Instructions for Use
SALSA® MLPA® Probemix P101 STK11

See also the MLPA General Protocol, the product description of the SALSA® MLPA® Reagent Kit, and the Coffalyser.Net Reference Manual.

Visit the SALSA® MLPA® Probemix P101 STK11 product page on our website to find Certificates of Analysis and a list of related products.

Product Name	**SALSA® MLPA® Probemix P101 STK11**
Version	C1
Catalogue numbers	P101-025R (25 reactions) P101-050R (50 reactions) P101-100R (100 reactions)
Basic UDI-DI	872021148P1015B
Ingredients	Synthetic oligonucleotides, oligonucleotides purified from bacteria, Tris-HCl, EDTA

Additional Test Components	**Catalogue Numbers**
SALSA® MLPA® Reagent Kit	EK1-FAM EK1-CY5 EK5-FAM EK5-CY5 EK20-FAM

Storage and Shelf Life

Recommended conditions		

A shelf life of until the expiry date is guaranteed, also after opening when stored in the original packaging under recommended conditions. For the exact expiry date, see the label on the vial. This product should not be exposed to more than 25 freeze-thaw cycles. Do not use the product if the packaging is damaged or opened. Leave chemicals in original containers. Waste material must be disposed of in accordance with the national and local regulations.

Regulatory Status	
IVD	EUROPE CE 2797 ISRAEL
RUO	ALL OTHER COUNTRIES

Label Symbols			
IVD	In Vitro Diagnostic	RUO	Research Use Only

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Any serious incident that has occurred in relation to this product should be reported to MRC Holland and the competent authority of the Member State or country in which the user and/or the patient is located.

Changes in this Product Version

As compared to version B4, two denaturation control probes have been replaced, two more denaturation control probes have been added, one target probe has been replaced, all reference probes have been replaced, and the lengths of certain probes were altered without the detected sequences having changed.

To be noted, throughout the rest of the document, the term **"denaturation control probes"** refers to the 136 nt, 161 nt, 166 nt, and 184 nt probes targeting GC-rich sequences in the *HMGB*, *JPH3*, and *SHANK3* genes. These probes provide further information of DNA denaturation status, and are **distinct from the denaturation fragments (D-fragments)**.

1. Intended Purpose

The SALSA MLPA Probemix P101 STK11 is an in vitro diagnostic (IVD)[1] or research use only (RUO) semi-quantitative manual assay[2] for the detection of deletions in the *STK11* gene in genomic DNA isolated from human peripheral whole blood specimens. P101 STK11 is intended to confirm a potential cause for Peutz-Jeghers syndrome and for molecular genetic testing of at-risk family members.

Copy number variations (CNVs) detected with the P101 STK11 probemix should be confirmed by another technique. In particular, CNVs detected by only a single probe always require confirmation by another method. Most defects in the *STK11* gene are point mutations, none of which will be detected by MLPA. It is therefore recommended to use this assay in combination with sequence analysis.

Assay results are intended to be used in conjunction with other clinical and diagnostic findings, consistent with professional standards of practice, including confirmation by alternative methods, clinical genetic evaluation, and counselling, as appropriate. The results of this test should be interpreted by a clinical molecular geneticist or equivalent.

This device is not intended to be used for standalone diagnostic purposes, pre-implantation or prenatal testing, population screening, or for the detection of, or screening for, acquired or somatic genetic aberrations, e.g from DNA extracted from formalin-fixed paraffin embedded (FFPE) or fresh tumour materials.

[1] Please note that this probemix is for IVD use in the countries specified on page 1 of this product description. In all other countries, this is a RUO product.

[2] To be used in combination with a SALSA MLPA Reagent Kit and Coffalyser.Net analysis software.

2. Sample Requirements

Specimen	50-250 ng purified human genomic DNA, free from heparin, dissolved in 5 µl $TE_{0.1}$ buffer, pH 8.0-8.5
Collection Method	Standard methods
Extraction Method	Methods tested by MRC Holland: • QIAGEN Autopure LS (automated) and QIAamp DNA mini/midi/maxi kit (manual) • Promega Wizard Genomic DNA Purification Kit (manual) • Salting out (manual)

Sample Types	
Test Sample	• Provided by user
Reference Samples (Required)	• Provided by user • Extraction method, tissue type, DNA concentration and treatment as similar as possible in all test and reference samples. • Have a normal copy number and ≤0.10 standard deviation for all probes. • At least three* independent reference samples required in each experiment for proper data normalisation. Derived from unrelated individuals from families without a history of Peutz-Jeghers syndrome.
No-DNA Control (Preferably)	• Provided by user • $TE_{0.1}$ buffer instead of DNA • To check for DNA contamination
Positive Control Samples (Preferably)	• Provided by user

*When testing >21 samples, include one extra reference for each 7 test samples.

