	The exon of the gene to which the probe is targeted.	No
Chromosomal band	The chromosomal band of the target sequence of the probe.	Yes
MV location	The location of the target sequence of the probe based on NCBI Map Viewer hg18.	Yes
Chromosome	The chromosome on which the target sequence of the probe is located.	Yes
MV start	The start location of the target sequence of the probe based on NCBI Map Viewer hg18.	Yes
MV end	The end location of the target sequence of the probe based on NCBI Map Viewer hg18.	Yes
Length (design)	The length on which the probe has been designed.	Yes
Length (Coffalyser)	The length of the probe as found by MRC Holland.	Yes
Manual_binset_lower_bound	The lower boundary of the probe's bin.	Yes
Manual_binset_upper_bound	The upper boundary of the probe's bin.	Yes

Table 8. Columns: Advanced

Name	Description	Mandatory: Yes / No
Report	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)	The number of copies in healthy individuals.	No
Copy number (SD_sample)	The number of copies in the SD sample (when applicable).	No
Probe_weight (target)	The weight of the probe in the slope correction procedure.	No – Not functional yet
Probe_weight (copy_number)	Tthe weight of the probe in the normalisation procedure.	No – Not functional yet

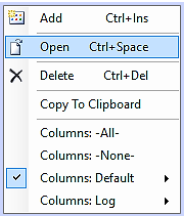
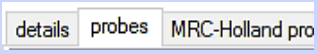
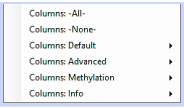
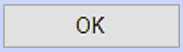
Table 9. Columns: Methylation

Name	Description	Mandatory: Yes / No
Normal methylation % (male)	The methylation percentage of the probe in healthy males.	Only mandatory when a value is present in the Normal Methylation % (female) field
Normal methylation % (female)	The methylation percentage of the probe in healthy females.	Only mandatory when a value is present in the Normal Methylation % (male) field
HhaI	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

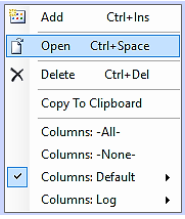
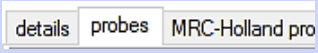
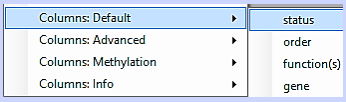
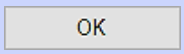
Table 10. Columns: Info

Name	Description	Mandatory: Yes / No
Probe number	The probe number.	N/A – This is MRC Holland's probe number
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	The details of the mutation targeted by a mutation-specific probe.	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.

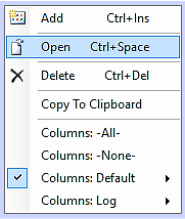
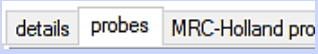
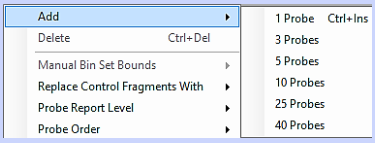
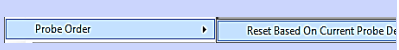
IMPORTANT NOTE:

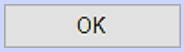
Coffalyser sheets should not be edited in a diagnostic setting. Changes made to a Coffalyser sheet will have an effect on the analysis results!

Add and delete probes in a Coffalyser sheet

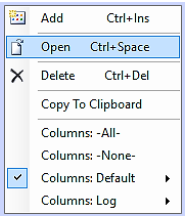
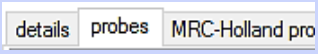
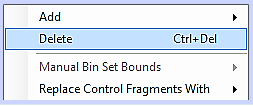
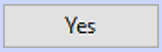
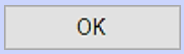
Coffalyser.Net allows you to add and remove probes from a Coffalyser sheet. These functions are predominantly useful when you have a customised MLPA probemix.

PROCEDURE: ADD PROBES TO A COFFALYSER SHEET

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*	

10.	Click **OK** to close the Coffalyser Work Sheet Editor window	

PROCEDURE: DELETE A PROBE FROM A COFFALYSER SHEET

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 probe you wish to delete and select *Delete*	
5.	Click **Yes** to confirm you want to delete the probe	
6.	Right click in the window	
7.	Select *Probe Order* and subsequently *Reset Based On Current Probe Details*	
8.	Click **OK** to close the Coffalyser Work Sheet Editor window	

Probe report levels

It is possible to change the probe labels 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 11 lists all possible probe report levels together with a description of each one of them.

