Product Description
SALSA® MLPA® Probemix P256-C1 FLCN

To be used with the MLPA General Protocol.

Version C1
As compared to version B4, a second probe for *FLCN* exon 1 has been included, a downstream flanking probe has been removed, and four target probes have been adjusted in length but not in sequence detected. The background signal of the c.1285delC mutation-specific probe has been reduced to 0%. In addition, seven reference probes have been replaced and one additional reference probe has been included. For complete product history see page 9.

Catalogue numbers:
- **P256-025R:** SALSA MLPA Probemix P256 FLCN, 25 reactions.
- **P256-050R:** SALSA MLPA Probemix P256 FLCN, 50 reactions.
- **P256-100R:** SALSA MLPA Probemix P256 FLCN, 100 reactions.

To be used in combination with a SALSA MLPA reagent kit and Coffalyser.Net data analysis software. MLPA reagent kits are either provided with FAM or Cy5.0 dye-labelled PCR primer, suitable for Applied Biosystems and Beckman/SCIEX capillary sequencers, respectively (see www.mrcholland.com).

Certificate of Analysis
Information regarding storage conditions, quality tests, and a sample electropherogram from the current sales lot is available at www.mrcholland.com.

Precautions and warnings
For professional use only. Always consult the most recent product description AND the MLPA General Protocol before use: 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.

Intended purpose
The SALSA MLPA Probemix P256 FLCN is an in vitro diagnostic (IVD)[1] or research use only (RUO) semi-quantitative assay[2] for the detection of deletions or duplications in the *FLCN* gene, as well as the presence of the two most common point mutations, c.1285delC and c.1285dupC, in genomic DNA isolated from human peripheral whole blood specimens. P256 FLCN is intended to confirm a potential cause for and clinical diagnosis of Birt-Hogg-Dubé syndrome (BHDS) and for molecular genetic testing of at-risk family members.

Copy number variations (CNVs) detected with P256 FLCN should be confirmed with a different technique. In particular, CNVs detected by only a single probe as well as the two common *FLCN* point mutations always require confirmation by another method. Most defects in the *FLCN* gene are point mutations, which will not be detected by MLPA, with exception of the two aforementioned *FLCN* point mutations. 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 in vitro diagnostic (IVD) use in the countries specified at the end of this product description. In all other countries, the product is for research use only (RUO).

[2]To be used in combination with a SALSA MLPA Reagent Kit, Coffalyser.Net analysis software, and SALSA Binning DNA SD032.

Clinical background
Birt-Hogg-Dubé syndrome (BHDS) is a rare inherited genodermatosis, characterised by hair follicle hamartomas, kidney tumours, pulmonary cysts, and spontaneous pneumothorax. BHDS is inherited in an autosomal dominant fashion and is associated with mutations in the *FLCN* gene that encodes a protein called folliculin. Folliculin acts as a tumour suppressor in the mTOR pathway and inactivation of FLCN leads to increased mitochondrial oxidative metabolism (Hartman et al. 2009).

In >95% of BHDS patients a germline mutation in *FLCN* is identified. In <8% of BHDS patients the causative mutation is a large CNV. Penetrance is high and there is extensive clinical variability. The prevalence is roughly estimated at ~1:200,000. A second (somatic) *FLCN* mutation or loss of heterozygosity is found in the majority (~70%) of BHDS-associated renal tumours, in line with the Knudsen two-hit model of tumorigenesis.

Notably, there is a hypermutable C8-tract in exon 11 that spawns the two most common *FLCN* mutations c.1285dupC and c.1285delC; ~20-24% of patients show a germline deletion or insertion of a cytosine at this site (Nickerson et al. 2002; Schmidt et al. 2005; Toro et al. 2007; Toro et al. 2008; Liu et al. 2017). A slippage-mediated mechanism during DNA replication is thought to be responsible for these frameshift mutations leading to protein truncation.

More information is available at: https://www.ncbi.nlm.nih.gov/books/NBK1522/

Gene structure
The *FLCN* gene spans 14 kilobases (kb) on chromosome 17p11.2 and contains 14 exons. The *FLCN* LRG_325 is available at www.lrg-sequence.org and is identical to GenBank NG_008001.2.