3. Test Procedure

See the MLPA General Protocol.

4. Quality Control, Data Analysis, and Troubleshooting

Quality Control Fragments in the Probemix	
Length (nt)	**Function**
64-70-76-82	DNA quantity control fragments
88-96	DNA denaturation control fragments
92	Benchmark fragment
100	Chromosome X presence control fragment
105	Chromosome Y presence control fragment

Coffalyser.Net should be used for data analysis in combination with the appropriate product and lot-specific Coffalyser sheet. See the Coffalyser.Net Reference Manual for details on data analysis and quality control.

For troubleshooting help, see the additional resources offered on our support portal.

5. Interpretation of Results

Determining Typical Values in Normal and Affected Populations

The typical final ratio (FR) values stated in the copy number tables were determined in a validation study with samples containing abnormal copy numbers. The standard deviation of each individual probe over all the reference samples was ≤0.10.

Expected Results of Reference Probes

Final Ratio (FR)	Copy Number	Description
0.80 – 1.20	2	Normal

Typical Results of Probes Targeting Two Copies (*STK11*)

Final Ratio (FR)	Copy Number	Description
0	0	Homozygous deletion
0.40 – 0.65	1	Heterozygous deletion
0.80 – 1.20	**2**	**Normal**
1.30 – 1.65	3	Heterozygous duplication
1.75 – 2.15	4	Homozygous duplication or Heterozygous triplication
All other values	-	Ambiguous

The tables illustrate the relationship between final probe ratio and corresponding copy number. Test results are expected to center around these values. Ambiguous values can indicate a technical problem, but may also reflect a biological cause such as mosaicism or a SNV influencing a single probe. It is important to use Coffalyser.Net to determine the significance of values found.

6. Performance Characteristics

Study	Description
Expected values for copy number in normal and affected populations	To determine the expected values in normal and affected populations a study was conducted on over 1500 MLPA reactions using samples with and without abnormal copy numbers. When the standard deviation of each individual probe over all the reference samples is ≤0.10, the ranges stated in the copy number table above can be used. Cut-off values for copy number determination were verified with SALSA MLPA Probemix P101 STK11 in 37 samples from healthy individuals with normal copy number and two samples with known CNVs. The expected FRs for the corresponding copy number were found in almost all tested samples. In one sample from a healthy individual two probes produced an ambiguous result. Ambiguous FRs would at most lead to delayed results as the assay may have to be repeated. No false positives or false negatives would ensue.
Limit of detection	A study using representative probemixes was conducted to evaluate the minimum and maximum amount of DNA acceptable as the assay input. Results support the use of 50-250 ng of human DNA as the recommend input amount. The use of insufficient or too much sample DNA can affect performance. These lower and higher limits of detection were verified using SALSA MLPA Probemix P101 STK11 on one sample with a known CNV and on one sample with normal copy number and expected results were obtained using both the lower and upper input amount of DNA.
Interfering substances	SNVs or other polymorphisms (e.g. indels) in the DNA target sequence and impurities in the DNA sample (e.g. NaCl or KCl, EDTA and hemoglobin) can affect the MLPA reaction. A study using SALSA MLPA Probemix P101 STK11 was performed to assess the potential for interference of endogenous and exogenous substances on genomic DNA on a sample with a known CNV and a sample with normal copy number.

Interferent	Source	Testing Concentration	Results*
EDTA	Exogenous – specimen collection tubes	1.5 mM	Copy number: Expected FR for 70/72 measurements
NaCl	Exogenous – DNA extraction	40 mM	Copy number: Expected FR for 25/72 measurements
KCl	Exogenous – DNA extraction	0.05 mM and 0.3 mM	Copy number: Expected FR for 131/132 measurements

<table>
<tr><th>Study</th><th>Description</th></tr>
<tr><td></td><td>
<table>
<tr><td>Fe^{3+} ($FeCl_3$)</td><td>Exogenous – DNA extraction</td><td>1 µM</td><td>Copy number: Expected FR for 66/72 measurements</td></tr>
<tr><td>Heparin</td><td>Exogenous – specimen collection tubes</td><td>0.02 U/mL</td><td>Copy number: Expected FR for 71/72 measurements</td></tr>
<tr><td>Hemoglobin</td><td>Endogenous – blood sample</td><td>0.02 µg/µl</td><td>Copy number: Expected FR for 34/72 measurements</td></tr>
</table>
* Results are summarised for 12 STK11 probes across the two samples tested.

