

# Coffalyser.Net Reference Manual



For Coffalyser.Net v.210226.1433

For Coffalyser.Net v.210604.1451

# Intended purpose

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Coffalyser.Net is an in vitro diagnostic (IVD)<sup>1</sup> or research use only (RUO) software designed for the analysis of (MS-)MLPA data generated using a SALSA® MLPA probemix<sup>2</sup> 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.

<sup>1</sup>Coffalyser.Net analysis software is registered for in vitro diagnostic use (IVD) in specific countries (see [www.mrcholland.com](http://www.mrcholland.com)). In all other countries, Coffalyser.Net analysis software is for research use only (RUO).

<sup>2</sup>To be used in combination with a SALSA MLPA Reagent Kit, SALSA Hhal, and SDOXX as specified in the product description of the application-specific probemix.

<b>INTENDED PURPOSE.....</b>	<b>2</b>
<b>1. INTRODUCTION.....</b>	<b>5</b>
<b>2. LOG IN TO COFFALYSER.NET.....</b>	<b>6</b>
<b>3. SHEET LIBRARY &amp; COFFALYSER SHEETS .....</b>	<b>7</b>
<b>4. CREATE A CAPILLARY ELECTROPHORESIS (CE) DEVICE .....</b>	<b>9</b>
<b>5. CREATE A PROJECT .....</b>	<b>10</b>
<b>6.A SET UP A COPY NUMBER ANALYSIS EXPERIMENT AND ANALYSE DATA.....</b>	<b>11</b>
<b>6.B SET UP A METHYLATION STATUS ANALYSIS EXPERIMENT AND ANALYSE DATA .....</b>	<b>16</b>
<b>7. VIEW ANALYSIS RESULTS.....</b>	<b>21</b>
<b>8. EXPORT RESULTS (OPTIONAL) .....</b>	<b>22</b>
<b>9. OPEN EXISTING EXPERIMENTS .....</b>	<b>23</b>
<b>10. QUALITY SCORES.....</b>	<b>24</b>
<b>APPENDIX I – NORMALISATION AND RESULT INTERPRETATION.....</b>	<b>25</b>
NORMALISATION.....	25
<i>Copy number analysis .....</i>	25
<i>Methylation-Specific MLPA analysis .....</i>	26
RESULTS AND INTERPRETATION .....	27
<i>Copy number analysis .....</i>	27
<i>Methylation-Specific MLPA analysis .....</i>	31
MUTATION SPECIFIC PROBES .....	35
<i>Sample DNA SD .....</i>	35
<i>Normalisation .....</i>	35
<b>APPENDIX II - QUALITY SCORES FRAGMENT ANALYSIS.....</b>	<b>38</b>
FRAGMENT RUN SEPARATION SCORE (FRSS) .....	40
<i>FRSS evaluations .....</i>	42
FRAGMENT MLPA REACTION SCORE (FMRS).....	46
<i>FMRS evaluations.....</i>	49
PROBE COUNTER.....	58
<i>MS-MLPA probemixes .....</i>	58
<i>Probemix with mutation specific probes.....</i>	59

Probe ID colours .....	60
<b>APPENDIX III - SHEET LIBRARY .....</b>	<b>61</b>
UPDATE OF THE SHEET LIBRARY .....	61
MANAGE THE SHEET LIBRARY .....	64
<i>Add Coffalyser sheets to the sheet library .....</i>	65
<i>Delete Coffalyser sheets from the sheet library .....</i>	68
DISPLAY OR HIDE COLUMNS IN THE SHEET LIBRARY .....	69
<b>APPENDIX IV - COFFALYSER SHEETS .....</b>	<b>70</b>
DISPLAY OR HIDE COLUMNS IN A COFFALYSER SHEET.....	73
EDIT COFFALYSER SHEETS.....	76
<i>Add and delete probes in a Coffalyser sheet.....</i>	76
<i>Control fragments in a Coffalyser sheet.....</i>	78
<i>Probe report levels .....</i>	79
<b>APPENDIX V - CE DEVICES.....</b>	<b>82</b>
ADD AND DELETE A CE DEVICE .....	84
EDIT A CE DEVICE .....	86
<b>APPENDIX VI - BIN SET.....</b>	<b>88</b>
INSPECT THE BIN SET .....	88
CREATE OR ADJUST A MANUAL BIN SET .....	92
<b>APPENDIX VII - USER ACCOUNTS.....</b>	<b>94</b>
USER ROLES .....	94
USER ACCOUNT INFORMATION.....	98
CREATE AND DELETE USER ACCOUNTS.....	100
EDIT USER ACCOUNTS.....	102
EDIT USER PROFILE .....	104
<b>CONTACT INFORMATION .....</b>	<b>106</b>
<i>Implemented Changes – compared to the previous version(s). .....</i>	107

# 1. Introduction

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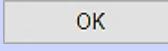
The purpose of this document is to provide a step-by-step guide for the analysis of MLPA data with Coffalyser.Net™. It comprises the procedures for login, software set up, data analysis and result export, which are presented in a chronological order. Instructions for installation of Coffalyser.Net™ can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

## IMPORTANT NOTES:

- Coffalyser.Net is registered for in vitro diagnostic (IVD) use in specific countries (see [www.mrcholland.com](http://www.mrcholland.com)). In all other countries, this product is for research use only (RUO). Please note that when Coffalyser.Net is used with RUO MLPA products, diagnostic decisions based on the results obtained with this program are for the sole responsibility of the user.
- When Coffalyser.Net is used in a diagnostic setting, the quality scores FRSS, FMRS and CAS of all samples should have 4 green bars. The quality scores are explained in chapter **10. Quality scores** of this document.
- Ensure to use the latest version of Coffalyser.Net and the Coffalyser.Net Reference Manual. The latest versions are available online at [www.mrcholland.com](http://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](http://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.

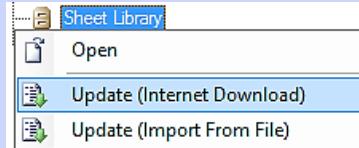
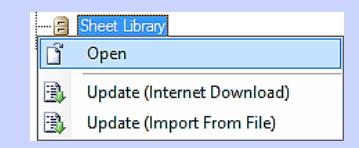
## 2. Log in to Coffalyser.Net

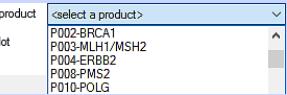
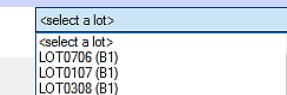
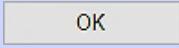
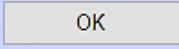
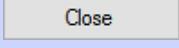
For storage of data (e.g. raw data, experiments, analysis results etc.), Coffalyser.Net uses a database, which is hosted on an SQL server. This SQL server is shown in the Server Selection window, which is displayed when Coffalyser.Net is started. In most cases, only one SQL server is visible in this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

1. Start Coffalyser.Net	
2. In the Server Selection window select the server	
3. Click <b>OK</b>	
 The Login window opens	
4. Enter your user name in the designated field	
5. Enter your password in the designated field	
6. Click <b>Login</b>	

### 3. Sheet library & Coffalyser sheets

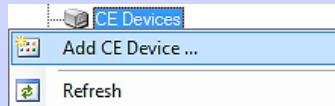
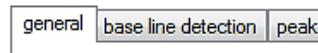
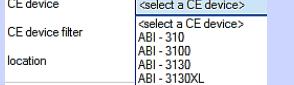
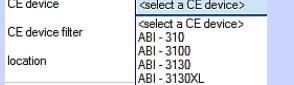
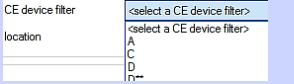
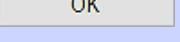
For reliable analysis and result interpretation, it is important that peaks in raw run data are properly recognised as signals coming from MLPA probes and fragments. Coffalyser.Net uses so-called Coffalyser sheets for this process. A Coffalyser sheet contains all necessary information that is specific for one lot of a probemix. Coffalyser sheets are stored in the sheet library. More information about the sheet library and Coffalyser sheets can be found in [Appendix III - Sheet library](#) on page 61 and [Appendix IV - Coffalyser sheets](#) on page 70).

1. Right click on <i>Sheet Library</i>	
2. Select <i>Update (Internet Download)</i>	
 The Download Updates (MRC-Holland) window opens	
3. Click <b>Start Update</b>	<b>Start Update</b>
4. In the Internet Permission window click <b>Yes</b> or <b>Always</b> to start the update	<b>Yes</b> <b>Always</b>
5. Once the update is finished, click <b>Close</b> to close the Download Updates (MRC-Holland) window	<b>Close</b>
6. Right click on <i>Sheet Library</i>	
7. Select <i>Open</i>	
 The Manage Coffalyser Work Sheets window opens	
8. Right click and select <i>Add</i>	
9. In the Add Coffalyser Work Sheet window select create a work sheet based on a MRC Coffalyser sheet	<input type="radio"/> create an empty work sheet <input type="radio"/> create a work sheet from file <input checked="" type="radio"/> 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 <b>OK</b>	
 The Coffalyser Work Sheet Editor window opens	
13. Leave everything in the Coffalyser sheet on default	
14. Click <b>OK</b> 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 <b>Close</b> to close the Manage Coffalyser Work Sheets window	

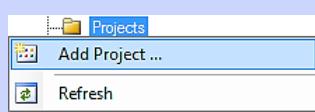
## 4. Create a Capillary Electrophoresis (CE) device

A CE device in Coffalyser.Net contains parameters for size-calling and peak recognition. Before data can be analysed, a CE device has to be created, which can then be linked to experiments later on. More information about CE devices in Coffalyser.Net can be found in **Appendix V - CE devices** on page 82.

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 <i>CE Devices</i>	
3. Select <i>Add CE Device ...</i>	
 The CE Device Properties window opens	
4. Navigate to the tab <b>GENERAL</b>	
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 <b>OK</b> 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 <i>Projects</i>	
2. Select <i>Add Project ...</i>	
 The Project window opens	
3. Navigate to the tab DETAILS	
4. Select a CE device from the drop down menu	CE device <input type="button" value="&lt;select a CE device&gt;"/>
5. Fill in the relevant text fields (only the field Title is mandatory)	
6. Click <b>OK</b> to save the project and close the Project window	<input type="button" value="OK"/>
7. Click <b>OK</b> to close the notification window	<input type="button" value="OK"/>

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

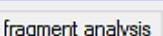
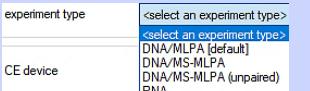
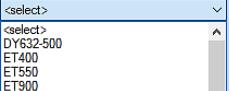
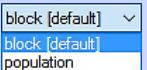
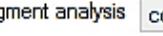
The deepest layer in the data storage structure is formed by experiments. Experiments hold the actual raw MLPA data as well as the analysis results. Per project an unlimited number of experiments can be created.

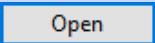
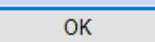
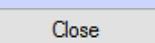
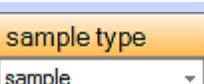
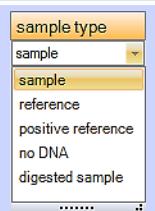
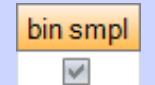
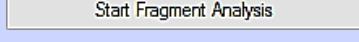
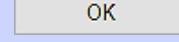
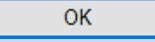
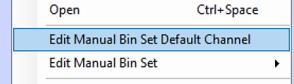
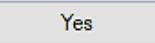
Two types of experiments exist in Coffalyser.Net: one for copy number analysis and one for the combined analysis of copy number and methylation status. In this chapter the procedure for copy number analysis is described. The combined analysis of copy number and methylation status is described in the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

### IMPORTANT NOTES:

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

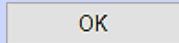
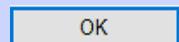
1. Expand the project by clicking the + sign	
2. Right click on the folder <i>Experiments</i>	
3. Select <i>Add Experiment ...</i>	
The Experiment properties window opens	
4. Check if the set CE device is correct. If not, select the appropriate device from the drop down menu	CE device      ABI - 3130XL

5. Fill in the relevant text fields (only the field Title is mandatory)	
6. Click <b>OK</b> to save the experiment and close the Experiment properties window	<b>OK</b>
 The Experiment window opens	
7. Navigate to the tab DETAILS in the window that opens	  
8. Select <i>DNA/MLPA [default]</i> as experiment type from the drop down menu	 experiment type <select an experiment type> <b>DNA/MLPA [default]</b> DNA/MS-MLPA DNA/MS-MLPA (Unpaired) RNA
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	 channel type <b>probes</b> size marker probes
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	 <select a sheet> 
11. Select the applicable Coffalyser sheet from the list	
12. Click <b>OK</b>	<b>OK</b>
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	 <select> <select> DY632-500 ET400 ET550 ET900
14. Leave the setting in the column ANALYSIS METHOD for the dye channel that contains the MLPA probes on default	 <b>block [default]</b> block [default] population
15. Go to the tab FRAGMENT ANALYSIS by clicking <b>Next &gt;&gt;</b>	  
16. Right click in the FRAGMENT ANALYSIS tab and select <i>Add (From File)</i>	 Open Ctrl+Space Edit Manual Bin Set Default Channel Edit Manual Bin Set Add (From File) Ctrl+A
 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 <b>Open</b>	
19. Click <b>OK</b> to confirm the import	
20. Click <b>Close</b> in the Import Files window	
21. In the column SAMPLE TYPE, click on the cell of a sample	
22. Click on the arrow head 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	
<p><b>i</b> If manual bin set has <i>not</i> been created before and is <i>not</i> present in the Coffalyser sheet, proceed with step 25.</p> <p>If a manual bin set has been created before and is present in the Coffalyser sheet, continue with step 35.</p>	
25. In case SALSA binning DNA has been included in the experiment, click the selection box of this sample in the column BIN SMPL.	
26. Click <b>Start Fragment Analysis</b>	
27. Leave all settings on default in the Fragment Analysis Settings window and click <b>OK</b>	
28. Click <b>OK</b> to close the fragment analysis confirmation message	
29. Right click in the window and select <i>Edit Manual Bin Set Default Channel</i>	
30. Click <b>Yes</b>	
 The Coffalyser Work Sheet Editor – Manual Bin Set window opens	

31.	Check if probes fall inside their bin. For more information see the section <b>Inspect the bin set</b> in <b>Appendix VI - Bin set</b> on page 88	
32.	When probe signals fall outside their bin, adjust the bin set according to the procedure as described in the section <b>Create or adjust a manual bin set</b> in <b>Appendix VI - Bin set</b> on page 92	
33.	Click <b>OK</b> to close the Coffalyser Work Sheet Editor – Manual Bin Set window	<b>OK</b>
	A dialog box opens	
34.	Click <b>Yes</b> to set the probe recognition method to manual	<b>Yes</b>
35.	Click <b>Start Fragment Analysis</b>	<b>Start Fragment Analysis</b>
36.	Confirm in the Fragment Analysis Settings window that the probe recognition method is set to <i>manual</i> , otherwise select it from the drop down menu	probe recognition method <input type="button" value="manual"/>
37.	Leave all other settings on default and click <b>OK</b>	<b>OK</b>
38.	Click <b>OK</b> to close the fragment analysis confirmation message	<b>OK</b>
<b>i</b>	When not all probes have been found as indicated by the probe counter, inspect the bin set ( <b>Inspect the bin set</b> in <b>Appendix VI - Bin set</b> on page 88) and adjust it when necessary ( <b>Create or adjust a manual bin set</b> in <b>Appendix VI - Bin set</b> on page 92). 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 <sup>(1)</sup> scores show 4 green bars, and if the DNA and DD columns show a green icon.	
<b>!</b>	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 <b>Next &gt;&gt;</b>	<b>analysis</b> <b>comparative analysis</b>
41.	Select samples that show 4 green bars for the FMRS to be included in the comparative analysis by clicking the selection box in the column ANALYSIS	<b>analy ...</b>
42.	Click <b>Start Comparative analysis</b>	<b>Start Comparative Analysis</b>

<sup>1</sup> See chapter 10. Quality scores for a description.