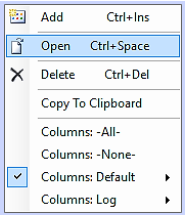
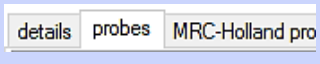
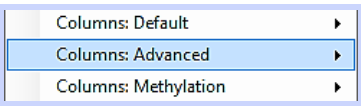
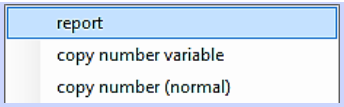
Table 11. Probe report level

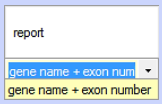
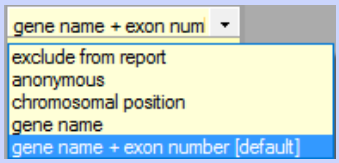
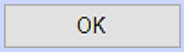
Probe report level	Result in screens and reports
Exclude from report	Probes are not visible*.
Anonymous	The role of the probe is displayed (i.e. Reference or Target).
Chromosomal position	The chromosomal position of the target location of the probe is displayed.
Gene name	The name of the gene that is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

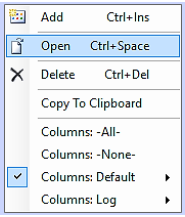
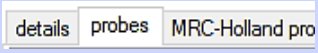
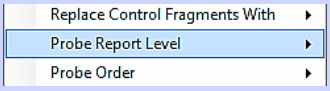
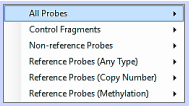
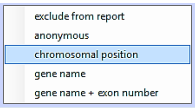
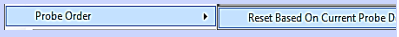
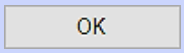
The probe report levels can be adjusted for each probe separately, but also for a subset of probes. The following subsets exist in Coffalyser.Net: 1. All probes, 2. Control fragments, 3. Non-reference Probes, 4. Reference Probes (Any Type), 5. Reference Probes (Copy Number), 6. Reference Probes (Methylation).

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 arrowhead 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	
7.	Right click in the window	
8.	Select *Probe Order* and subsequently *Reset Based On Current Probe Details*	
9.	Click **OK** to close the Coffalyser Work Sheet Editor window	

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 12 presents a list with all supported instruments and formats of the raw data files.

Table 12.

Instrument	Format raw data files
ABI Genetic Analyzer devices	.fsa
Beckman Coulter CEQ devices	.esd
Hitachi DS3000	.fsa
MegaBACE 1000 devices	.rsd
Promega Spectrum Compact	.fsa

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 13 contains an overview of common filter sets and their corresponding dyes for the MLPA probes and size marker.

Table 13.

Instrument	Filter set	Dye MLPA probes	Dye size marker
ABI Genetic Analyzers	C	6-FAM™	TAMRA
	D	6-FAM™	ROX
	G5	6-FAM™	LIZ™
Beckman Coulter	Cy3-Cy5	Cy5	Cy3
Hitachi DS3000	AB 5-dye	6-FAM™	LIZ™
	Filter3 4-dye	6-FAM™	ROX
MegaBACE 1000	FilterSet 1	6-FAM™	ET-ROX
	FilterSet 2	6-FAM™	ET-ROX
Promega Spectrum Compact	T 5-dye	6-FAM™	LIZ™
	Filter3 4-dye	6-FAM™	ROX

Box 5: CE Device Properties window – general tab

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

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

CE device*
The type of the CE device.

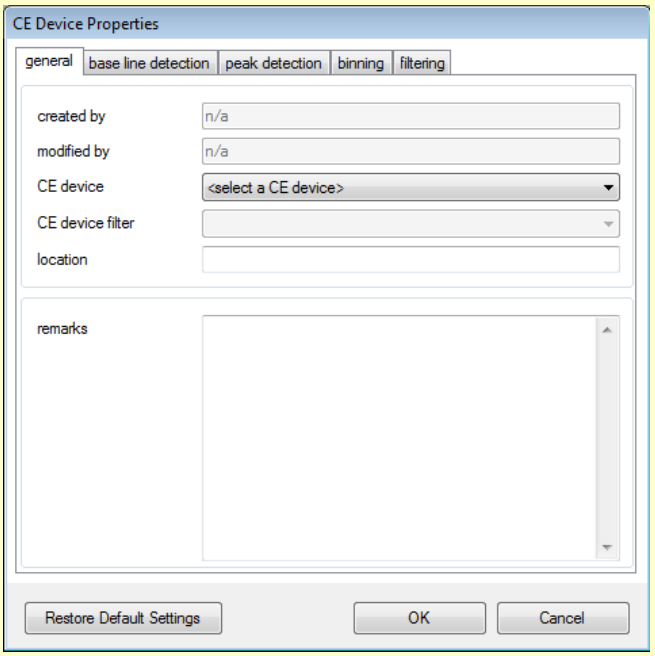
CE device filter*
The filter set of the CE device.

Location
The location or other identification of the CE device.

Remarks
Remarks about the CE device.