EDTA, Fe^{3+}, heparin, and hemoglobin all lead to ambiguous FRs being obtained in the sample harbouring a STK11 heterozygous deletion. Ambiguous FRs would at most lead to delayed results, as the assay may have to be repeated. No false positives or false negatives would ensue. In the sample with a normal STK11 copy number, one ambiguous measurement was obtained with Fe^{3+}, which would at most lead to delayed results. All measurements obtained with hemoglobin for this sample were aberrant or ambiguous, which would lead to false positives. DNA extraction methods from blood remove hemoglobin. Therefore, it is only when hemoglobin is in excess that deviating probe signals can be found. Importantly, warnings or errors were obtained in all affected samples using Coffalyser.Net software.

From the salt compounds, NaCl had the largest effect on FRs in both the STK11 heterozygous deletion sample and the wildtype sample. Denaturation warnings were obtained for both samples, and the denaturation control probes all produced low signals (FR ≤ 0.65). KCl only led to one ambiguous measurement being obtained in the STK11 heterozygous deletion sample at the lower concentration of 0.05 mM. Since the ambiguous measurement was just outside the FR range for the correct copy number on the higher end of the cut-off bracket, this is likely not due to denaturation issues. For the wildtype sample, all measurements were within the expected range at both KCl concentrations. No Coffalyser.Net denaturation warning was obtained in the presence of KCl, and none of the four denaturation control probes produced significantly low ratios. Overall, KCl does not seem to induce incomplete denaturation of the STK11 gene at the tested concentrations.

In addition to these interfering substance tests, internal testing was carried out on P101 STK11 in order to gain more insight on the effect of NaCl on probe performance. NaCl concentrations of 10 mM up to 50 mM were tested in duplicate on samples from healthy individuals. Ambiguous FRs were produced at 25 mM, while from 30 mM and above, FRs yielding a false result are obtained. At concentrations of 25 mM and 30 mM, Coffalyser.Net did not issue a denaturation warning, yet at least two out of the four denaturation control probes produce low signals (FR ≤ 0.65). From 35 mM, Coffalyser.Net did issue a denaturation warning, and all denaturation control probes produce low signals (FR ≤ 0.65). See Figure A1 and the information box in the Appendix for details regarding DNA denaturation checks.

To minimise variability across samples, all samples tested, including reference DNA samples, should be derived from the same tissue type, handled using the same procedure, and prepared using the same DNA extraction method when possible.
</td></tr>
<tr><td>Cross-reactivity</td><td>Cross-reactivity is the potential for probes to bind to homologous regions (e.g. pseudogenes) or other cross-reactive sequences. Quality tests were carried out to determine whether probes are specific to their target sequence and all probes met the quality criteria for specificity.</td></tr>
<tr><td>Accuracy</td><td>Results of accuracy are derived from trueness and precision studies. For trueness, two previously genotyped samples were tested using SALSA MLPA Probemix P101 STK11 and the expected results were found. Assay precision was tested by repeatedly testing samples with known copy number over multiple days, and by multiple operators. Results showed a correct call in 344/348 data points, leading to a precision of 99%.</td></tr>
<tr><td>Clinical validity*</td><td>STK11: 15-20% of Peutz-Jeghers syndrome is caused by deletions in STK11(Wu et al. 2020)

*Based on a 2021-2024 literature review</td></tr>
</table>

Summary of Safety and Performance (SSP)
The SSP is available in the European database on medical devices (Eudamed), https://ec.europa.eu/tools/eudamed, or upon request.