43. Leave all settings on default in the Comparative Analysis Settings window and click <b>OK</b>	
44. Click <b>OK</b> to close the comparative analysis confirmation message	
45. Check if the CAS <sup>(2)</sup> score shows 4 green bars for each sample that has been included in the comparative analysis	
46. Check the PSLP, FSLP, RSQ and RPQ <sup>(2)</sup> of samples that do not show 4 green bars by hovering the cursor over these quality checks	
<p> Only the results of samples that show 4 green bars for the CAS can reliably be interpreted.</p>	

<sup>2</sup> See chapter 10. Quality scores for a description.

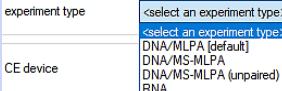
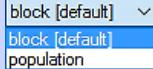
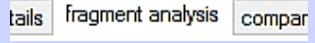
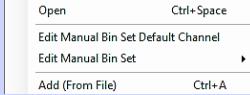
## 6.b Set up a methylation status analysis experiment and analyse data

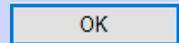
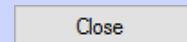
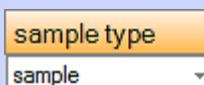
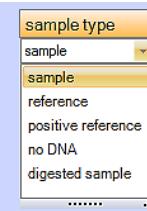
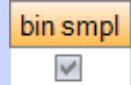
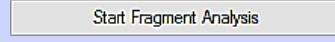
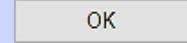
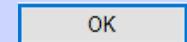
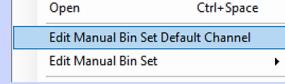
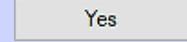
Two types of experiments exist in Coffalyser.Net: one for copy number analysis and one for the combined analysis of copy number and methylation status. In this chapter the combined analysis of copy number and methylation status is described. The procedure for copy number analysis is described in the 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 have to be left on their default values in a diagnostic setting. These parameters have been determined after extensive testing and changing them might lead to inclusion of samples with a lower quality in the analysis. Adjusting these parameters should only be done in a research setting.
- When Coffalyser.Net is used in a diagnostic setting, the quality scores FRSS, FMRS and CAS of all samples should have 4 green bars.

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

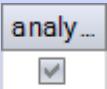
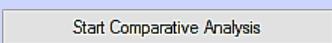
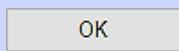
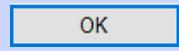
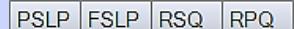
6. Click <b>OK</b> to save the experiment and close the Experiment properties window	
 The Experiment window opens	
7. Navigate to the tab DETAILS in the window that opens	
8. Select DNA/MS-MLPA as experiment type from the drop down menu	
9. Check for each dye channel if the type of fragments is set 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 <b>OK</b>	
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 <b>Next &gt;&gt;</b>	
16. Right click in the FRAGMENT ANALYSIS tab and select <i>Add (From File)</i>	
 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 <b>Open</b>	

19. Click <b>OK</b> to confirm the import	
20. Click <b>Close</b> in the Import Files window	
21. In the column SAMPLE TYPE, click on the cell of a sample	
22. Click on the arrow head to expand the list with sample types	
23. Select a sample type from the list. All samples <sup>(3)</sup> to which the restriction enzyme Hhal has been added should be defined as digested sample	
24. Repeat steps 21 to 23 for the other samples	
<p><b>i</b> If manual bin set is <u>not</u> present in the Coffalyser sheet, proceed with step 25.        If a manual bin set is present in the Coffalyser sheet, continue with step 35.</p>	
25. In case SALSA binning DNA has been included in the experiment, click the selection box of this sample in the column BIN SMPL	
26. Click <b>Start Fragment Analysis</b>	
27. Leave all settings on default in the Fragment Analysis Settings window and click <b>OK</b>	
28. Click <b>OK</b> to close the fragment analysis confirmation message	
29. Right click in the window and select <i>Edit Manual Bin Set Default Channel</i>	
30. Click <b>Yes</b>	
 The Coffalyser Work Sheet Editor – Manual Bin Set window opens	

<sup>3</sup> 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 Hhal restriction enzyme is added should be defined as 'No DNA'.

31.	Check if probes fall inside their bin. For more information see the section <b>Inspect the bin set</b> in <b>Appendix VI - Bin set</b> on page 88	
32.	When probe signals fall outside their bin, adjust the bin set according to the procedure as described in the section <b>Create or adjust a manual bin set</b> in <b>Appendix VI - Bin set</b> on page 92	
33.	Click <b>OK</b> to close the Coffalyser Work Sheet Editor – Manual Bin Set window	<b>OK</b>
	 A dialog box opens	
34.	Click <b>Yes</b> to set the probe recognition method to manual	<b>Yes</b>
35.	Click <b>Start Fragment Analysis</b>	<b>Start Fragment Analysis</b>
36.	Confirm in the Fragment Analysis Settings window that the probe recognition method is set to <i>manual</i> , otherwise select it from the drop down menu	probe recognition method <input type="button" value="manual"/>
37.	Leave all other settings on default and click <b>OK</b>	<b>OK</b>
38.	Click <b>OK</b> to close the fragment analysis confirmation message	<b>OK</b>
<b>i</b> When not all probes have been found as indicated by the probe counter, inspect the bin set ( <b>Inspect the bin set</b> in <b>Appendix VI - Bin set</b> on page 88 and adjust it when necessary ( <b>Create or adjust a manual bin set</b> in <b>Appendix VI - Bin set</b> on page 92)). 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 <sup>(4)</sup> 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.	
<b>!</b> 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 <b>Next &gt;&gt;</b>	<b>analysis</b> <b>comparative analysis</b>
41.	In the column DIGESTED, click on the cell of a (undigested) sample	<input type="button" value="digested"/> <input type="button" value="(none)"/>
42.	Click on the arrow head to expand the list with digested samples	<input type="button" value="digested"/> <input type="button" value="(none)"/> 126-REF-DIG-ME028-C1-0118-KTU

<sup>4</sup> See chapter 10. Quality scores for a description.

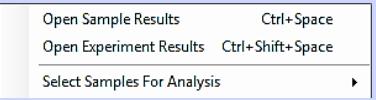
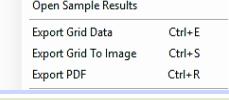
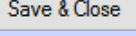
43.	Select the digested sample corresponding to the undigested sample	
44.	Repeat steps 41 to 43 for the other samples	
45.	Select samples to be included in the comparative analysis by clicking the selection box in the column ANALYSIS	
46.	Click <b>Start Comparative analysis</b>	
47.	Leave all settings on default in the Comparative Analysis Settings window and click <b>OK</b>	
48.	Click <b>OK</b> to close the comparative analysis confirmation message	
49.	Check if the CAS <sup>(5)</sup> score shows 4 green bars for each sample that has been included in the comparative analysis	
50.	Check the PSLP, FSLP, RSQ and RPQ <sup>(5)</sup> of samples that do not show 4 green bars by hovering the cursor over these quality checks	
<b>⚠ Only the results of samples that show 4 green bars for the CAS can reliably be interpreted.</b>		

<sup>5</sup> See chapter 10. Quality scores for a description.

## 7. View analysis results

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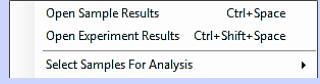
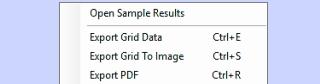
Coffalyser.Net offers the possibility to view the analysis results of all samples at once, but results of individual samples can also be viewed. It is recommended to first view the results of the complete experiment. In this way samples of interest can easily be spotted and subsequently be assessed in 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 25.

1. Right click in the window and select <i>Open Experiment Results</i> to open the results of the complete experiment	
 The Comparative analysis experiment explorer opens	
2. Right click on a sample of interest and select <i>Open Sample Results</i> to open the sample results	
 The Comparative Analysis Sample Results Explorer opens	
3. Close both results explorers by clicking <b>X</b> in the top right corners	
4. Click <b>Save &amp; Close</b> to save the analysis results and to close the experiment	

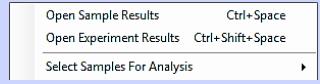
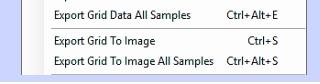
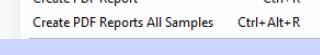
## 8. Export results (optional)

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](#)). The instructions below have the Comparative Analysis tab as starting point.

### EXPORT RESULTS OF A COMPLETE EXPERIMENT

1. Navigate to the tab COMPARATIVE ANALYSIS	
2. Right click in the window and select <i>Open Experiment Results</i> 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 <i>Open Sample Results</i> 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 <i>Create PDF Report</i> to export the results to a pdf file when desired	

## 9. Open existing experiments

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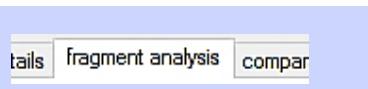
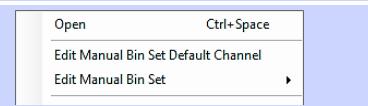
1. Expand the organisation that holds the experiment by clicking the + sign	 Organisation 1
2. Expand the project that holds the experiment by clicking the + sign	 Project 1
3. Expand the folder Experiments by clicking the + sign	 Experiments
4. Right click on the experiment you want to open	
5. Select Open	

## 10. Quality scores

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

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

1. Navigate to the tab <b>FRAGMENT ANALYSIS</b>	
2. Right click on a sample and select <b>Open</b>	
 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

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

## Normalisation

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### 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 ( $T_{p1}$ ) is divided by the signal intensity of reference probe 1 ( $R_{p1}$ ) in sample 1. The same is done in reference sample 1. The first value is then divided by the second value.

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

$$\text{Median} \left( \frac{(T_{p1} \text{ in sample 1}/R_{p1} \text{ in sample 1})}{(T_{p1} \text{ in reference sample 1}/R_{p1} \text{ in reference sample 1})}, \dots, \frac{(T_{p1} \text{ in sample 1}/R_{pn} \text{ in sample 1})}{(T_{p1} \text{ in reference sample 1}/R_{pn} \text{ in reference sample 1})} \right)$$

#### 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 in a probemix and samples in an experiment.

## No reference samples defined in analysis

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

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

## Methylation-Specific MLPA analysis

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

Simplified, the second part of the normalisation works as follows:

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

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

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

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

This procedure is repeated for all probes and samples.

## Results and interpretation

### IMPORTANT NOTES:

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

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

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

## Copy number analysis

### 95% confidence interval

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

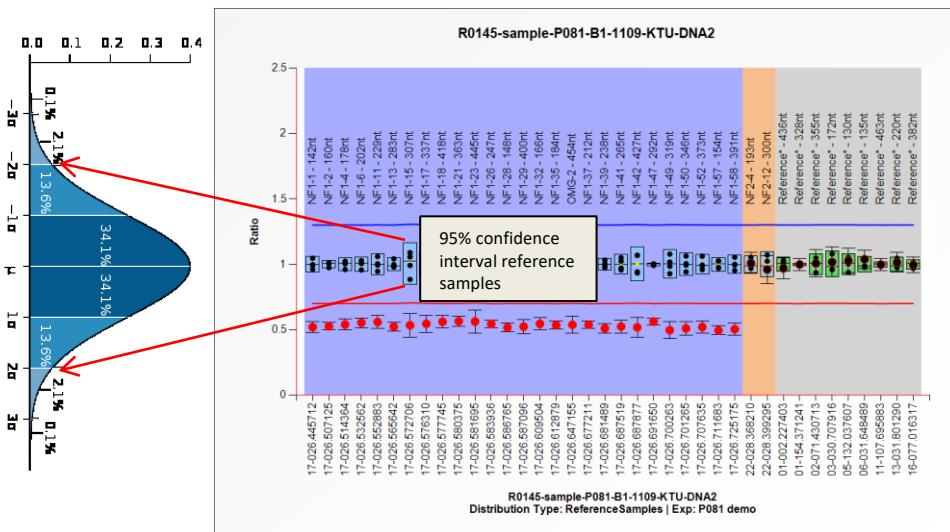
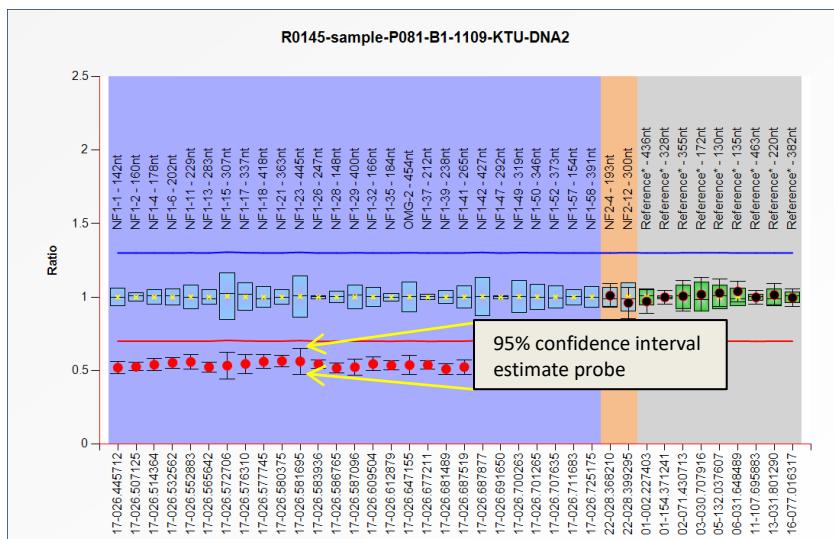


Figure 1. 95% confidence intervals over the reference samples.