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

* Mandatory



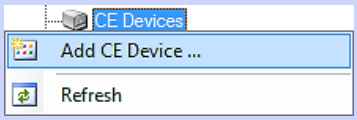
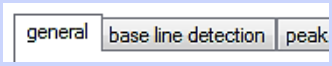
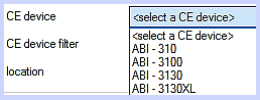
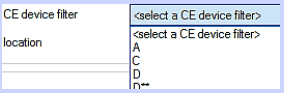
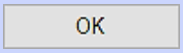
OK button
Saves (changes in) the CE device and closes the CE Device Properties window.

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

Add and delete a CE device

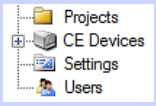
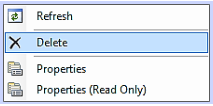
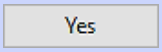
To be able to analyse MLPA data, a CE device has to be created within an organisation. An organisation can hold multiple CE devices. CE devices can only be created and deleted by Organisation Administrators and Server Administrators. Note that a CE device can only be deleted when it is not selected in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

PROCEDURE: CREATE CE DEVICE

1.	Right click on the folder *CE Devices*	
2.	Select *Add CE Device …*	
	The CE Device Properties window opens	
3.	Navigate to the tab GENERAL	
4.	Select the CE device type used for electrophoresis from the CE device drop-down menu	
5.	Select the filter set used during electrophoresis from the CE device filter drop-down menu [6]	
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	

[6] 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	
2.	Right click on the CE device you want to remove and select *Delete*	
3.	Click **Yes** to confirm you want to delete the selected CE device	

Edit a CE device

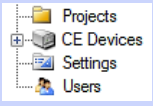
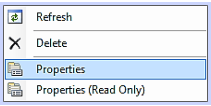
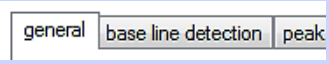
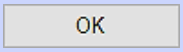
It is possible to adjust the properties of any created CE device. This functionality is reserved for Organisation Administrators and Server Administrators. The parameters for size calling and peak recognition can easily be reset to their default values, in case these have been changed.

IMPORTANT NOTES:

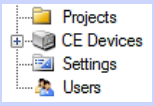
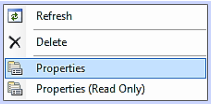
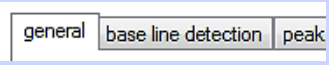
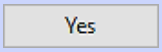
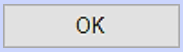
- Only the properties in the tab *General* of a CE device should be edited.
- The other tabs contain the parameters for size calling and peak recognition. These parameters have been set after extensive testing and should not be changed in a diagnostic setting.

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

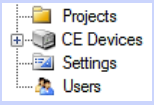
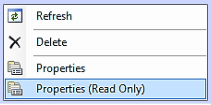
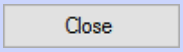
1.	Expand the folder CE Devices by clicking the **+** sign	
2.	Right click on the CE device you want to edit and select *Properties*	
	The CE Device Properties window opens	
3.	Navigate to the tab GENERAL	
4.	Edit the information in the relevant fields	
5.	Click **OK** to save the changes and close the window	

PROCEDURE: RESTORE DEFAULT SETTINGS

1.	Expand the folder CE Devices by clicking the **+** sign	
2.	Right click on the CE device you want to edit and select *Properties*	
	The CE Device Properties window opens	
3.	Navigate to the tab GENERAL	
4.	Click *Restore Default Settings*	
5.	Click **Yes** to confirm you want reset all settings to the default settings	
6.	Click **OK** to close the CE Device Properties window	

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.

1.	Expand the folder CE Devices by clicking the **+** sign	
2.	Right click on the CE device you want to edit and select *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	

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, a fragment might have a length of 140.25 nt in one sample, whereas its length may be 140.45 in another sample.

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 nucleotides (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

A bin set can be checked at two places: in the Sample Results Explorer and in the Edit Manual Bin Set window. 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 10: in all bins a signal has been found, resulting in the bins to be green.