Content – Probe Details Sorted by Chromosomal Position

Chr. position	Target	Exon	Distance to next probe	Length (nt)	Probe number	Warnings
13q12.3	*HMGB* - DNA denaturation			161	23243-L32905	* ∫
16q24.2	*JPH3* - DNA denaturation			136	S1293-L32906	* ∫
19p13.3	*CDC34*		314.6 kb	265	01737-L01313	¬ «
19p13.3	*ELANE*		64.4 kb	214	07916-L07646	¬ «
19p13.3	*KISS1R*		285.3 kb	202	11955-L18080	¬ «
19p13.3	*STK11*	Exon 1	0.8 kb	247	02215-L10041	« Ø
19p13.3	*STK11*	Exon 1	0.4 kb	274	23215-L32830	« *
19p13.3	*STK11*	Exon 1	11.5 kb	195	03124-L03988	«
19p13.3	*STK11*	Exon 2	0.9 kb	221	03125-L18081	«
19p13.3	*STK11*	Exon 3	1.1 kb	143	22797-L25647	« ¥
19p13.3	*STK11*	Exon 4	0.2 kb	238	03127-L03338	«
19p13.3	*STK11*	Exon 5	0.7 kb	283	16639-L32148	« ¥
19p13.3	*STK11*	Exon 6	0.7 kb	299	03129-L30673	« ¥
19p13.3	*STK11*	Exon 7	1.2 kb	317	16638-L19168	«
19p13.3	*STK11*	Exon 8	3.4 kb	337	03131-L02583	«
19p13.3	*STK11*	Exon 9	1.1 kb	373	22798-L32789	« ¥
19p13.3	*STK11*	Exon 10		172	03891-L32904	« ¥
22q13.33	*SHANK3* - DNA denaturation			184	19609-L28703	* ∫
22q13.33	*SHANK3* - DNA denaturation			166	14719-L15794	* ∫ Δ
1p	Reference			208	13233-L14566	*
4q	Reference			355	15081-L16844	*
5q	Reference			130	00797-L21056	*
6q	Reference			231	14934-L16667	*
7q	Reference			257	04594-L03773	*
8q	Reference			178	06830-L06424	*
9q	Reference			291	08722-L08733	*
11q	Reference			150	10301-L30389	*
12q	Reference			328	16583-L18726	*
18q	Reference			391	12522-L13572	*

Probe lengths may vary slightly depending on capillary electrophoresis instrument settings. Please see the most up to date Coffalyser sheet for exact probe lengths obtained at MRC Holland.
The *STK11* exon numbers are derived from MANE project and are based on MANE Select transcript. For more information, see the probe sequences document available on the product page at www.mrcholland.com.
Chromosomal bands are based on: hg18.

7. Precautions and Warnings

Probe changes

*	New probes.
¥	Probes changed in this product version. Minor alteration, no change in sequence detected.

Probe warnings

¬	These probes are flanking probes, included to help determine the extent of a deletion/duplication. Copy number alterations of flanking probes are unlikely to be related to the condition tested.
«	These probes are located in or near a GC-rich region. A low signal can be caused by NaCl contamination in the DNA sample leading to incomplete DNA denaturation.
∫	These probes are located in or near a GC-rich region. A low signal (FR ≤0.65) for at least two out of these four probes in combination with an apparent *STK11* deletion indicates NaCl contamination in the DNA sample leading to incomplete DNA denaturation. These probes will not generate a denaturation warning in Coffalyser.Net and therefore require an additional visual check (see information box in the **Appendix**). Please note that the 184 nt and 166 nt probes are more NaCl-sensitive than the 161 nt and 136 nt probes and may be the only two DNA denaturation probes to produce a low signal (FR ≤0.65) at mild NaCl concentrations (see **Figure A1** of the **Appendix**).
Ø	This probe targets a sequence in the promoter region of *STK11*. This promoter region overlaps with the UTR in exon 1.
Δ	This probe may be sensitive to certain experimental variations. Aberrant results should be treated with caution.

Probemix-specific precautions

1. This product is not known to contain any harmful agents. Based on the concentrations present, none of the ingredients are hazardous as defined by the Hazard Communication Standard. **A Safety Data Sheet (SDS) is not required for this product**: none of the ingredients contain dangerous substances at concentrations requiring distribution of an SDS (as per Regulation (EC) No 1272/2008 [EU-GHS/CLP] and 1907/2006 [REACH] and amendments).
2. Sample or technical artefacts may appear as a (mosaic) copy number change of the whole/partial gene. Whole/partial gene deletions or duplications should therefore be confirmed by analysis of an independent DNA sample, to exclude false positive results.
3. Small changes (e.g. SNVs, small indels) in the sequence targeted by a probe can cause false positive results, even when >20 nt from the probe ligation site. Sequence changes can reduce the probe signal by preventing ligation of the

probe oligonucleotides or by destabilising the binding of a probe oligonucleotide to the sample DNA. Deviations detected by this product should be confirmed, and single-probe deviations always require confirmation. Sequencing of the target region is recommended. Please contact MRC Holland for more information: info@mrcholland.com.