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



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

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

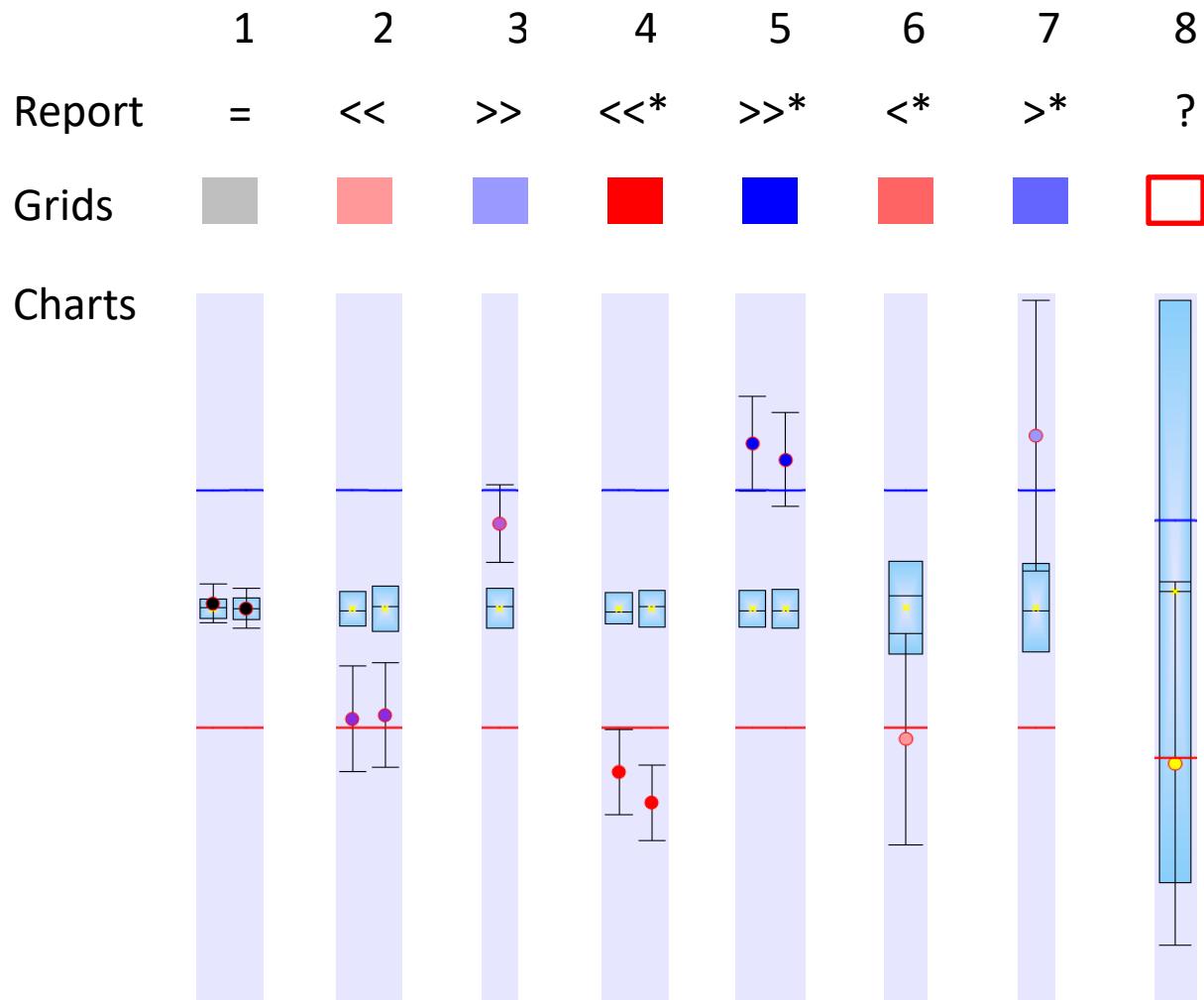
### Arbitrary borders

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

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

## Display of results in Coffalyser.Net

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



**Figure 3.** Probe results in Coffalyser.Net.

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

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

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

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

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

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

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

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

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

## Methylation-Specific MLPA analysis

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

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

**Table 1** MS-MLPA results

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

To determine whether the obtained results indicate an aberration, they should be compared to the results obtained for the reference samples, which are expected to have a 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 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 Hhal restriction site, experimental variation etc.), therefore, the arbitrary borders are not straight lines.

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

## Display of results in Coffalyser.Net

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

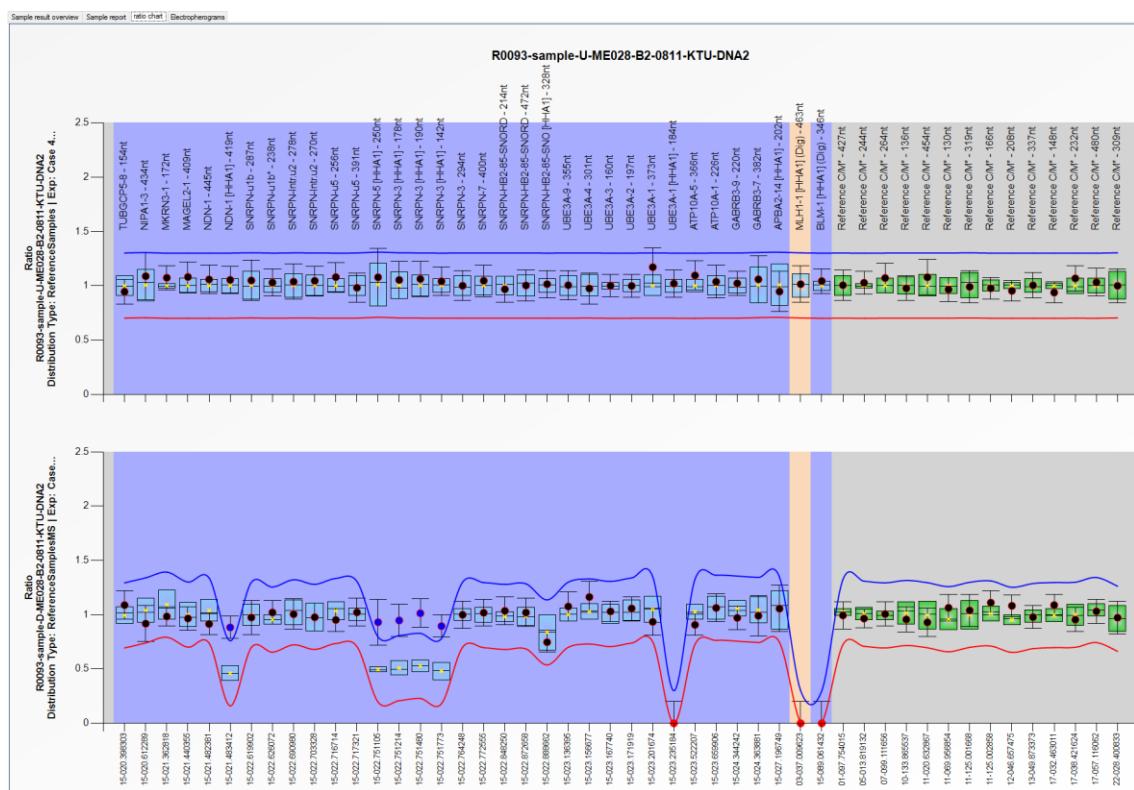
In the *Comparative analysis experiment explorer* Coffalyser.Net by default displays the results of the undigested and digested counterparts of a sample directly adjacent to each other (see Figure 4).

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

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

**A:** Sample R0093 **B:** Sample R0400

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

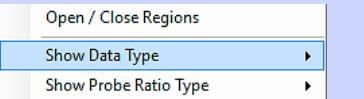
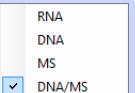


**Figure 5** Ratio charts of a sample in the Comparative Analysis Sample Results Explorer. The upper chart displays the results of the undigested counterpart / the copy number analysis and the lower chart displays the results of the digested counterpart / the methylation status analysis.

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

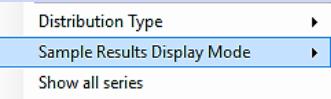
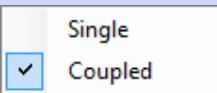
#### PROCEDURE: CHANGE THE DISPLAY OF RESULTS IN THE TAB RATIO OVERVIEW OF THE COMPARATIVE ANALYSIS EXPERIMENT EXPLORER

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1. In the tab ratio overview of the Comparative analysis experiment explorer right click and select <i>Show Data Type</i>	
2. From the appearing list select <i>DNA</i> or <i>MS</i> 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 <i>DNA/MS</i>	

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

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1. In the tab ratio chart of the Comparative Analysis Sample Results Explorer right click on a chart and select <i>Sample Results Display Mode</i>	
2. From the appearing list select <i>Single</i>	
3. To see the combined results again, follow steps 1 and 2 and select <i>Coupled</i>	

## Mutation specific probes

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

## Sample DNA SD

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

For most MLPA probemixes that contain mutation specific probes, a special Sample DNA (SD) will be supplied with the probemix. In this SD, DNA target sequences for all probes are present (including mutation specific sequences). The MLPA data of the SD sample is extremely useful for creating a manual bin set. See section **6.a Set up a copy number analysis experiment and analyse data** (step 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 88 contains instructions for creating a manual bin set.

## Normalisation

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

### Reference samples included in experiment

#### ➤ No signal for mutation specific probe present in reference samples

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

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

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

#### ➤ Signal for mutation specific probe present in reference samples

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

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

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

#### No reference samples included in experiment

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

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<sup>6</sup> Note that this percentage does not indicate the percentage of cells carrying the mutation!

In samples where the signal of the mutation specific probe is lower than 10% of the median signal of the reference probes, Coffalyser.Net displays the intra-normalised ratio of the mutation specific probe in the Experiment results window and PDF report as percentage<sup>7</sup>. In the ratio chart the intra-normalised ratio is presented as an orange box. In case only one sample contains a signal of the mutation specific probe the final ratio will be 1.

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

#### IMPORTANT NOTE:

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

<sup>7</sup> Note that this percentage does not indicate the percentage of cells carrying the mutation!

## Appendix II - Quality scores Fragment Analysis

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

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	REFERENCE1-P081-B1-1109	reference	[bar chart]	[bar chart]	[bar chart]	38/38	[checkmark]	[checkmark]	[checkmark]	[checkmark]	A11
2	REFERENCE2-P081-B1-1109	reference	[bar chart]	[bar chart]	[bar chart]	38/38	[checkmark]	[checkmark]	[checkmark]	[radio button]	C11
3	REFERENCE3-P081-B1-1109	reference	[bar chart]	[bar chart]	[bar chart]	38/38	[checkmark]	[checkmark]	[checkmark]	[checkmark]	E11
4	REFERENCE4-P081-B1-1109	reference	[bar chart]	[bar chart]	[bar chart]	38/38	[checkmark]	[checkmark]	[checkmark]	[radio button]	G11
5	noDNA-P081-B1-1109	no DNA	[bar chart]	[bar chart]	[bar chart]	0/0	[radio button]	[radio button]	[radio button]	[radio button]	F10
6	SAMPLE2-P081-B1-1109	sample	[bar chart]	[bar chart]	[bar chart]	38/38	[checkmark]	[checkmark]	[checkmark]	[radio button]	F11

**Figure 6.** Experiment – fragment analysis tab.

The following items 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 the DNA concentration (also part of the FMRS).