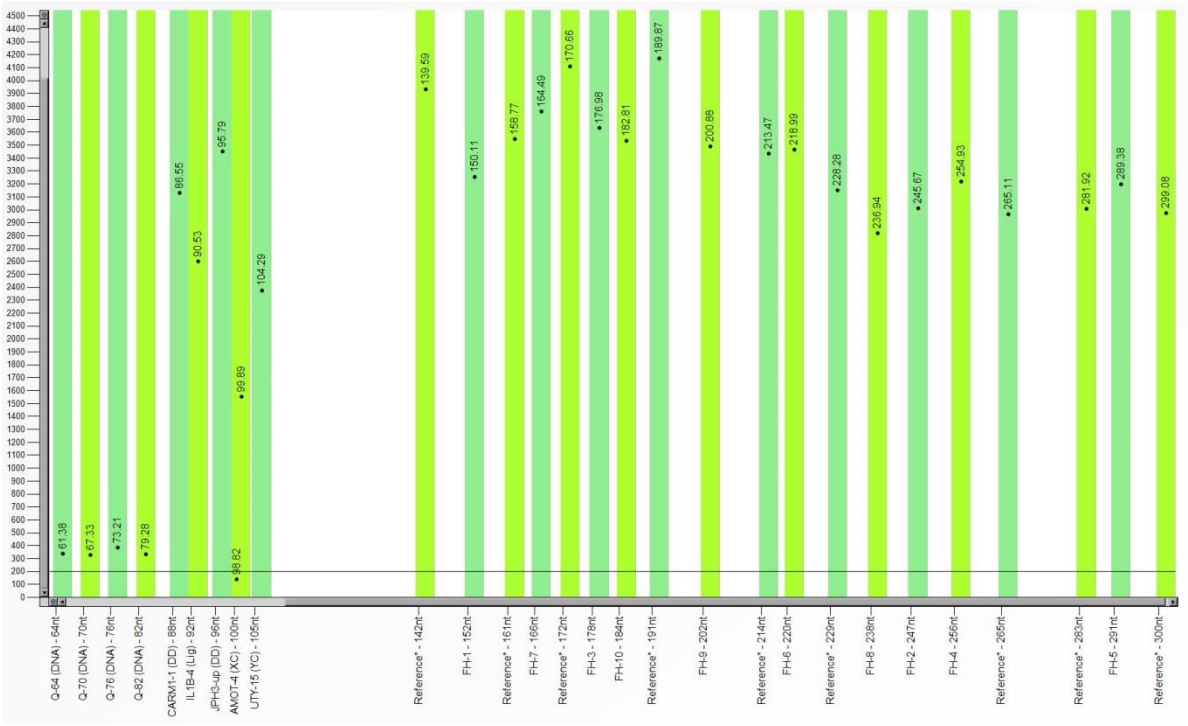


Figure 10. Correct bin set in Coffalyser.Net

When no signal has been found, the bin will be red. See Figure 11 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 will be present outside the bins!

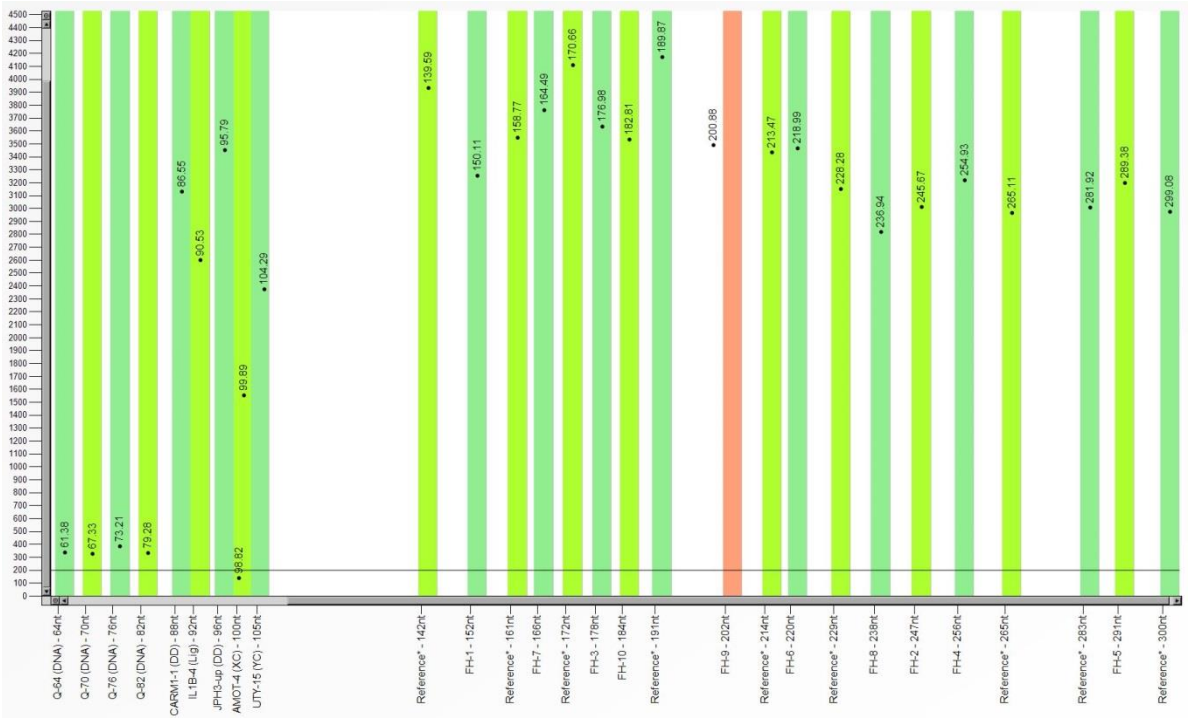


Figure 11. Incorrect bin set in Coffalyser.Net. The signal of the *FH* exon 9 probe falls outside its bin.