Transcript variants
For *FLCN,* multiple variants have been described but only one transcript variant that encodes the full length protein, isoform 1 (NM_144997.7; 3667 nt; coding sequence 485-2224; http://www.ncbi.nlm.nih.gov/gene/201163). This sequence is a reference standard in the NCBI RefSeq project. The ATG translation start site is located after exon 1 and the stop codon is located in exon 14.

Exon numbering
The *FLCN* exon numbering used in this P256-C1 FLCN product description is the exon numbering from the LRG_325 sequence. The exon numbering of the NM_144997.7 that was used for determining a probe's ligation site does not always correspond to the exon numbering obtained from the LRG sequences. As changes to the databases can occur after release of this product description, the NM_ sequence and exon numbering may not be up-to-date.

Probemix content
The SALSA MLPA Probemix P256-C1 FLCN contains 27 MLPA probes with amplification products between 129 and 380 nucleotides (nt). This includes 15 copy number probes for the *FLCN* gene and two probes specific for the c.1285delC and c.1285dupC *FLCN* mutations, which will only generate a signal when the mutation is present. In addition, ten reference probes are included that detect autosomal chromosomal locations. Complete probe sequences and the identity of the genes detected by the reference probes are available online (www.mrcholland.com).

This probemix contains nine quality control fragments generating amplification products between 64 and 105 nt: four DNA Quantity fragments (Q-fragments), two DNA Denaturation fragments (D-fragments), one Benchmark fragment, and one chromosome X and one chromosome Y-specific fragment (see table below). More information on how to interpret observations on these control fragments can be found in the MLPA General Protocol and online at www.mrcholland.com.

Length (nt)	Name
64-70-76-82	Q-fragments (only visible with <100 ng sample DNA)
88-96	D-fragments (low signal indicates incomplete denaturation)
92	Benchmark fragment
100	X-fragment (X chromosome specific)
105	Y-fragment (Y chromosome specific)

MLPA technique
The principles of the MLPA technique (Schouten et al. 2002) are described in the MLPA General Protocol (www.mrcholland.com).

MLPA technique validation
Internal validation of the MLPA technique using 16 DNA samples from healthy individuals is required, in particular when using MLPA for the first time, or when changing the sample handling procedure, DNA extraction method or instruments used. This validation experiment should result in a standard deviation ≤0.10 for all probes over the experiment. Note that the peaks of the mutation-specific probes are expected to be absent in the majority of samples from healthy individuals.

Required specimens
Extracted DNA from peripheral blood, free from impurities known to affect MLPA reactions. For more information please refer to the section on DNA sample treatment found in the MLPA General Protocol.

Reference samples
A sufficient number (≥3) of reference samples should be included in each MLPA experiment for data normalisation. 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. Reference samples should be derived from different unrelated individuals who are from families without a history of BHDS. More information regarding the selection and use of reference samples can be found in the MLPA General Protocol (www.mrcholland.com).

Positive control DNA samples
MRC Holland cannot provide positive DNA samples. Inclusion of a positive sample in each experiment is recommended. Coriell Institute (https://catalog.coriell.org) and Leibniz Institute DSMZ (https://www.dsmz.de/) have diverse collections of biological resources which may be used as positive control DNA samples in your MLPA experiments. Sample ID numbers NA08146, NA13476, NA18319, NA18320, NA18322, NA18324, NA18326 and NA20743 from the Coriell Institute have been tested with this P256-C1 probemix at MRC-Holland and can all be used as positive control samples to detect a heterozygous deletion of the whole *FLCN* gene. The quality of cell lines can change; therefore samples should be validated before use.

SALSA Binning DNA SD032
The SD032 Binning DNA provided with this probemix can be used for binning of all probes including the two mutation-specific probes: the188 nt probe 08598-L31913 detecting the c.1285delC mutation, and the 195 nt probe 08598-L31789 detecting the c.1285dupC mutation. SD032 Binning DNA is a mixture of genomic DNA from healthy individuals and plasmid DNA that contains the target sequences detected by the above mentioned probes. Inclusion of one reaction with 5 µl SD032 Binning DNA in initial MLPA experiments is essential as it can be used to aid in data binning of the peak pattern using Coffalyser.Net software. Furthermore, Binning DNA should be included in the experiment whenever changes have been applied to the set-up of the capillary electrophoresis device (e.g. when capillaries have been renewed). Binning DNA should never be used as a reference sample in the MLPA data analysis, neither should it be used in quantification of mutation signal(s). It is strongly advised that all samples tested are extracted with the same method and derived from the same source of tissue. For further details, please consult the SD032 Binning DNA product description, available online: www.mrcholland.com.