- **DD** - check for the DNA denaturation (also part of the FMRS).
- **DIG** - check for the 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 the X chromosome control fragment.
- **Y** - Indicates the presence of the 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)

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

**Table 2. Overview FRSS scores**

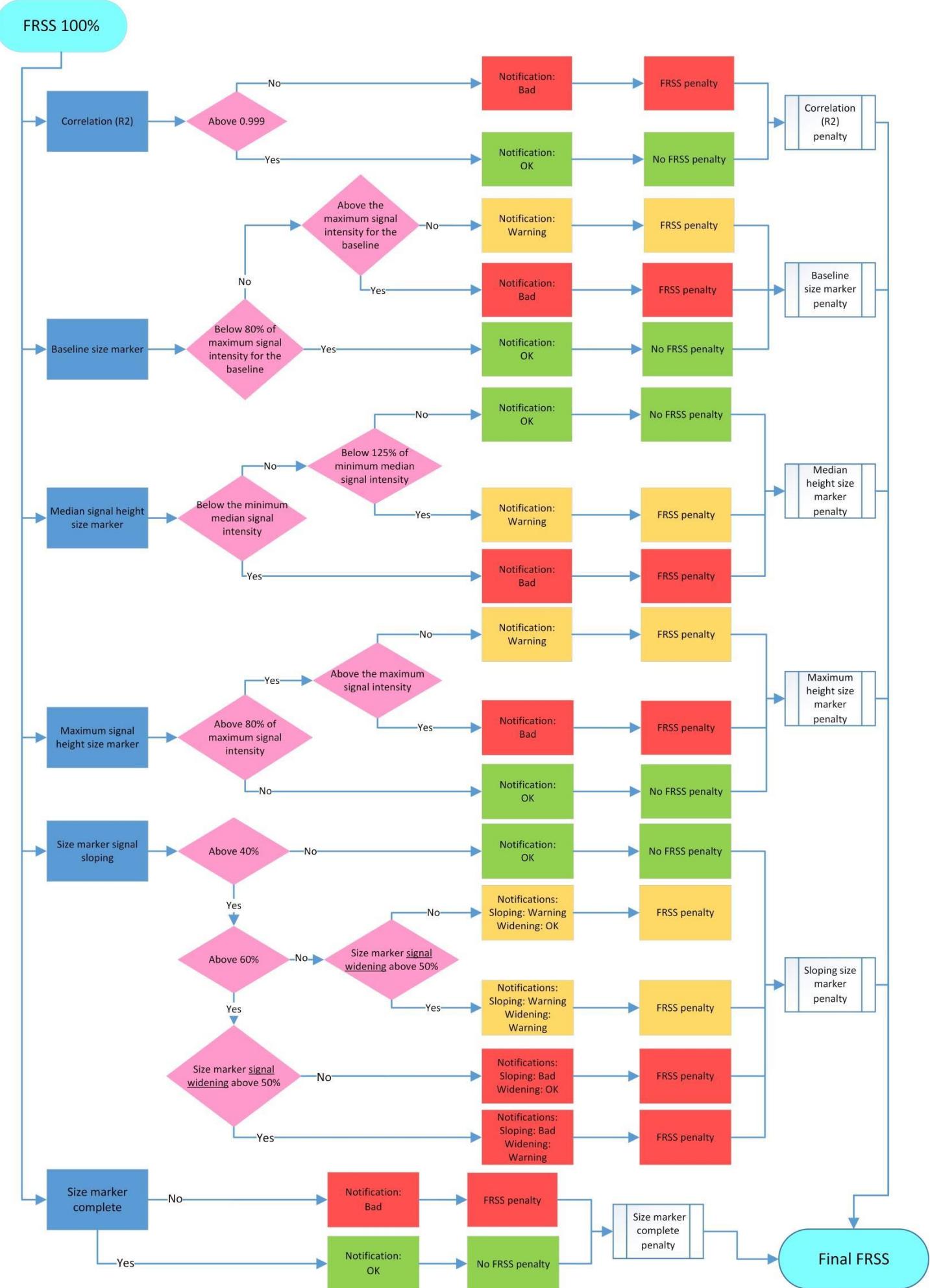
FRSS	Points
	≥ 90
	≥ 75 and < 90
	≥ 45 and < 75
	> 25 and < 45
	≤ 25

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

**Table 3. Overview thresholds size marker signal intensities**

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

## Diagram calculation Fragment Run Separation Score (FRSS)



## FRSS 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 <b>Table 3</b> )	Ok	No penalty
	Between 80% and 100% of the Size marker maximum signal of the baseline specified for the Capillary Electrophoresis instrument (see <b>Table 3</b> )	Warning	10 points
	Above 100% of the Size marker maximum signal of the baseline specified for the Capillary Electrophoresis instrument (see <b>Table 3</b> )	Bad	15 points

#### Related issues and solutions:

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

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

### Median signal height

#### Background:

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

#### Conditions:

		Notification	Penalty
	Above 125% of the Size marker minimum median signal specified for the Capillary Electrophoresis instrument (see <b>Table 3</b> )	Ok	No penalty
	Between 100% and 125% of the Size marker minimum median signal specified for the Capillary Electrophoresis instrument (see <b>Table 3</b> )	Warning	10 points
	Below 100% of the Size marker minimum median signal specified for the Capillary Electrophoresis instrument (see <b>Table 3</b> )	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 <b>Table 3</b> )	Ok	No penalty
	Between 80% and 100% of the Size marker maximum signal specified for the Capillary Electrophoresis instrument (see <b>Table 3</b> )	Warning	10 points
	Above 100% of the Size marker maximum signal specified for the Capillary Electrophoresis instrument (see <b>Table 3</b> )	Bad	15 points

#### Related issues and solutions:

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

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

### **Size marker signal sloping**

#### Background:

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

#### Conditions:

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

#### Related issues and solutions:

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

### **Size marker signal widening**

#### Background:

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

Conditions:

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

Related issues and solutions:

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

**Size marker complete**

Background:

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

Conditions:

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

Related issues and solutions:

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

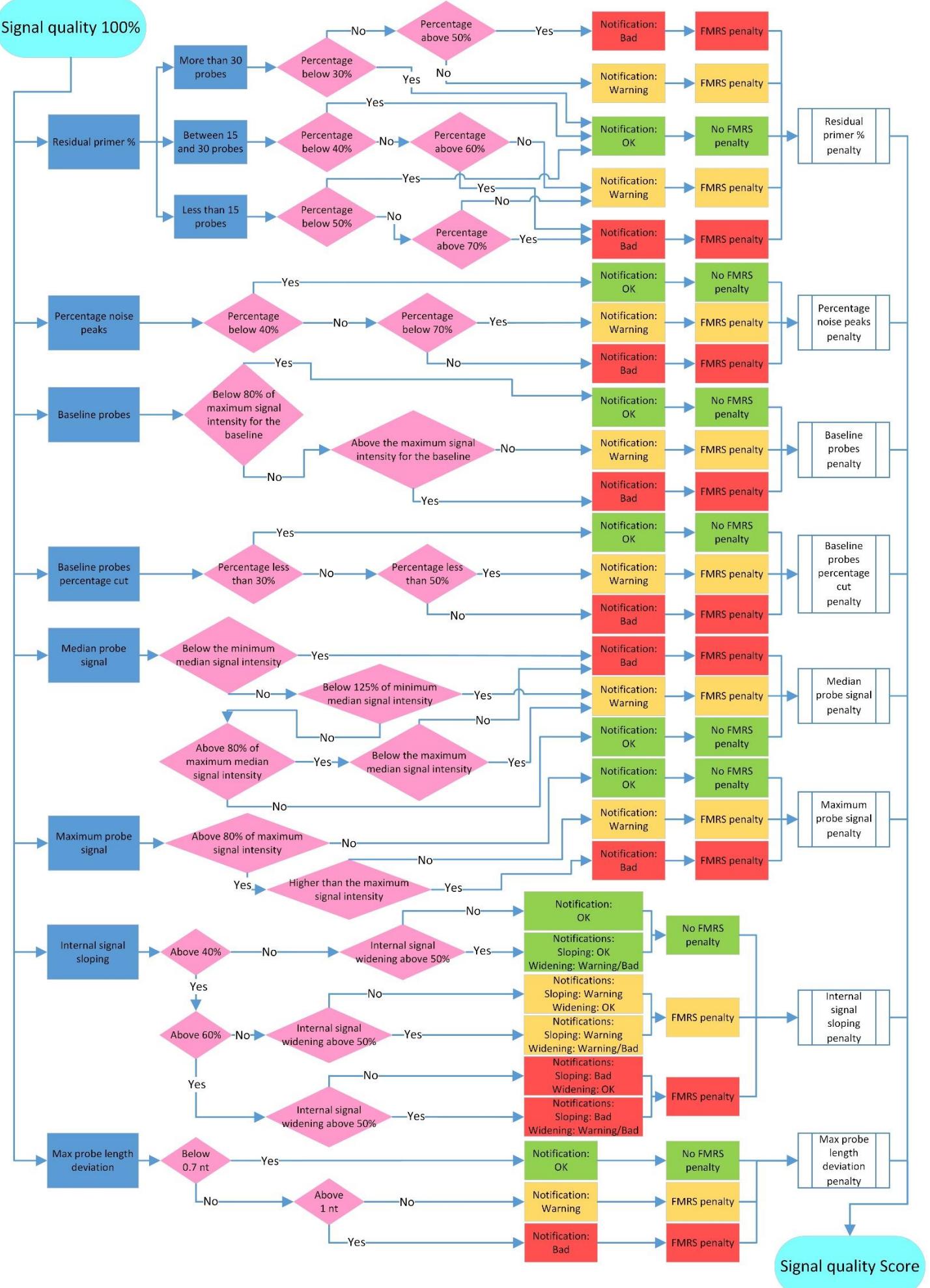
**Table 5. Overview probe peak signal intensities**

	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	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

## Diagram calculation Fragment MLPA Reaction Score (FMRS)



Diagram Signal quality



## FMRS evaluations

### Ligation

#### Background:

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

#### Conditions:

		Notification	Penalty
	Above 125% of the minimum median signal and below 80% of the maximum median signal intensity specified for the Capillary Electrophoresis instrument (see <b>Table 5</b> )	Ok	No penalty
	Between 100% and 125% of the minimum median signal specified for the Capillary Electrophoresis instrument (see <b>Table 5</b> )	Warning	20 points
	Between 80% and 100% of the maximum signal specified for the Capillary Electrophoresis instrument (see <b>Table 5</b> )	Warning	20 points
	Below 100% of the minimum median signal specified for the Capillary Electrophoresis instrument (see <b>Table 5</b> )	Bad	60 points
	Above 100% of the maximum signal specified for the Capillary Electrophoresis instrument (see <b>Table 5</b> )	Bad	60 points

#### Related issues and solutions:

When this fragment is too low or too high, it is usually accompanied by other phenomena affecting the quality of the raw data.

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 92.

### Concentration

#### Background:

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

#### Conditions:

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

#### Related issues and solutions:

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

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

#### **Denaturation**

##### Background:

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

#### Conditions:

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

#### Related issues and solutions:

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

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

#### **Digestion (only DNA/MS-MLPA)**

##### Background:

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

##### Conditions:

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

##### Related issues and solutions:

A signal of the Digestion control probes in samples to which the Hhal 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 Hhal 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 more than 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 more than 30 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 <b>Table 5</b> )	Ok	No penalty
	Between 80% and 100% of the Probes maximum signal of the baseline specified for the Capillary Electrophoresis instrument (see <b>Table 5</b> )	Warning	10 points
	Above 100% of the Probes maximum signal of the baseline specified for the Capillary Electrophoresis instrument (see <b>Table 5</b> )	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:

		<b>Notification</b>	<b>Penalty</b>
	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 3x the signal of the baseline.

Conditions:

		<b>Notification</b>	<b>Penalty</b>
	Above 125% of the Probes minimum median signal specified for the Capillary Electrophoresis instrument (see <b>Table 5</b> )	Ok	No penalty
	Below 80% of the Probes maximum median signal specified for the Capillary Electrophoresis instrument (see <b>Table 5</b> )	Ok	No penalty
	Between 100% and 125% of the Probes minimum median signal specified for the Capillary Electrophoresis instrument (see <b>Table 5</b> )	Warning	10 points
	Between 80% and 100% of the Probes maximum median signal specified for the Capillary Electrophoresis instrument (see <b>Table 5</b> )	Warning	10 points
	Below 100% of the Probes minimum median signal specified for the Capillary Electrophoresis instrument (see <b>Table 5</b> )	Bad	20 points
	Above 100% of the Probes maximum median signal specified for the Capillary Electrophoresis instrument (see <b>Table 5</b> )	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:

		<b>Notification</b>	<b>Penalty</b>
	Below 80% of the Probes maximum signal specified for the Capillary Electrophoresis instrument (see <b>Table 5</b> )	Ok	No penalty
	Between 80% and 100% of the Probes maximum signal specified for the Capillary Electrophoresis instrument (see <b>Table 5</b> )	Warning	10 points
	Above 100% of the Probes maximum signal specified for the Capillary Electrophoresis instrument (see <b>Table 5</b> )	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:

		<b>Notification</b>	<b>Penalty</b>
	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:

		<b>Notification</b>	<b>Penalty</b>
	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 above 1.0 nt	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, possibly the bin set is not correct. This can happen when changes have been made to the capillary electrophoresis device or electrophoresis, 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, it 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

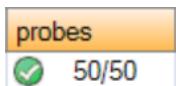
---

### 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 signals of the reference probes.
- 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.

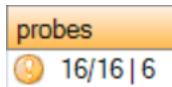


**Figure 7.** Example of the probe counter of a probemix. 50 probes have been found and 50 probes are expected.

## MS-MLPA probemixes

For most MS-MLPA probemixes, the number of expected probes in digested samples may be 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 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, that 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.

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 detected 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 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 have to add the Coffalyser sheet to the active section. The reason for this setup is that more than 400 probemixes are available with numerous lots. As the active section only holds the Coffalyser sheets that you have added, it is easier to find the correct sheet for your experiment. In addition, it is possible to make adjustments to the sheets in the active section (e.g. adding synthetic probes to the sheet), which are not saved in the original Coffalyser sheet in the hidden section. It is therefore possible to add the original Coffalyser sheet, as provided by MRC Holland, to the active section of the library again.

## Update of the sheet library

---

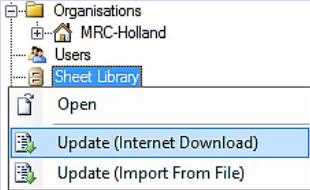
When a new lot of a probemix or a completely new probemix is released, its corresponding Coffalyser sheet is made available for downloading. Coffalyser sheets are also sometimes updated, for example to add product notifications. In order to get these new or modified Coffalyser sheets, you'll need to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

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

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

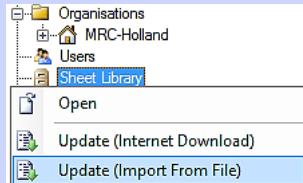
NOTE: The software gives a notification when the update of the sheet library has successfully been completed. This notification includes the number of updated items. In case it states that 0 items were updated, this indicates that the library was already up to date.