In case a signal has been detected in a bin, but more than 0.7 nt away from the centre of the bin, the bin will be coloured purple (see Figure 12).

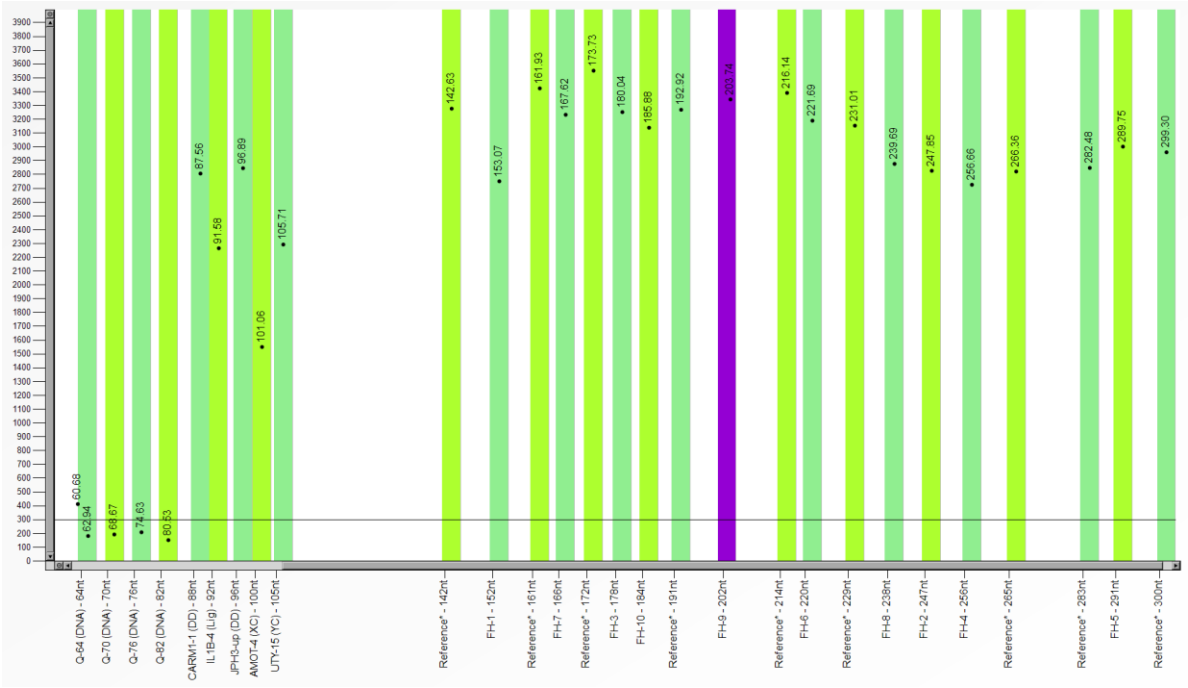
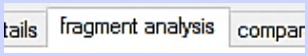
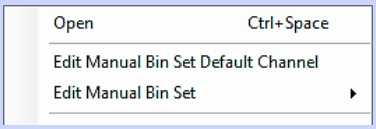
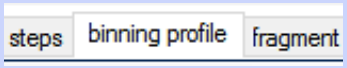
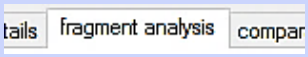
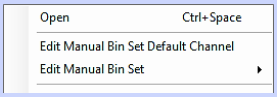


Figure 12. Bin set with the signal of the FH exon 9 probe too far away from the centre of its bin.

PROCEDURE: INSPECT BIN SET IN THE SAMPLE RESULTS EXPLORER

1.	Navigate to the tab FRAGMENT ANALYSIS of an experiment	
2.	Right click on a sample	
3.	Select *Open*	
	The Sample Results Explorer window opens	
4.	Navigate to the tab BINNING PROFILE	
5.	Check the graph for purple and red bins	
6.	Inspect other samples by selecting them from the list on the left side of the window when desired	

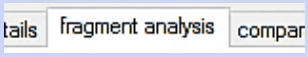
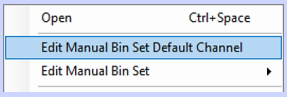
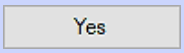
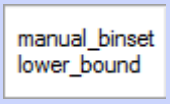
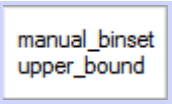
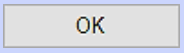
PROCEDURE: INSPECT BIN SET IN THE EDIT MANUAL BIN SET WINDOW