Performance characteristics

In >95% of patients with a clinical diagnosis of BHDS, a germline mutation in the *FLCN* gene is identified. <8% of all BHDS patients have a large deletion or duplication of *FLCN* and the c.1285dupC or c.1285delC mutations are present in the genome of ~20–24 % of patients (Sattler et al. 2018, GeneReview BHDS https://www.ncbi.nlm.nih.gov/books/NBK1522/). Considering these mutation frequencies, the diagnostic sensitivity of P256 is estimated at 28-32%. Analytical performance for the detection of deletions/duplications in *FLCN* is very high and can be considered >99% (based on a 2010-2021 literature review).

Analytical performance can be compromised by: SNVs or other polymorphisms in the DNA target sequence, impurities in the DNA sample, incomplete DNA denaturation, the use of insufficient or too much sample DNA, the use of insufficient or unsuitable reference samples, problems with capillary electrophoresis or a poor data normalisation procedure and other technical errors. The MLPA General Protocol contains technical guidelines and information on data evaluation/normalisation.

Data analysis

Coffalyser.Net software should be used for data analysis in combination with the appropriate lot-specific MLPA Coffalyser sheet. For both, the latest version should be used. Coffalyser.Net software is freely downloadable at www.mrcholland.com. Use of other non-proprietary software may lead to inconclusive or false results. For more details on MLPA quality control and data analysis, including normalisation, see the Coffalyser.Net Reference Manual.

Interpretation of results

The expected results for all copy number probes are allele copy numbers of 2 (normal), 1 (heterozygous deletion) or 3 (heterozygous duplication). In rare cases, copy numbers of 0 (homozygous deletion) or 4 (heterozygous triplication/homozygous duplication) can be obtained. The standard deviation of each individual target probes (with exception of the mutation-specific probes) over all the reference samples should be ≤0.10 and the final ratio (FR) of each individual reference probe in the patient samples should be between 0.80 and 1.20. When these criteria are fulfilled, the following cut-off values for the FR of the copy number probes can be used to interpret MLPA results for autosomal chromosomes or pseudo-autosomal regions:

Copy number status	Final ratio (FR)
Normal	0.80 < FR < 1.20
Homozygous deletion	FR = 0
Heterozygous deletion	0.40 < FR < 0.65
Heterozygous duplication	1.30 < FR < 1.65
Heterozygous triplication/homozygous duplication	1.75 < FR < 2.15
Ambiguous copy number	All other values

Note: The term “dosage quotient”, used in older product description versions, has been replaced by “final ratio” to become consistent with the terminology of the Coffalyser.Net software. (Calculations, cut-offs and interpretation remain unchanged.) Please note that the Coffalyser.Net software also shows arbitrary borders as part of the statistical analysis of results obtained in an experiment. As such, arbitrary borders are different from the final ratio cut-off values shown here above.

The above mentioned FR values do not apply to the mutations-specific probes. The peaks of the mutation-specific probes are expected to be absent (188 nt) or very low (195 nt) in the majority of samples tested and therefore their standard deviation cannot be determined. In healthy samples without the c.1285dupC mutation, the FLCN 195 nt probe will generate a background signal of 5-10% of the median peak height of all reference probes, which indicates absence of the mutation. Clear signal (>20% of the median peak height of all reference probes in that sample) for the mutation-specific probes indicates that the mutation is present.

- Arranging probes according to chromosomal location facilitates interpretation of the results and may reveal more subtle changes such as those observed in mosaic cases. Analysis of parental samples may be necessary for correct interpretation of complex results.