#### PROCEDURE: UPDATE SHEET LIBRARY (INTERNET DOWNLOAD)

1. Right click on <i>Sheet Library</i>	
2. Select Update (Internet Download)	
 The Download Updates (MRC-Holland) window opens	
3. Click <b>Start Update</b>	<b>Start Update</b>
4. In the Internet Permission window click <b>Yes</b> or <b>Always</b>	<b>Yes</b> <b>Always</b>
 Wait for the update process to complete	
5. Click <b>Close</b> to close the Download Updates (MRC-Holland) window	<b>Close</b>

#### PROCEDURE: UPDATE SHEET LIBRARY (IMPORT FROM FILE)

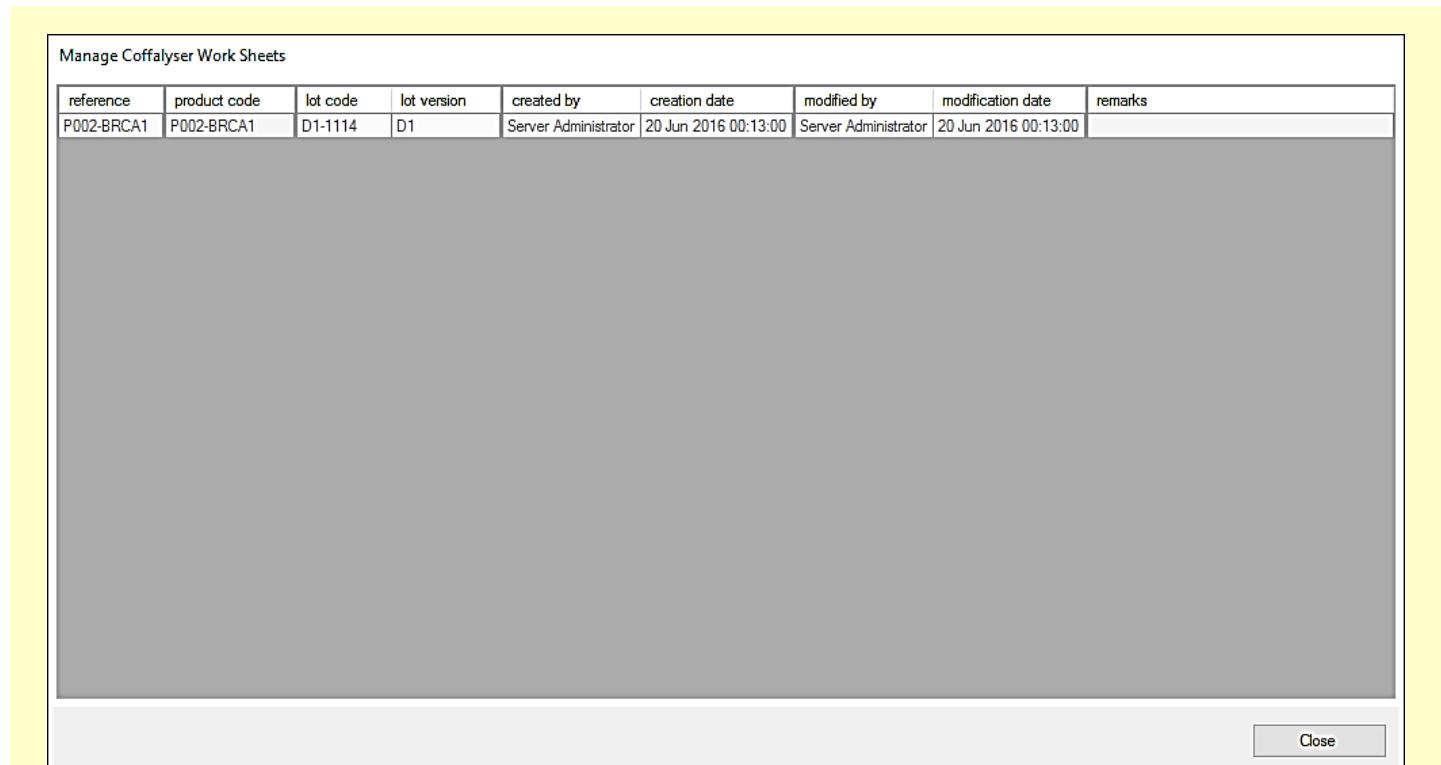
1. Sign in to your MRC Holland account on <a href="http://www.mrcholland.com">www.mrcholland.com</a>	
2. Click the icon to open the account menu	
3. Select <i>Software</i> from the account menu	
4. Click on the Coffalyser.Net logo	

5. Under the header Downloads, click on <i>Coffalyser.Net – sheet library update</i> and save the file	<a href="#">Coffalyser.Net – sheet library update</a>
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 <i>Sheet Library</i>	
9. Select <i>Update (Import From File)</i>	
 The Download Updates (MRC-Holland) window opens	
10. Click <b>Start Update</b>	<b>Start Update</b>
 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 <b>Open</b>	<b>Open</b>
13. Click <b>Close</b> to close the Download Updates (MRC-Holland) window	<b>Close</b>

## Manage the sheet library

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

**Box 1: Manage Coffalyser Work Sheet window**



### Reference column

The reference name of the probemix is displayed here.

### Product code column

The product code of the probemix is displayed here.

### Lot code column

The lot number of the probemix is displayed here.

### Lot version column

The version number of the probemix is displayed here.

### Created by column

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

### Creation date column

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

### Modified by column

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

### Modification date column

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

### Remarks column

Remarks made in the Coffalyser sheet are displayed here.

### Close button

Closes the Manage Coffalyser Work Sheets window.

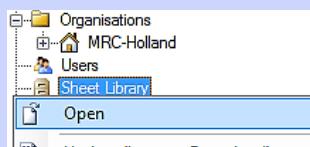
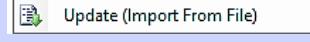
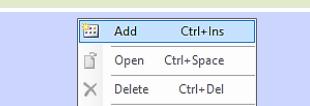
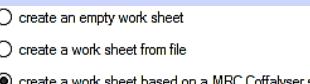
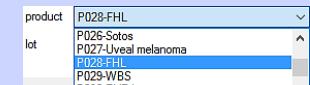
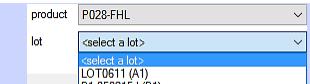
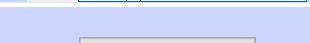
## Add Coffalyser sheets to the sheet library

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

1. The hidden section of the sheet library. This is the most common and recommended option. It requires that the sheet library is up to date.
2. A Coffalyser sheet file. This is useful when you want to share a (modified) Coffalyser sheet between two computers that are not in the same network.

Coffalyser.Net also allows you to add empty Coffalyser sheets (or 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.

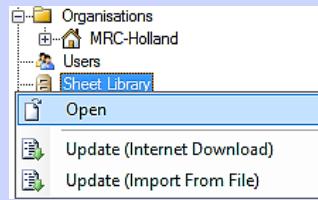
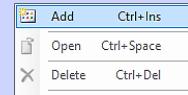
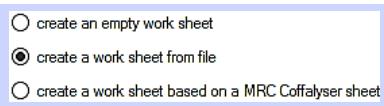
### PROCEDURE: ADD A COFFALYSER SHEET FROM THE HIDDEN SECTION (CREATE A WORK SHEET BASED ON A MRC COFFALYSER SHEET)

1. Right click on <i>Sheet Library</i>	
2. Select Open	
 The Manage Coffalyser Work Sheets window opens	
3. Right click in the window and select <i>Add</i>	
 The Add Coffalyser Work Sheet form appears	
4. Select <i>create a work sheet based on a MRC Coffalyser sheet</i>	
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 <b>OK</b>	

 The Coffalyser Work Sheet Editor window opens	
8. Click <b>OK</b> to save the Coffalyser sheet and close the Coffalyser Work Sheet Editor window	

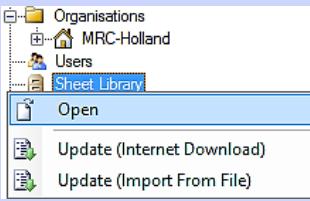
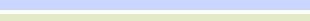
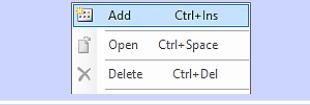
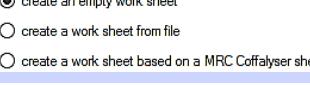
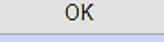
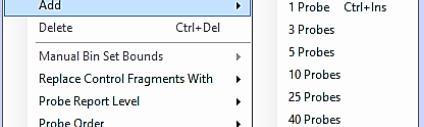
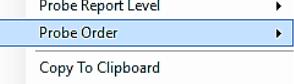
#### PROCEDURE: IMPORT A COFFALYSER SHEET FILE (CREATE A WORK SHEET FROM FILE)

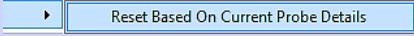
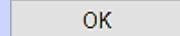
---

1. Right click on <i>Sheet Library</i>	
2. Select <i>Open</i>	
 The Manage Coffalyser Work Sheets window opens	
3. Right click in the window and select <i>Add</i>	
 The Add Coffalyser Work Sheet form appears	
4. Select <i>create a work sheet from file</i>	
5. Click <b>OK</b>	
 A dialog box opens	
6. Navigate to the location where the Coffalyser sheet file is stored	
7. Select the Coffalyser sheet file and click <b>Open</b>	
 The Coffalyser Work Sheet Editor window opens	
8. Click <b>OK</b> to save the Coffalyser sheet and close the Coffalyser Work Sheet Editor window	

## PROCEDURE: ADD AN EMPTY COFFALYSER SHEET

---

1. Right click on <i>Sheet Library</i>	
2. Select <i>Open</i>	
 The Manage Coffalyser Work Sheets window opens	
3. Right click in the window and select <i>Add</i>	
4. Select <i>create an empty work sheet</i>	
5. Click <b>OK</b>	
 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 <i>Add</i>	
10. Select the desired number of probes you want to add	
11. Enter the required information in the applicable fields (see <b>Appendix IV - Coffalyser sheets</b> )	
12. Right click in the window and select <i>Probe Order</i>	

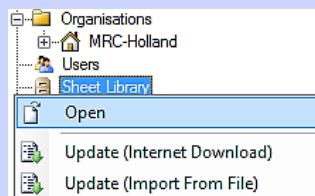
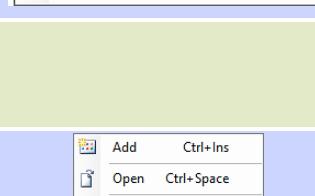
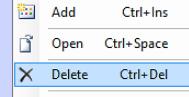
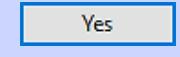
13. Select <i>Reset Based On Current Probe Details</i>	
14. Click <b>OK</b> to save the Coffalyser sheet and close the window	

## 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 <i>Sheet Library</i>	
2. Select <i>Open</i>	
 The Manage Coffalyser Work Sheets window opens	
3. Right click on the Coffalyser sheet you wish to delete and select <i>Delete</i>	
4. Click <b>Yes</b> to confirm you want to delete the Coffalyser sheet	

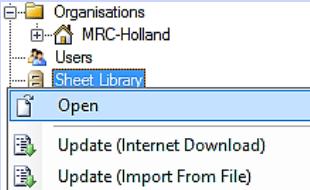
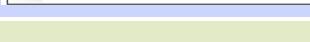
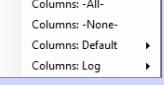
## Display or hide columns in the sheet library

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.

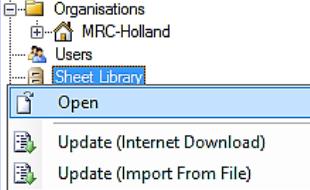
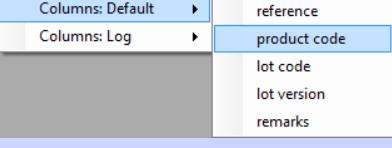
Four groups exist:

- **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 <i>Sheet Library</i>	
2. Select <i>Open</i>	
 The Manage Coffalyser Work Sheets window opens	
3. Right click in the window and click on one of the columns groups	

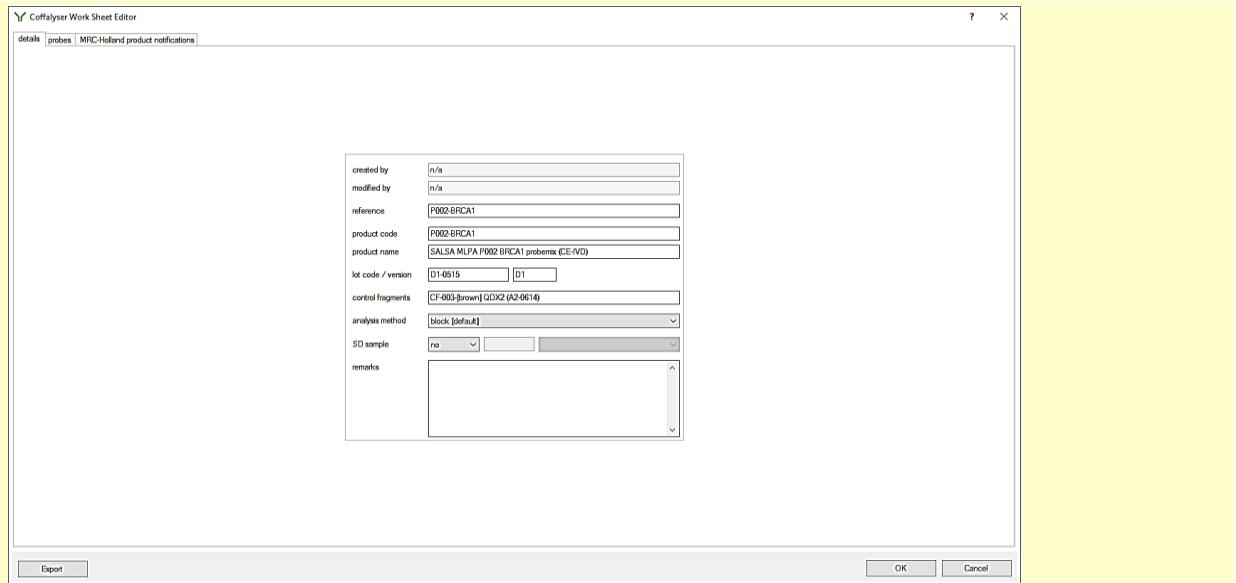
### PROCEDURE: DISPLAY OR HIDE A SINGLE COLUMN

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

# Appendix IV - Coffalyser sheets

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

Box 2: Coffalyser Work Sheet Editor window – details tab



## Created by

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

## Modified by

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

## Reference

The reference name of the probemix is displayed here.

## Product code

The product code of the probemix is displayed here.

## Lot code / version

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

## Control fragments

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

## Analysis method

The analysis method is displayed here.