4. Copy number alterations of reference probes are unlikely to be related to the condition tested.
5. This probemix is particularly prone to denaturation problems (see information box in the **Appendix**). The use of DNA samples containing 25 mM or more NaCl can result in false positive deletion results due to incomplete denaturation. A low signal of the 88 nt and 96 nt DNA denaturation control fragments will generate a warning for denaturation in Coffalyser.Net in case the sample contains 35 mM or more NaCl. When the sample contains NaCl concentrations of ~25-30 mM, *STK11* probes may show reduced probe signals even in the absence of an incomplete DNA denaturation warning in the Coffalyser.Net software.
6. Four extra probes at 136 nt, 161 nt, 166 nt, and 184 nt located in GC-rich regions are present in this probemix. A low signal (FR ≤ 0.65) for at least two out of these four probes in combination with an apparent *STK11* deletion indicates NaCl contamination in the DNA sample leading to incomplete DNA denaturation. These probes will not generate a denaturation warning in Coffalyser.Net and therefore require an additional visual check (see information box in the **Appendix**). Please note that the 184 nt and 166 nt probes are more NaCl-sensitive than the 161 nt and 136 nt probes and may be the only two DNA denaturation probes to produce a low signal (FR ≤ 0.65) at mild NaCl concentrations (see **Figure A1** of the **Appendix**).
7. One study suggests that 1-2% of patients with clinical features of Peutz-Jeghers syndrome could have a mosaic *STK11* mutation (McKay et al. 2016). In case of apparent mosaic *STK11* deletions, check for denaturation problems (see information box in the **Appendix**). Mosaic *STK11* deletions detected with the P101 STK11 probemix must be confirmed by analysis of a second, independently collected DNA sample or a different technique, in order to exclude a false positive mosaic result.

Technique-specific precautions
See the MLPA General Protocol.

8. Limitations

Technique-specific limitations
See the MLPA General Protocol.

9. References Cited in this IFU

1. McKay V et al. (2016). First report of somatic mosaicism for mutations in STK11 in four patients with Peutz-Jeghers syndrome. Fam Cancer. 15:57-61.
2. Wu BD et al. (2020). Clinical and Genetic Analyses of 38 Chinese Patients with Peutz-Jeghers Syndrome. Biomed Res Int. 2020:9159315.

Implemented changes in the product description
Version C1-01 – 9 April 2025 (03S) - Product description adapted to a new product version and a new template. - Compared to the previous product version, the intended purpose now excludes *STK11* duplications and the function of confirming a clinical diagnosis of Peutz-Jeghers syndrome. - Probemix is now IVDR certified.

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10. Appendix

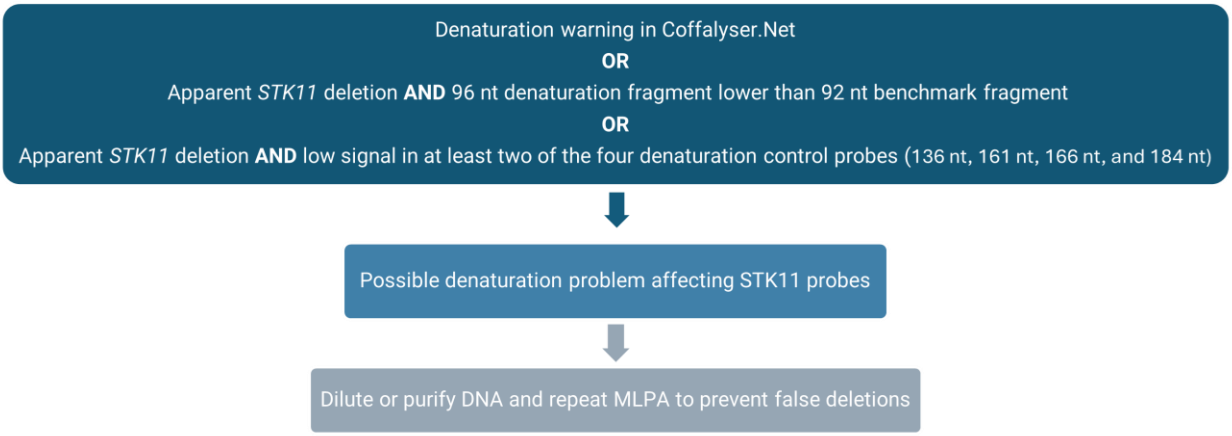
ratio overview | statistics | chart statistics