- False positive results: Please note that abnormalities detected by a single probe (or multiple consecutive probes) still have a considerable chance of being a false positive result. Sequence changes (e.g. SNVs, point mutations) in the target sequence detected by a probe can be one cause. Incomplete DNA denaturation (e.g. due to salt contamination) can also lead to a decreased probe signal, in particular for probes located in or near a GC-rich region. The use of an additional purification step or an alternative DNA extraction method may resolve such cases. Additionally, contamination of DNA samples with cDNA or PCR amplicons of individual exons can lead to an increased probe signal (Varga et al. 2012). Analysis of an independently collected secondary DNA sample can exclude these kinds of contamination artefacts.
- Normal copy number variation in healthy individuals is described in the database of genomic variants: http://dgv.tcag.ca/dgv/app/home. Users should always consult the latest update of the database and scientific literature when interpreting their findings.
- Not all abnormalities detected by MLPA are pathogenic. In some genes, intragenic deletions are known that result in very mild or no disease (as described for *DMD* by Schwartz et al. 2007). For many genes, more than one transcript variant exists. Copy number changes of exons that are not present in all transcript variants may not have clinical significance. Duplications that include the first or last exon of a gene (e.g. exons 1-3) might not result in inactivation of that gene copy.
- Copy number changes detected by reference probes or flanking probes are unlikely to have any relation to the condition tested for.
- False results can be obtained if one or more peaks are off-scale. For example, a duplication of one or more exons can be obscured when peaks are off-scale, resulting in a false negative result. The risk on off-scale peaks is higher when probemixes are used that contain a relatively low number of probes. Coffalyser.Net software warns for off-scale peaks while other software does not. If one or more peaks are off-scale, rerun the PCR products using either: a lower injection voltage or a shorter injection time, or a reduced amount of sample by diluting PCR products.

P256 specific notes

- The two c.1285delC and c.1285dupC mutation-specific probes are only intended to determine the presence (or absence) of the mutation.
- In healthy samples without the c.1285dupC mutation, the FLCN 195 nt probe will generate a background signal of 5-10% of the median peak height of all reference probes, which indicates absence of the mutation. The presence of a clear signal for the 195 nt probe (at least 20% of the median peak height of all reference probes in the sample), indicates the presence of the FLCN c.1285dupC mutation. The percentage obtained for the mutation-specific probe can vary between samples and does not determine whether the mutation is present in heterozygous or homozygous state.

Limitations of the procedure

- In most populations, the major cause of genetic defects in the *FLCN* gene are small (point) mutations, most of which will not be detected by using SALSA MLPA Probemix P256 FLCN. Only the presence of the two most common point mutations (c.1285dupC and c.1285delC) in the *FLCN* gene can be detected, but other point mutations cannot.
- The results of mutation specific probes, should always be confirmed visually in the size called peak pattern and / or raw run data. Detection of a background signal in reference or patient samples may lead to false positive results.
- MLPA cannot detect any changes that lie outside the target sequence of the probes and will not detect copy number neutral inversions or translocations. Even when MLPA did not detect any aberrations, the possibility remains that biological changes in that gene or chromosomal region *do* exist but remain undetected.
- Sequence changes (e.g. SNVs, point mutations) in the target sequence detected by a probe can cause false positive results. Mutations/SNVs (even when >20 nt from the probe ligation site) 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.

Confirmation of results

Copy number changes detected by only a single probe as well as the two common *FLCN* point mutations always require confirmation by another method. Because the two mutation-specific probes are only intended to determine the presence of the mutation, positive results obtained for either of these probes need to be confirmed by sequence analysis to determine the zygosity of the mutation.

An apparent deletion detected by a single copy number probe can be due to e.g. a mutation/polymorphism that prevents ligation or destabilises the binding of probe oligonucleotides to the DNA sample. Sequence analysis can establish whether mutations or polymorphisms are present in the probe target sequence. The finding of a heterozygous mutation or polymorphism indicates that two different alleles of the sequence are present in the sample DNA and that a false positive MLPA result was obtained.

Copy number changes detected by more than one consecutive probe should be confirmed by another independent technique such as long range PCR, qPCR, array CGH or Southern blotting, whenever possible. Deletions/duplications of more than 50 kb in length can often be confirmed by FISH.

***FLCN* mutation database**

We strongly encourage users to deposit positive results in the *FLCN* LOVD database: https://databases.lovd.nl/shared/genes/FLCN. Recommendations for the nomenclature to describe deletions/duplications of one or more exons can be found on http://varnomen.hgvs.org/.

Please report copy number changes detected by the reference probes, false positive results due to SNVs and unusual results (e.g., a duplication of *FLCN* exons 6 and 8 but not exon 7) to MRC Holland: info@mrcholland.com.