## SD sample

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

## Remarks

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

## Export button

Exports the Coffalyser sheet as .bin file.

## OK button

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

## Cancel button

Closes the Coffalyser Work Sheet editor without saving changes.

### Box 3: Coffalyser Work Sheet Editor window – probes tab

Coffalyser Work Sheet Editor													
details		probes		MRC-Holland product notifications									
status	order	function(s)	gene	GenBank Exon	chromosomal band	MV location	chromosome	MV start	MV end	length (design)	length (Coffalyser)	manual_binset_lower_bound	manual_binset_upper_bound
info	1	quantity control	Q-64		unknown					64	61.2		
info	2	quantity control	Q-70		unknown					70	67.2		
info	3	quantity control	Q-76		unknown					76	73.5		
info	4	quantity control	Q-82		unknown					82	79.8		
green	1	denaturation control 1	CARM1	1	13p13.2	19:010:843569	19	10843569	10843616	88	86.1		
green	2	ligation control	IL1B	4	02q13	02:113:307442	02	113307442	113307492	92	90.9		
green	3	denaturation control 2	JPH3	up	16q24.2	16:088:192972	16	86192972	86193028	96	96.4		
green	4	X presence control	AMOT	4	Xq23	X:111:945310	X	111945310	111945369	100	100.8		
green	5	Y presence control	UTY	15	Yq11.221	Y:013:975840	Y	13975840	13976005	105	105.6		
green	6	probe, reference probe (C.N.)	IL4	1	05q31.1	05:132:037610	05	132037610	132037671	130	128.8		
green	7	probe, reference probe (C.N.)	FBN1	62	15q21.1	15:046:501165	15	46501165	46501234	136	134.6		
green	8	probe	BRCA1	11	17q21.31	17:038:497038	17	38497038	38497107	142	141.5		
green	9	probe	BRCA1	24	17q21.31	17:038:451166	17	38451166	38451235	149	148.3		
green	10	probe	BRCA1	1a	17q21.31	17:038:530812	17	38530812	38530870	154	153.5		
green	11	probe	BRCA1	16	17q21.31	17:038:476716	17	38476716	38476774	160	159		
green	12	probe	BRCA1	up	17q21.31	17:038:531705	17	38531705	38531763	166	164.6		
green	13	probe	BRCA1	2	18q21.32	18:055:720355	18	55720355	55720920	172	170.5		
green	14	probe	BRCA1	2	17q21.31	17:038:529588	17	38529588	38529649	178	177.2		
green	15	probe	BRCA1	23	17q21.31	17:038:451316	17	38451316	38452521	184	183.1		
green	16	probe	BRCA1	5	17q21.31	17:038:476701	17	38476701	38512075	190	189		
green	17	probe	BRCA1	16	17q21.31	17:038:476705	17	38476705	38476750	195	195.7		
green	18	probe	BRCA1	13	17q21.31	17:038:487591	17	38487591	38487625	202	202		
green	19	probe, reference probe (C.N.)	PICKCA	2	03q26.32	03:180:396905	03	180396905	180396977	208	207.3		
green	20	probe	BRCA1	19	17q21.31	17:038:469358	17	38469358	38469325	214	213.3		
green	21	probe	BRCA1	up	17q21.31	17:038:506693	17	38506693	38507023	220	219.7		
green	22	probe	BRCA1	21	17q21.31	17:038:455598	17	38455598	38456558	226	226.2		
green	23	probe	BRCA1	11	17q21.31	17:038:490203	17	38490203	38490984	233	228.8		
green	24	probe	BRCA1	9	17q21.31	17:038:502751	17	38502751	38502832	238	238.6		
green	25	probe, reference probe (C.N.)	ATP7B	14	13q14.3	13:051:416301	13	5146301	5146372	244	244.6		
green	26	probe	BRCA1	10	17q21.31	17:038:501402	17	38501402	38501466	251	251		
green	27	probe	BRCA1	18	17q21.31	17:038:493436	17	38493436	38496500	256	256		
green	28	probe	BRCA1	11	17q21.31	17:038:502073	17	38500273	38500234	263	262.4		
green	29	probe	BRCA1	14	17q21.31	17:038:482059	17	38482059	38482124	269	269.1		
green	30	probe	STIL	6	01q33	01:047:538303	01	47538303	47538384	275	275.4		
green	31	probe	BRCA1	11	17q21.31	17:038:497460	17	38497460	38497521	281	280.6		
green	32	probe	BRCA1	1b	17q21.31	17:038:530571	17	38530571	38530629	289	288		
green	33	probe	BRCA1	11	17q21.31	17:038:493436	17	38493436	38499514	296	295.3		

#### Status

Displays if all relevant probe information is entered correctly.

#### Order

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

#### Function(s)

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

#### Gene

Displays the gene to which the probe is targeted.

#### GenBank Exon

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

#### Chromosomal band

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

#### MV location

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

#### Chromosome

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

#### MV start

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

#### MV end

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

#### Length (design)

Displays the length on which the probe has been designed.

#### Length (Coffalyser)

Displays the length of the probe as found by MRC Holland.

#### Manual\_binset\_lower\_bound

Displays the lower boundary of the probe's bin.

#### Manual\_binset\_upper\_bound

Displays the upper boundary of the probe's bin.

#### OK button

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

#### Cancel button

Closes the Coffalyser Work Sheet editor without saving changes.

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

The screenshot shows the 'Coffalyser Work Sheet Editor' window with the 'MRC-Holland product notifications' tab selected. The main area displays several bullet points of text regarding probe locations and mutations:

- Probes for exons 1 and 2 are located in a CpG island. Incomplete DNA denaturation, due to the presence of sat in DNA samples, can result in false positive deletions of this region.
- The signal of the 160 nt probe 20022-L27333 may be influenced by the pathogenic mutation c.4689 C>G. In case of apparent deletions, it is recommended to sequence the region targeted by this probe.
- Please note that we use the traditional BRCA1 exon numbering (24 exons; no exon 4). A new BRCA1 exon numbering has been proposed recently and is used in the LRG sequence with 23 exons.
- This probe may show a false deletion when the c.5096G>A, p.Arg1699Gln (rs41293459) mutation is present.
- The exon 13(12) probes 02603-L27340 and 18169-L23037 may give variable results and need additional confirmation in case a deletion/duplication of a single probe is found.

At the bottom of the window are 'OK' and 'Cancel' buttons. To the left of the window, there is descriptive text for the 'MRC-Holland product notifications' tab and the 'Export' button. To the right, there is descriptive text for the 'OK' and 'Cancel' buttons.

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

**Export button**  
Exports the Coffalyser sheet as .bin file.

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

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

## Display or hide columns in a Coffalyser sheet

---

When a Coffalyser sheet is opened, Coffalyser.Net only shows a subset of columns. However, it allows you to select other columns to be displayed as well. It is possible to display multiple columns at once by selecting a group or by selecting individual columns from these groups. Columns can be hidden in a similar fashion. In Table 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.

**Table 7. Columns: default**

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

**Table 8. Columns: Advanced**

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

**Table 9. Columns: Methylation**

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

**Table 10. Columns: Info**

Name	Description	Mandatory: Yes / No
Probe number	Displays the probe number.	N/A – This is the 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	Displays the details of the mutation targeted by a mutation specific probe.	Yes – Only for mutation specific probes

## PROCEDURE: DISPLAY OR HIDE MULTIPLE COLUMNS AT ONCE

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

## PROCEDURE: DISPLAY OR HIDE A SINGLE COLUMN

1. Right click on the sheet	
2. Select Open	
The Coffalyser Work Sheet Editor window opens	
3. Navigate to the tab PROBES	
4. Right click in the sheet	
5. Select <i>Columns: Default</i> , <i>Columns: Advanced</i> , <i>Columns: Methylation</i> or <i>Columns: Info</i> 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.

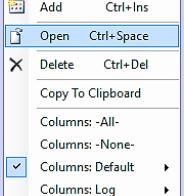
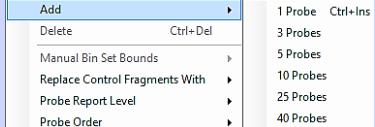
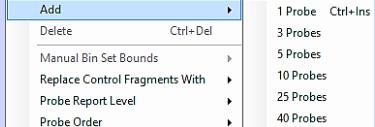
### IMPORTANT NOTE:

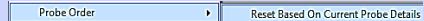
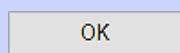
Coffalyser sheets should not be edited in a diagnostic setting. Changes made to a Coffalyser sheet will have an effect on the analysis results!

## 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.

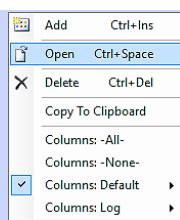
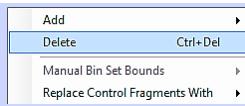
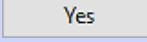
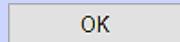
### PROCEDURE: ADD PROBES TO A COFFALYSER SHEET

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 <i>Probe Order</i> and subsequently <i>Reset Based On Current Probe Details</i>	
10. Click <b>OK</b> to close the Coffalyser Work Sheet Editor window	

#### PROCEDURE: DELETE A PROBE FROM A COFFALYSER SHEET

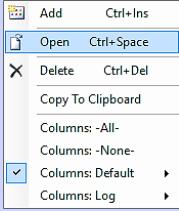
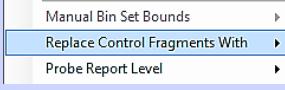
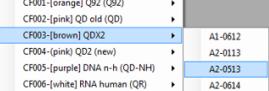
---

1. Right click on the sheet	
2. Select <i>Open</i>	
3. Navigate to the tab PROBES	
4. Right click on the probe you wish to delete and select <i>Delete</i>	
5. Click <b>Yes</b> to confirm you want to delete the probe	
6. Right click in the window	
7. Select <i>Probe Order</i> and subsequently <i>Reset Based On Current Probe Details</i>	
8. Click <b>OK</b> to close the Coffalyser Work Sheet Editor window	

## Control fragments in a Coffalyser sheet

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

### PROCEDURE: CHANGE CONTROL FRAGMENT SET IN A COFFALYSER SHEET

1. Right click on the sheet	
2. Select Open	
 The Coffalyser Work Sheet Editor window opens	
3. Navigate to the tab PROBES	
4. Right click in the window and select <i>Replace Control Fragments with</i>	
5. Select the appropriate set of control fragments and lot number	
6. Click <b>Yes</b> to confirm you want to replace the control fragment set	
7. Right click in the window	
8. Select <i>Probe Order</i> and subsequently <i>Reset Based On Current Probe Details</i>	
9. Click <b>OK</b> to close the Coffalyser Work Sheet Editor window	

## Probe report levels

It is possible to change the probe identities in screens and reports. For instance, the name of the gene targeted by a probe or the chromosomal position can be displayed. This is done via the Probe report level functionality in the Coffalyser sheet. Table 11 lists all possible probe report levels together with a description of each one of them.

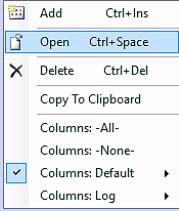
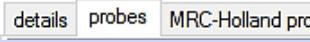
**Table 11. Probe report level**

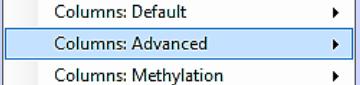
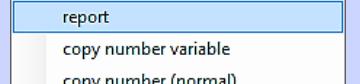
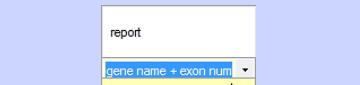
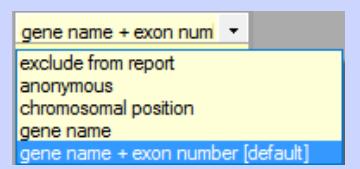
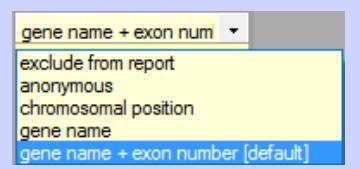
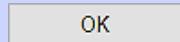
Probe report level	Result in screens and reports
Exclude from report	Probes are not visible*.
Anonymous	The role of the probe is displayed (i.e. Reference or Target).
Chromosomal position	The chromosomal position of the target location of the probe is displayed.
Gene name	The name of the gene that is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

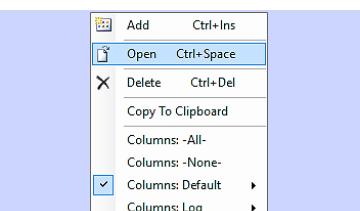
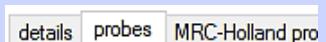
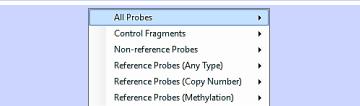
### PROCEDURE: CHANGE THE PROBE REPORT LEVEL OF INDIVIDUAL PROBES

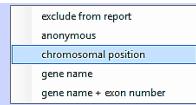
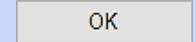
1. Right click on the sheet	
2. Select Open	
 The Coffalyser Work Sheet Editor window opens	
3. Navigate to the tab PROBES	

4. Right click in the window and select <i>Columns: Advanced</i>	
5. Select <i>report</i> from the appearing list	
6. In the column report, click on the cell of the corresponding probe	
7. Click on the arrow head to expand the list with report levels	
8. Select a report level from the list	
9. Right click in the window	
10. Select <i>Probe Order</i> and subsequently <i>Reset Based On Current Probe Details</i>	
11. Click <b>OK</b> 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 <i>Open</i>	
 The Coffalyser Work Sheet Editor window opens	
3. Navigate to the tab PROBES	
4. Right click in the window and select <i>Probe Report Level</i>	
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 <i>Probe Order</i> and subsequently <i>Reset Based On Current Probe Details</i>	
9. Click <b>OK</b> to close the Coffalyser Work Sheet Editor window	

# Appendix V - CE devices

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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
MegaBACE 1000 devices	.rsd

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

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

## Filter set

A filter set defines which fluorescent dyes are recognised in each dye channel. Table 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
MegaBACE 1000	FilterSet 1	6-FAM™	ET-ROX
	FilterSet 2	6-FAM™	ET-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 device are displayed here.

### **Modified by**

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

### **CE device\***

The type of the CE device is displayed here.