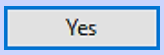
1.	Navigate to the tab FRAGMENT ANALYSIS of an experiment	
2.	Right click in the window and select *Edit Manual Bin Set*	
3.	Select the channel that contains the MLPA probes	
	The Coffalyser Work Sheet Editor – Manual Bin Set window opens	
4.	Check the graph for purple and red bins	
5.	Inspect other samples by selecting them from the list on the left side of the window when desired	

Create or adjust a manual bin set

To ensure that peaks are properly linked to their corresponding probes, it is important that the bin set is correct. In case a probe signal falls outside its bin, Coffalyser.Net won't detect this signal and issues a warning for missing probes. Also, when a probe signal falls within its bin, but more than 0.7 nt away from the centre, Coffalyser.Net warns for this and gives a penalty on the quality check Max probe length deviation. Both can be solved by creating or adjusting a manual bin set.

PROCEDURE: CREATE OR ADJUST A MANUAL BIN SET

1.	Navigate to the tab FRAGMENT ANALYSIS of an experiment	
2.	Right click in the window and select *Edit Manual Bin Set Default Channel*	
3.	When applicable, click **Yes** to replace manual bin set with the autobin results of the last analysis	
	The Coffalyser Work Sheet Editor – Manual Bin Set window opens	
	When adjustments to the bin set (auto bin set and manual bin set) are necessary, proceed with step 4. When no adjustments to the bin set are necessary, continue with step 9.	
4.	Select a sample in which the bin set is incorrect	
5.	Look up in the chart the actual length of the fragment that falls outside its bin	
6.	In the column MANUAL BINSET LOWER BOUND change the value to a value below the actual length of the fragment	
7.	In the column MANUAL BINSET UPPER BOUND change the value to a value above the actual length of the fragment	
	Do not make the bin larger than 4 nt and ensure that it does not overlap with other bins	
8.	When necessary, repeat steps 4 to 7 to adjust the bin for other fragments	
9.	Click **OK** to close the Coffalyser Work Sheet Editor – Manual Bin Set window	
	A dialog box opens	

10. Click **Yes** to set the probe recognition method to manual	

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 14.

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 14. 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]			

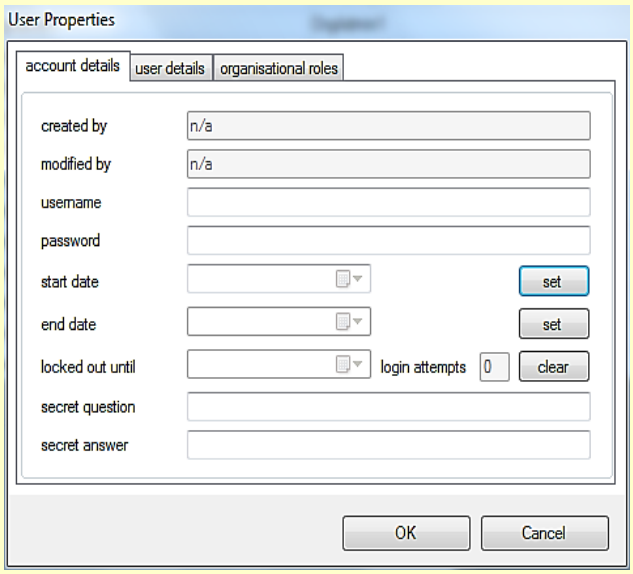
	Server Administrator	Organisation Administrator	Organisation User	Project Administrator	Project User
View the properties of an organisation (read only)	●	●	●	●	●
Create a CE device	●	●			
Delete a CE device	●	●			
Adjust the properties of a CE device	●	●			
View the properties of a CE device (read only)	●	●	●	●	●
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]	●	●
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 *
The surname of the user.

Given name(s)
The given name(s) of the user.

E-mail address
The email address of the user.

Department
The department of the user.

Function
The function of the user.

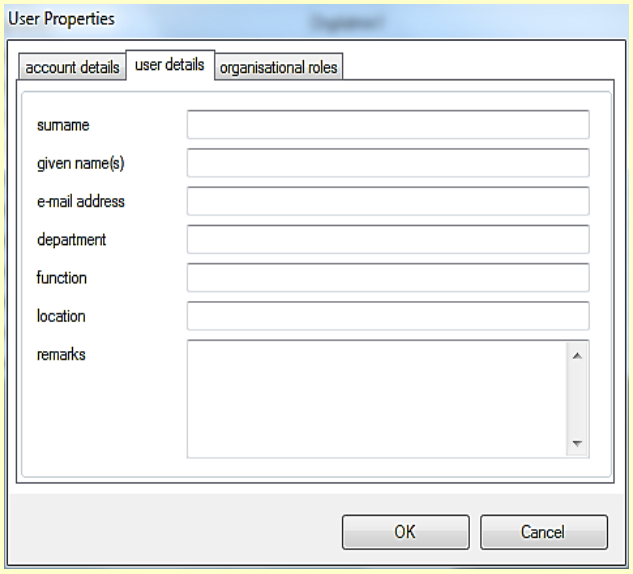
Location
The location/room of the user.