		Probe target info	10 mM NaCl	20 mM NaCl	25 mM NaCl	30 mM NaCl	35 mM NaCl	40 mM NaCl	45 mM NaCl	50 mM NaCl	ReferenceSamples		
			AllSamples										
			P101-C1-...	P101-C1-...	P101-C1-...	P101-C1-...	P101-C1-...	P101-C1-...	P101-C1-...	P101-C1-...	P101-C1-...	P101-C1-...	P101-C1-...
FRSS (n=7)		n/a	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%
CAS (n=5)	EMRS	n/a	100%	100%	90%	100%	30%	30%	30%	30%	100%	90%	100%
	PSLP - Relati...	n/a	OK	OK	OK	OK	OK	OK	OK	OK	OK	OK	OK
	FSLP - Relati...	n/a	OK	OK	OK	OK	OK	OK	OK	OK	OK	OK	OK
	RSQ - Refere...	n/a	OK	OK	OK	OK	OK	OK	OK	OK	OK	OK	OK
	RPQ - Refere...	n/a	OK	OK	OK	OK	OK	OK	OK	OK	OK	OK	OK
Denaturation control (n=2)	HMGB1-1	13-029,937180	1,01	0,99	0,93	0,7	0,15	8%	7%	8%	0,98	1,01	1,01
	JPH3-up	16-086,193300	1,02	1,05	1,05	0,52	0,13	9%	9%	9%	1	1,01	0,99
STK11 region (n=15)	CDC34-5	19-000,492450	0,95	0,86	0,91	0,8	0,8	0,74	0,79	0,73	0,99	0,98	1,03
	ELANE-5	19-000,807080	1,06	1,03	1,01	1,05	0,96	0,93	0,78	0,73	1,01	0,99	1
	KISS1R-5	19-000,871480	1,01	1,06	0,98	0,99	0,94	1	0,96	0,86	1	0,99	1,01
	STK11-1	19-001,156790	0,97	0,97	0,93	0,88	0,85	0,86	0,84	0,79	0,98	0,99	1,03
	STK11-1	19-001,157580	1,04	1	0,92	0,91	0,86	0,83	0,81	0,76	1,04	0,96	1
	STK11-1	19-001,157970	1,03	1,01	1,02	0,89	0,85	0,82	0,85	0,77	0,99	1	1,01
	STK11-2	19-001,169440	1	0,96	0,89	0,72	0,65	0,61	0,64	0,58	1,02	1,01	0,97
	STK11-3	19-001,170340	1,01	1,05	0,97	0,73	0,6	0,66	0,58	0,53	1,01	0,99	1
	STK11-4	19-001,171400	0,99	0,99	0,9	0,67	0,57	0,56	0,54	0,49	1,01	0,98	1,01
	STK11-5	19-001,171590	0,98	0,94	0,79	0,65	0,57	0,56	0,55	0,54	1,01	0,94	1,05
	STK11-6	19-001,172250	0,94	0,9	0,8	0,58	0,52	0,47	0,53	0,43	1,01	0,94	1,05
	STK11-7	19-001,172950	0,98	0,98	0,89	0,62	0,54	0,49	0,49	0,46	1,01	0,97	1,02
	STK11-8	19-001,174120	0,95	0,91	0,78	0,51	0,44	0,42	0,42	0,37	1,02	0,95	1,04
	STK11-9	19-001,177510	0,96	0,91	0,83	0,43	0,28	0,25	0,25	0,23	1	0,96	1,03
	STK11-10	19-001,179620	0,98	0,97	0,86	0,46	0,2	0,23	0,19	0,17	1	1,01	0,99
Denaturation control (n=2)	SHANK3-12	22-049,482960	1,04	0,93	10%	8%	7%	7%	6%	5%	1,03	1,02	0,96
	SHANK3-13	22-049,484000	0,99	0,9	0,22	0,15	0,14	0,16	0,11	10%	0,97	1,04	1
References (n=10)	Reference	01-103,117960	0,98	1	1,07	0,99	1,02	0,98	0,99	0,96	0,97	1,02	1,01
	Reference	04-145,852610	0,99	0,93	0,99	0,9	0,98	0,97	0,98	0,99	1,01	0,96	1,03
	Reference	05-132,037630	1,02	1,04	1,03	0,99	0,98	1,06	1	0,98	1,02	1,02	0,96
	Reference	06-129,808520	0,99	1	1,02	1,01	1,01	0,97	1,05	0,98	1,02	1	0,98
	Reference	07-091,681910	1,01	0,99	0,99	1	0,99	1,01	1,01	1,05	1,01	1,01	0,99
	Reference	08-118,894320	0,99	1,06	0,97	1,04	1,01	1,07	0,97	1,02	0,97	1,03	1
	Reference	09-077,993890	1,04	0,98	1	1,02	0,98	0,99	0,99	1,06	1	0,97	1,03
	Reference	11-077,638990	1,01	1,08	1,09	1,07	1,04	1,08	1,02	1,05	1,01	1,02	0,97
	Reference	12-118,109200	0,97	0,94	0,95	0,98	0,97	1	0,97	0,95	1,02	0,96	1,03
	Reference	18-051,045400	0,98	1	0,94	0,94	1,01	0,96	1,05	0,98	1,01	0,95	1,05