### **CE device filter\***

The filter set of the CE device is displayed here.

### **Location**

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

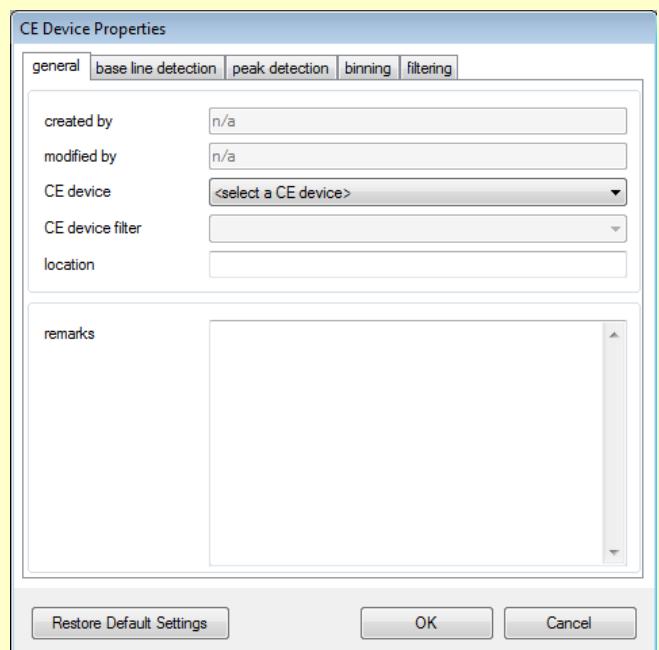
### **Remarks**

Remarks about the CE device are displayed here.

### **Restore default settings button**

Returns all settings to their default value.

\* Mandatory



### **OK button**

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

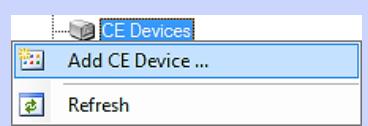
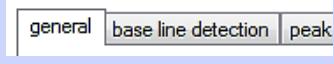
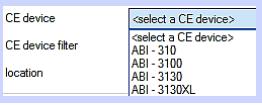
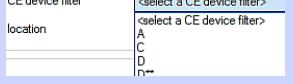
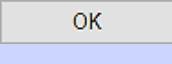
### **Cancel button**

Closes the CE Device Properties window without saving changes.

## Add and delete a CE device

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

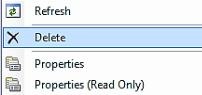
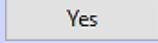
### PROCEDURE: CREATE CE DEVICE

1. Right click on the folder <i>CE Devices</i>	
2. Select <i>Add CE Device ...</i>	
 The CE Device Properties window opens	
3. Navigate to the tab <b>GENERAL</b>	
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 <sup>(8)</sup>	
6. Fill in the Location text field when desired	
7. Fill in the Remarks text field when desired	
8. Click <b>OK</b> to save the CE device and close the window	

<sup>8</sup> 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

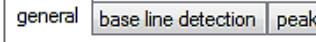
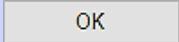
It is possible to adjust the properties of any created CE device. This functionality is reserved for Organisation administrators and Server administrators. The parameters for size calling and peak recognition can easily be reset to their default values, in case these have been changed.

### 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.

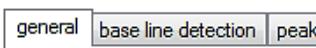
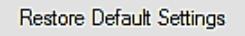
Changing the settings of a CE device will influence size calling and peak recognition. This will have an effect on the analysis results!

### 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 <i>Properties</i>	
 The CE Device Properties window opens	
3. Navigate to the tab GENERAL	
4. Edit the information in the relevant fields	
5. Click <b>OK</b> 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 <i>Properties</i>	
 The CE Device Properties window opens	
3. Navigate to the tab GENERAL	
4. Click <b>Restore Default Settings</b>	
5. Click <b>Yes</b> to confirm you want reset all settings to the default settings	
6. Click <b>OK</b> 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 <i>Properties (Read Only)</i>	
 The CE Device Properties window opens	
3. Navigate to the tabs of interest	
4. Click <b>Close</b> to close the CE Device Properties window	

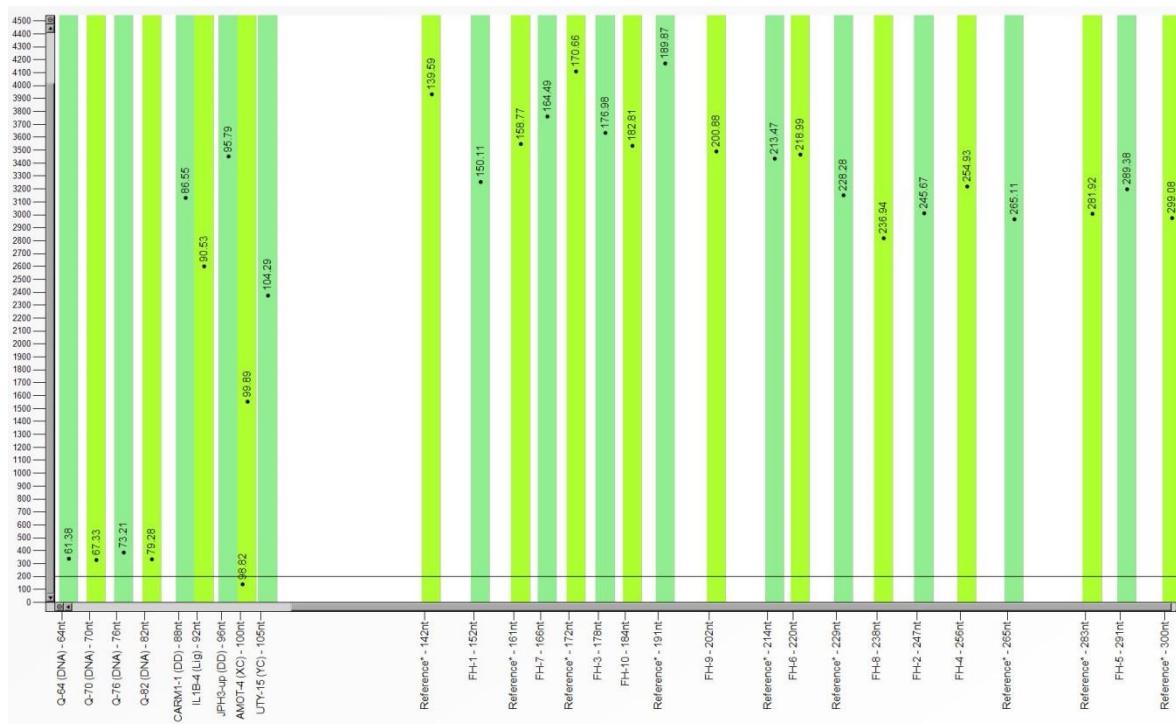
# Appendix VI - Bin set

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 in one sample a fragment has a length of 140.25 nt, whereas in another sample it is 140.45.

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

## Inspect the bin set

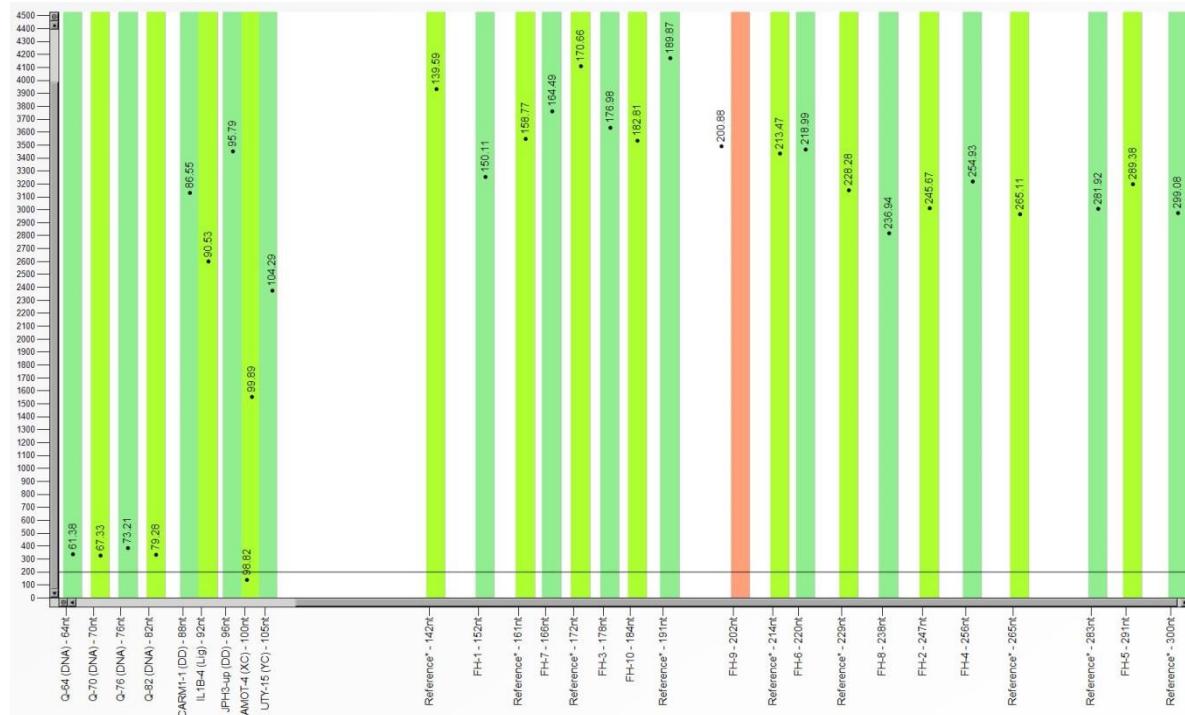
In Coffalyser.Net, each bin is displayed as a vertical bar and each signal as a black dot with a size label. When a signal has been found in a bin, this bin will be green. An example of a correct bin set is presented in Figure 10: in all bins a signal has been found, resulting in the bins to be green.



**Figure 10.** Correct bin set in Coffalyser.Net

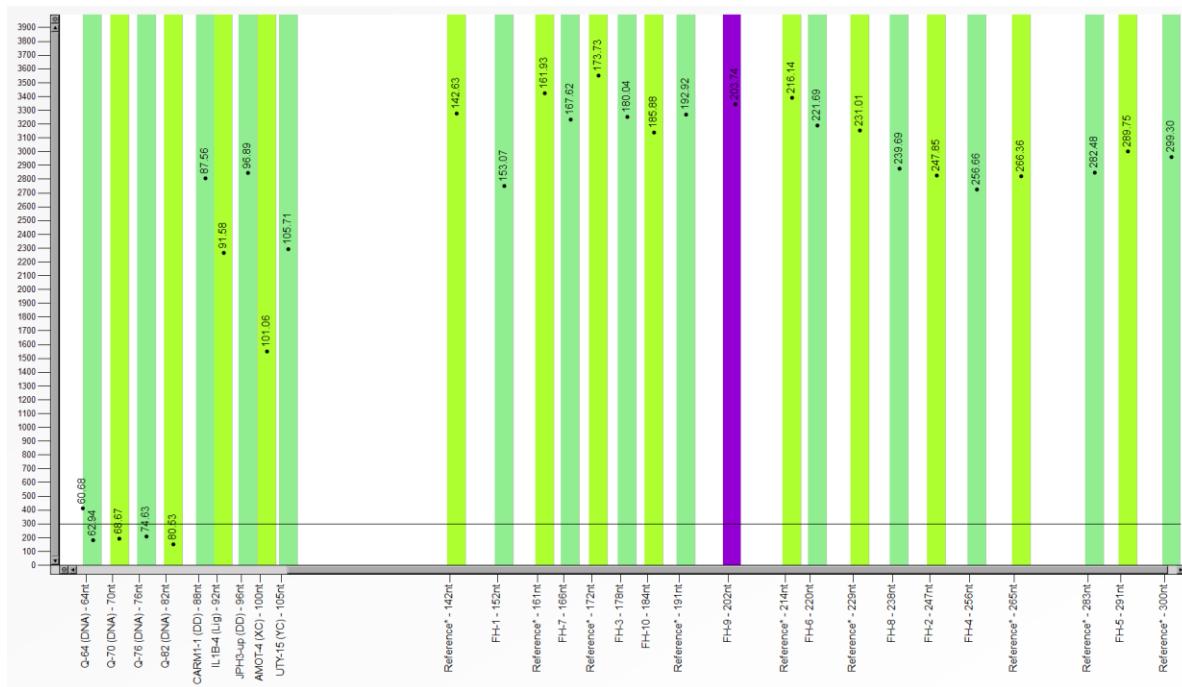
When no signal has been found, the bin will be red. See Figure 11 for an example of an incorrect bin set. In this case no signal has been detected in one bin in this sample. This bin has turned red and the probe signal lies just outside of it.

*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 is 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.

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

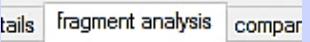
#### PROCEDURE: INSPECT BIN SET IN THE SAMPLE RESULTS EXPLORER

1. Navigate to the tab FRAGMENT ANALYSIS of an experiment	
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	

- |   |  |
|---|--|
| <p>6. Inspect other samples by selecting them from the list on the left side of the window when desired</p> |  |
|---|--|

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

---

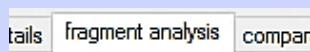
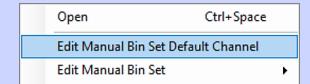
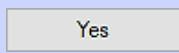
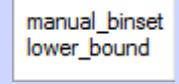
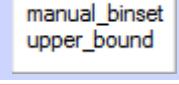
<p>1. Navigate to the tab FRAGMENT ANALYSIS of an experiment</p>	
<p>2. Right click in the window and select <i>Edit Manual Bin Set</i></p>	
<p>3. Select the channel that contains the MLPA probes</p>	
<p> The Coffalyser Work Sheet Editor – Manual Bin Set window opens</p>	
<p>4. Check the graph for purple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

## 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 <i>Edit Manual Bin Set Default Channel</i>	
3. When applicable, click <b>Yes</b> 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 <b>OK</b> to close the Coffalyser Work Sheet Editor – Manual Bin Set window	<b>OK</b>
 A dialog box opens	
10. Click <b>Yes</b> to set the probe recognition method to manual	<b>Yes</b>

# Appendix VII - User accounts

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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

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### 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	●	● <sup>1</sup>			
Change the organisational role of a user	●	● <sup>2</sup>			
Create an organisation	●				
Delete an organisation	●				
Adjust the properties of an organisation	●	● <sup>2</sup>			
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	●	●	● <sup>3</sup>	●	
Adjust the properties of a project, including user roles	●	●	● <sup>3</sup>	●	
View contents of a project	●	●	●	●	●
View the properties of a project (read only)	●	●	●	●	●
Create an experiment	●	●	● <sup>4</sup>	●	●
Delete an experiment	●	●	● <sup>3</sup>	●	
Open an experiment	●	●	● <sup>4</sup>	●	●
Adjust the properties of an experiment	●	●	● <sup>4</sup>	●	●

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

<sup>1</sup> 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.