Remarks
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

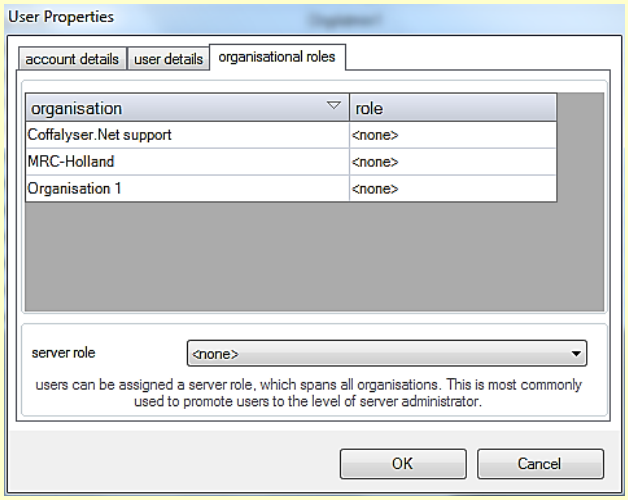
Organisation column
List with Organisations.

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.

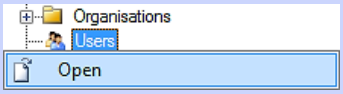
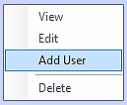
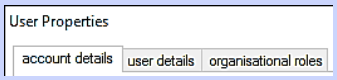
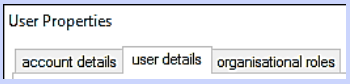
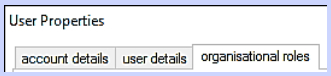
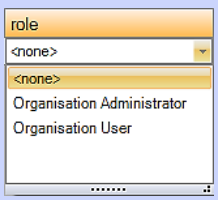
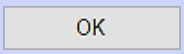
Cancel button
Closes the User Properties menu without saving changes.

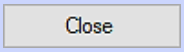


Create and delete user accounts

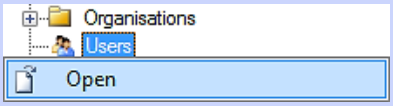
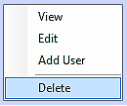
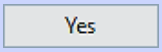
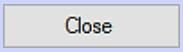
User accounts can be created and deleted by Server Administrators only. Both can be done in the *database* folder 'Users'. Once a user account has been created, it is recommended that the Server Administrator informs the user to change the password of this account and to enter a secret question + answer. 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	
8.	Click on the arrowhead 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	

12. Click **Close** to close the Users window	

PROCEDURE: DELETE USER ACCOUNT

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

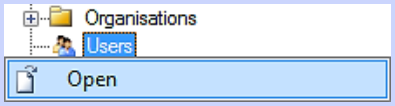
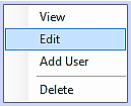
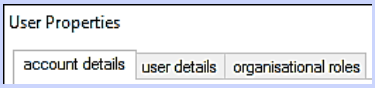
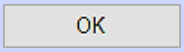
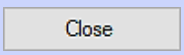
Edit user accounts

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.

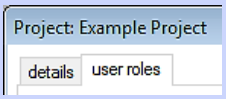
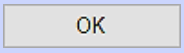
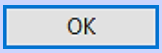
Before users can access the projects created by other users, they must first be granted permission to do so. This can be done by Project Administrators, Organisation Administrators and Server Administrators in the Project properties.

PROCEDURE: EDIT USER ACCOUNT

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

PROCEDURE: EDIT PROJECT USER ROLE

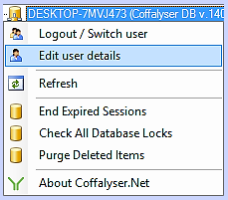
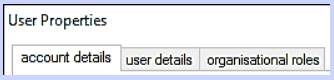
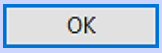
This part describes how access rights to a project can be adjusted for Organisation Users. This can be done by Project Administrators, Organisation Administrators and Server Administrators.

1.	Expand the folder *Projects* in an organisation by clicking the **+** sign next to this folder	
2.	Right click on a project	
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	
5.	Click **OK** to save the changes	
6.	Click **OK** to confirm you have read the notification and to close the notification window	

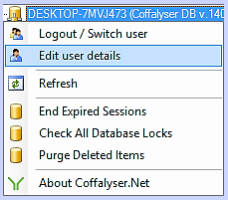
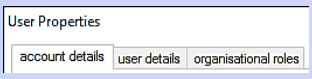
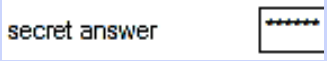
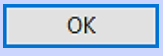
Edit user profile

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*.