Figure A1. Coffalyser.Net analysis from additional internal tests including samples containing 10 mM to 50 mM NaCl compared with three reference samples. Please note that Coffalyser.Net gives a warning for incomplete denaturation (low 88 and 96 nt D-fragments) from NaCl concentrations of 35 mM onwards.

Extra instructions for SALSA MLPA Probemix P101 STK11 in order to prevent detection of false positive *STK11* deletions

Visual denaturation check in case of apparent *STK11* deletions: The *STK11* gene is (one of) the hardest-to-denature genes due to an extremely high GC content (~63%). Impaired sample denaturation, for instance due to a high NaCl concentration, can inhibit probe-to-sample binding, thereby causing MLPA probe signals to be reduced. In most cases, these denaturation problems will be successfully identified and warned for by the Coffalyser.Net software. **However, at certain NaCl concentrations (<35 mM), *STK11*, in particular the last exons of *STK11*, may be affected by incomplete denaturation without the software issuing a warning. Therefore, an additional visual check of the 96 nt denaturation fragment, as well as the signals of the denaturation control probes (136 nt, 161 nt, 166 nt, and 184 nt) needs to be performed by the user. In case of denaturation problems, the DNA sample needs to be diluted or purified and the MLPA reaction repeated to exclude false positive results.**



Context: Usually, Coffalyser.Net issues an incomplete DNA denaturation warning when the 88 nt and/or 96 nt denaturation fragments are too low in one or several samples. However, as the *STK11* gene is more difficult to denature than other gene regions, **an additional visual check is needed when interpreting the results**. In case of apparent (partial) deletions, the 96 nt denaturation fragment should be visually examined, as this control fragment is most sensitive to denaturation problems. When the 96 nt denaturation fragment is lower than the 92 nt benchmark fragment (in Coffalyser.Net, open *Samples results explorer*; if D2-fragment(s) ratio < 1.00) the sample is likely affected by poor denaturation. The signals of the denaturation control probes (136 nt, 161 nt, 166 nt, and 184 nt) should also be visually examined, as these probes are sensitive to denaturation problems. When at least two out of four of these signals are low (FR ≤ 0.65), the sample is likely affected by incomplete denaturation. Please note that the 184 nt and 166 nt probes are more NaCl-sensitive than the 161 nt and 136 nt probes and may be the only two DNA denaturation probes to produce a low signal (FR ≤ 0.65) at milder NaCl concentrations (25 mM).

Dilution and purification: If DNA quantity allows for this, the sample DNA can be diluted with $TE_{0.1}$ (MLPA General Protocol) to reduce NaCl concentrations and the MLPA experiment should be repeated. If this does not resolve the issue or if it is not possible to further dilute the sample DNA, the use of an additional purification step or an alternative DNA extraction method may resolve cases with high NaCl concentrations. When using silica column-based DNA purification, NaCl concentrations can often be reduced by inclusion of a wash step with 85% ethanol before the elution step.