<sup>2</sup> Only possible in the organisation of which the user is Organisation administrator.

<sup>3</sup> Only possible when the Organisation user is also Project administrator of the project.

<sup>4</sup> 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.

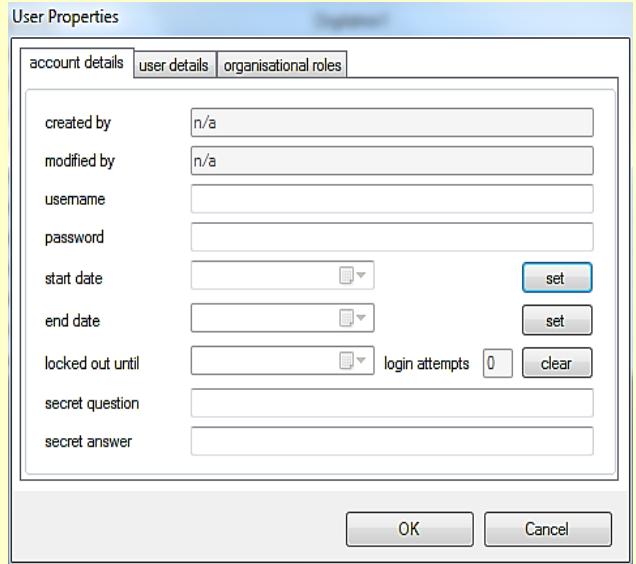
**Secret question**  
Input field for a question that will be asked in case of forgotten password.

**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



The screenshot shows the 'User Properties' dialog box with the 'account details' tab selected. The form contains the following fields:

- created by: n/a
- modified by: n/a
- username: (empty)
- password: (empty)
- start date: (date picker) with a 'set' button to its right
- end date: (date picker) with a 'set' button to its right
- locked out until: (date picker) with a 'login attempts' field containing '0' and a 'clear' button to its right
- secret question: (empty)
- secret answer: (empty)

At the bottom right of the dialog are 'OK' and 'Cancel' buttons.

**Box 7: User properties window – user details tab**

**Surname \***  
Input field for the surname of the user.

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

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

**Department**  
Input field for the department of the user.

**Function**  
Input field for the function of the user.

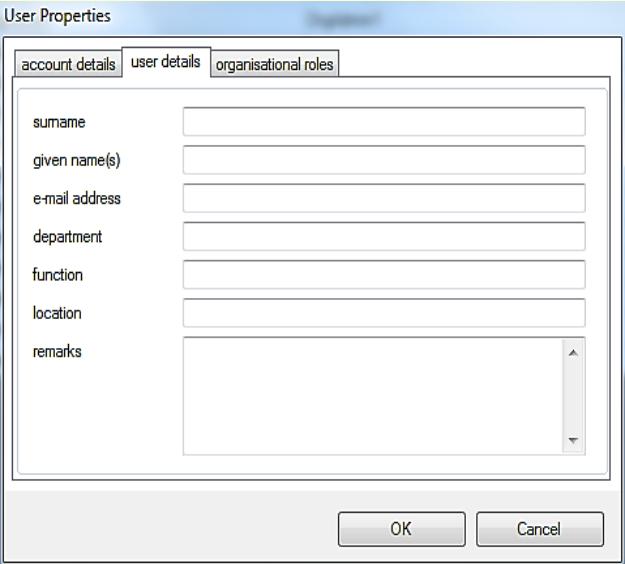
**Location**  
Input field for the location/room of the user.

**Remarks**  
Input field for additional remarks.

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

**Cancel button**  
Closes the User Properties menu without saving changes.

\* Mandatory



**Box 8: User properties window – organisational roles tab**

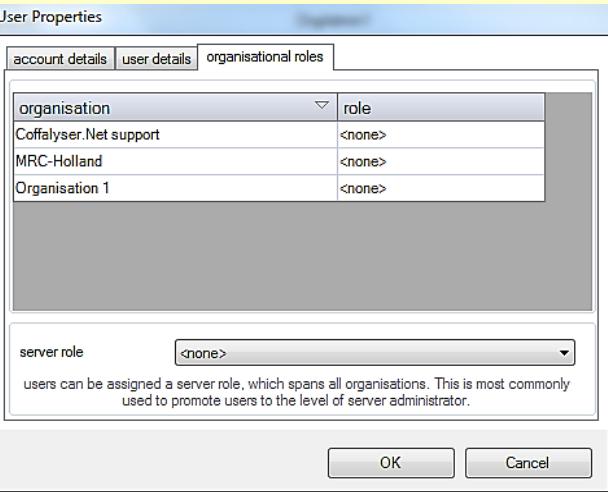
**Organisation column**  
List with Organisations.

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

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

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

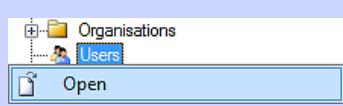
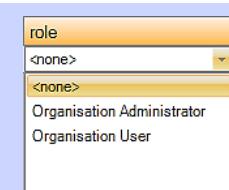
**Cancel button**  
Closes the User Properties menu without saving changes.



## Create and delete user accounts

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

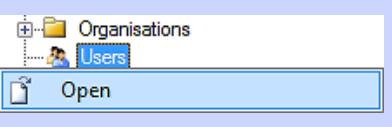
### PROCEDURE: CREATE USER ACCOUNT

1. Right click on <i>Users</i> in the database	
2. Select <i>Open</i>	
 The Users window opens	
3. Right click and select <i>Add user</i>	
 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 arrow head to expand the list with organisational roles	
9. Select a role from the list	

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

#### PROCEDURE: DELETE USER ACCOUNT

---

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

## Edit user accounts

---

Server administrators can grant or deny user accounts access to organisations by changing the organisational role.

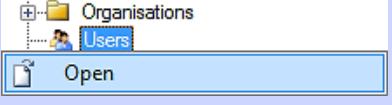
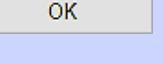
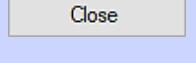
Next to this, they can reset the password of user accounts when the user forgot his password and didn't enter a secret question and answer. These actions can be performed in the *database* folder 'Users'.

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

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

### PROCEDURE: EDIT USER ACCOUNT

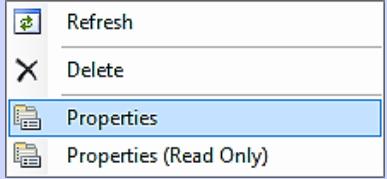
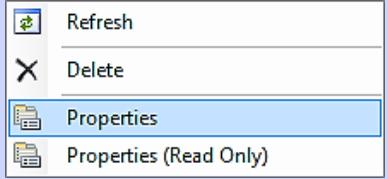
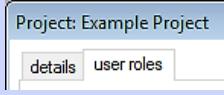
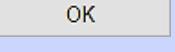
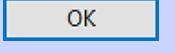
---

1. Right click on <i>Users</i>	
2. Select Open	
 The Users window opens	
3. Right click on a user account and select <i>Edit</i>	
 The User Properties form opens	
4. Edit the information in the respective tabs as desired	
5. Click <b>OK</b> to save the changes and close the USER PROPERTIES form	
6. Click <b>Close</b> 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 <i>Projects</i> 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 <b>USER ROLES</b> and adjust the role for one or more Organisation users	
5. Click <b>OK</b> to save the changes	
6. Click <b>OK</b> to confirm you have read the notification and to close the notification window	

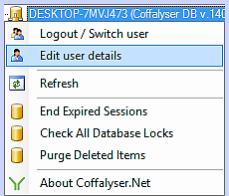
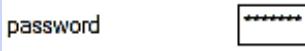
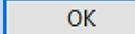
## Edit user profile

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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.

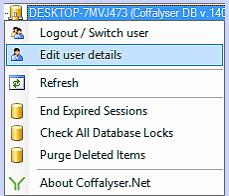
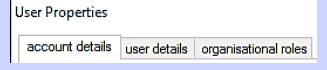
### PROCEDURE: CHANGE PASSWORD

---

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

### PROCEDURE: CHANGE SECRET QUESTION + ANSWER

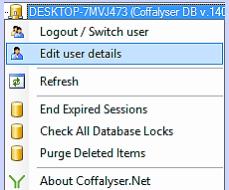
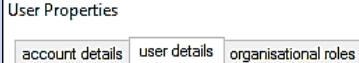
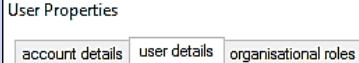
---

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

4. Enter a new secret question in the designated text field	<input type="text" value="secret question"/> <input type="password" value="*****"/>
5. Enter the answer to the secret question in the designated text field	<input type="text" value="secret answer"/> <input type="password" value="*****"/>
6. Click <b>OK</b> to confirm you have read the notification and to close the notification window and User properties form	<input type="button" value="OK"/>

#### PROCEDURE: ADD OR CHANGE USER INFORMATION

---

1. Right click on the database icon at the top of the tree structure in the solution explorer	
2. Select <i>Edit user details</i>	
 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 <b>OK</b> to confirm you have read the notification and to close the notification window and User properties form	<input type="button" value="OK"/>

# Contact Information

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More information: [www.mrcholland.com](http://www.mrcholland.com); [www.mrcholland.eu](http://www.mrcholland.eu)

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Phone	+31 888 657 200

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

### Version 07 – June 2021

- This manual can be used for Coffalyser v.210226.1433 AND v.210604.1451.

### Version 06 – May 2021

- Changes have been made to the FMRS evaluation Ligation:
  - Penalties are added.
  - Text in the section Related issues and solutions has been adjusted to reflect the new version of the software.
- The procedure Create or adjust a manual bin set has been adjusted to reflect the new version of the software.
- The Important notes in the chapter Probe counter have been adjusted. Peaks should meet the same criteria in both reference samples and patient/test samples to be recognised as probe.
- Adjusted the first condition of the FMRS evaluation Max probe length deviation so it now states no penalty is given when the Max probe length deviation is 0.7 nt or smaller.
- Small textual and visual changes.

### Version 05 – March 2021

- Intended purpose has been updated.
- References to the website (URLs, pictures, text) have been updated to the new website.
- Screenshots of the old version have been replaced with ones of the new version.
- Changes have been made to chapters 6.a Set up a copy number analysis experiment and analyse data and 6.b Set up a methylation status analysis experiment and analyse data:
  - Sentence has been added to the Important notes that the creation and use of a manual bin set is mandatory.
  - Sentence has been added to the Important notes that only the probe recognition method in the fragment analysis settings should be adjusted and that other settings should be left at their default value.
  - Adjusted the procedures in such a way that they can be followed when a manual bin set has already been created.
  - Added a screenshot of the fragment analysis overview screen after fragment analysis .
  - The procedures now state that only samples should be included in the comparative analysis that have green icons in the DNA and DD columns in addition to 4 green bars for the FRSS and the FMRS.
- The information about the display of the results of mutation specific probes has been updated to reflect the new version of the software.
- Added the following remark to Appendix II - Quality scores Fragment Analysis: "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." and a screenshot of the fragment analysis overview screen after fragment analysis.
- A section about the probe counter has been added to Appendix II - Quality scores Fragment Analysis. Existing information about the probe counter has been moved here and new information has been added.
- The column Probes minimum peak amplitude (in RFU) has been added to the table Overview probe peak signal intensities.
- The Diagram Signal quality has been updated with the quality check Max probe length deviation.
- A description of the quality check Max probe length deviation has been added to the section FMRS evaluations in Appendix II - Quality scores Fragment Analysis.
- The description of the quality check Signal quality – Residual primer % in the section FMRS evaluations in Appendix II - Quality scores Fragment Analysis has been corrected. It is now described more clearly that the amount of fluorescence of the primer is compared to the total fluorescence of the detected MLPA probe peaks in a sample instead of the number of probes in a probemix.

- In the section Display or hide columns in a Coffalyser sheet in Appendix IV - Coffalyser sheets, GenBank is removed from the table Columns: Info.
- A screenshot of a bin set in which a signal deviates more than 0.7 nt from the center of its bin has been added to the section Inspect the bin set in Appendix VI - Bin set.
- Small textual and visual changes.

#### *Version 04 – November 2018*

- A text has been added about which type of results should be visually confirmed in the electropherogram / raw data
- A warning has been added that a bin should not be made larger than 4 basepairs
- “Every MS-MLPA probemix contains one or more Digestion control probes” has been replaced by “Most MS-MLPA probemixes contain one or more Digestion control probes”
- A note has been added that an update of the sheet library only updates the Coffalyser sheets in the hidden section of the sheet library. The sheets in the active section remain unaffected.
- Filter set A has been removed from Table 11 as ROX labelled primers for MLPA probes are discontinued.
- A warning has been added that a CE device can only be deleted when not linked to an experiment.
- A warning has been added in a footnote that the percentage mentioned for mutation specific probes is NOT the percentage of cells carrying the mutation.
- Adjusted some texts in the Important note boxes 1, 2 and 3
- Some minor textual adjustments have been made.
- Description of the normalization in copy number analysis has been adjusted, so it now describes the actual calculations correctly.
- Description of the normalization for mutation specific probes has been adjusted, so it now describes the actual calculations correctly.
- Descriptions of MS-MLPA normalization and result interpretation have been added. These parts are not included in version 03.
- It is mentioned now that No DNA samples to which HhaI has been added should be defined as ‘NoDNA’

#### *Version 03 – March 2017*

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

#### *Version 02 – November 2016*

- Coffalyser.Net Reference Manual has completely been rewritten

#### *Version 01*

- New document