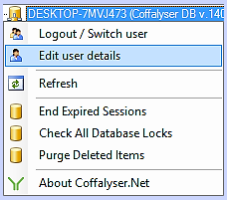
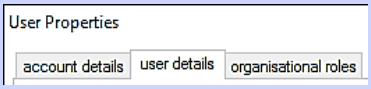
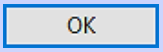
PROCEDURE: CHANGE PASSWORD

1.	Right click on the database icon at the top of the tree structure in the solution explorer	
2.	Select *Edit user details*	
	The User Properties form opens	
3.	Navigate to the tab ACCOUNT DETAILS	
4.	Enter a new password in the designated text field	
5.	Click **OK** to confirm you have read the notification and to close the notification window and User properties form	

PROCEDURE: CHANGE SECRET QUESTION + ANSWER

1.	Right click on the database icon at the top of the tree structure in the solution explorer	
2.	Select Edit user details	
	The User Properties form opens	
3.	Navigate to the tab ACCOUNT DETAILS	
4.	Enter a new secret question in the designated text field	
5.	Enter the answer to the secret question in the designated text field	
6.	Click **OK** to confirm you have read the notification and to close the notification window and User properties form	

PROCEDURE: ADD OR CHANGE USER INFORMATION

1.	Right click on the database icon at the top of the tree structure in the solution explorer	
2.	Select *Edit user details*	
	The User Properties form opens	
3.	Navigate to the tab USER DETAILS	
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	

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IVD	EUROPE* CE ISRAEL
RUO	ALL OTHER COUNTRIES

*comprising EU (candidate) member states, members of the European Free Trade Association (EFTA), and the UK. The product is for RUO in all other European countries.

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

Version 04 – For Coffalyser.Net v.220513.1739, v.240129.1959 and v.250317.1029 – March 2025

- This manual can now also be used for Coffalyser v.250317.1029.

Version 03 – For Coffalyser.Net v.220513.1739 and v.240129.1959 – February 2024

- This manual can now also be used for Coffalyser v.240129.1959.
- Various corrections to the lay-out.
- Small textual changes.

Version 02 – For Coffalyser.Net v.220513.1739 – June 2022

- Added a column *Size marker minimum peak amplitude (in RFU)* to the table Overview thresholds size marker signal intensities.
- Added the following sentence to the FRSS evaluation Median signal height: "This evaluation is only relevant for those devices for which the *Size marker minimum peak amplitude* is smaller than the *Size marker minimum median signal* (see **Table 3**)."
- *Size marker minimum peak amplitude* has been added to the FRSS evaluation Size marker complete.
- Small textual and visual changes.
- The section Methylation-specific MLPA analysis in Appendix I still mentioned percentages for methylation specific probes. This has now been changed to ratios.

Version 01 – For Coffalyser.Net v.220513.1739 – May 2022

- This manual can be used for Coffalyser v.220513.1739.
- Sentence about brand names and logos has been added to the title page.
- Throughout the manual it is now mentioned that the FRSS, FMRS and CAS of all samples should have 4 green bars after the comparative analysis.
- The Important Notes box in the chapter Introduction now mentions that:
  - Serious incidents that have occurred in relation to the device shall be reported to MRC Holland and the competent authority of the Member State in which the user and/or the patient is established.
  - The Summary of Safety and Performance will be available in the European database on medical devices (Eudamed), https://ec.europa.eu/tools/eudamed.
- The information about the display of the results of Methylation-Specific MLPA analysis has been updated to reflect the new version of the software (probe results are displayed as final ratios instead of percentages).
- The information about the display of the results of mutation-specific probes has been updated to reflect the new version of the software.
- Figure 3 and its legend have been updated to reflect the new version of the software.
  - Colours have been updated.
  - New situations have been added
- The section Final ratio vs. Intra ratio percentage has been added.
- The CE devices ABI SeqStudio Flex, Promega Spectrum Compact and Hitachi DS3000 have been added.
- The name of the FMRS evaluation 'Ligation' has been changed to 'Benchmark'. It is now the same as in the software.

- The descriptions of the conditions of the FMRS evaluation Signal quality – Residual primer % have been corrected.
- The Important Notes box in the section Probe counter has been adjusted and now states that peaks will be recognised as probe signals when they are higher than the Probes minimum peak amplitude (in RFU) specified for each instrument type (see Table 5.) AND when they are higher than 10% of the median signal intensity over the reference probes.
- Section about Control fragments in a Coffalyser sheet has been removed.
- Small textual and visual changes.