Table 1. SALSA MLPA Probemix P256-C1 FLCN

Length (nt)	SALSA MLPA probe	Chromosomal position (hg18)[a]	
		Reference	*FLCN*
64-105	Control fragments – see table in probemix content section for more information		
129 *	Reference probe 18709-L26847	5q	
143	**FLCN probe** 08591-L23693		**Exon 5**
153	**FLCN probe** 08595-L08596		**Exon 9**
165 *	Reference probe 13816-L28133	2q	
172	**FLCN probe** 08599-L08602		**Exon 12**
178 *	Reference probe 07032-L28099	14q	
188 ¥ § ±	**FLCN probe** 08598-L31913		**c.1285delC**
195 ¥ § ±	**FLCN probe** 08598-L31789		**c.1285dupC**
203 *	Reference probe 17177-L31937	15q	
214	**FLCN probe** 08594-L08595		**Exon 8**
220 *	Reference probe 08940-L31919	11p	
227	**FLCN probe** 08588-L08589		**Exon 2**
238	**FLCN probe** 08596-L08597		**Exon 10**
250	**FLCN probe** 08590-L08591		**Exon 4**
257	Reference probe 10692-L11274	6p	
265	**FLCN probe** 08600-L08603		**Exon 13**
275 *	Reference probe 04489-L03878	1p	
285 ¥	**FLCN probe** 08593-L31925		**Exon 7**
301	**FLCN probe** 08601-L08604		**Exon 14**
310	Reference probe 18380-L25673	10q	
321	**FLCN probe** 08589-L08590		**Exon 3**
328 *	Reference probe 16275-L22420	19p	
337	**FLCN probe** 08597-L23694		**Exon 11**
346 * +	**FLCN probe** 22584-L31783		**Exon 1**
355	**FLCN probe** 08592-L08593		**Exon 6**
366 ¥	**FLCN probe** 22580-L31776		**Exon 1**
380 *	Reference probe 16932-L19875	4q	

[a] See section Exon numbering on page 2 for more information.

* New in version C1.
¥ Changed in version C1. Minor alteration, no change in sequence detected.
§ Mutation-specific probe. The probes at 188 nt and 195 nt will only generate a signal when the c.1285delC or c.1285dupC mutations are present, respectively. Clear signal (at least 20% of the median peak height of all reference probes in that sample) for one of these probes indicates that the mutation is present.
± SNP rs41459448 could increase the background signal on wildtype DNA for both 188 nt and 195 nt mutation specific probes. In case of an apparent mutation, it is recommended to sequence the region targeted by this probe.
+ The ligation site of this probe is located in an alternative exon present in alternative transcript NM_001353229.1. The significance of deletions/duplications of only this alternative exon is not clear as this exon is non-coding and alternative transcript variants using other transcription start sites are known.

SNVs located in the target sequence of a probe can influence probe hybridization and/or probe ligation. Please note: not all known SNVs are mentioned in the tables above. Single probe aberration(s) must be confirmed by another method.

Table 2. *FLCN* probes arranged according to chromosomal location

Length (nt)	SALSA MLPA probe	Gene exon[a]	Ligation site NM_144997.7	Partial sequence[b] (24 nt adjacent to ligation site)	Distance to next probe
366	22580-L31776	Exon 1	42-43	GTCGCTCCTGGT-TCTGCCAGCTCC	0.5 kb
346 +	22584-L31783	Exon 1	294 nt after exon 1, reverse	CAGAGGACCCTC-CTGAACGCCAAA	3.8 kb
227	08588-L08589	Exon 2	359-360	AAGCGTGATTCT-GCTGAGTGTCAG	0.9 kb
321	08589-L08590	Exon 3	32 nt before exon 3	TTCTCTTCAAAT-GTCTGTTTTCTG	3.9 kb
		start codon	*485-487 (Exon 4)*		
250	08590-L08591	Exon 4	645-646	TGGCATTCAGAT-GAACAGTCGGAT	1.7 kb
143	08591-L23693	Exon 5	770-771	ACCCGGGATATA-TCAGCCATGATA	2.2 kb
355	08592-L08593	Exon 6	957-958	CACCTTCTTCAT-CAAGGACAGCCT	1.5 kb
285	08593-L31925	Exon 7	1151-1152	CTCAGAGGATGA-ACACAGCCTTCA	1.0 kb
214	08594-L08595	Exon 8	1309-1310	AAGCTCCTGGAA-GGTGCTCCGACC	2.4 kb
153	08595-L08596	Exon 9	Intron 8 - 1356	TCTGTCTTGCAG-ATTTAGAAGAGG	2.1 kb
238	08596-L08597	Exon 10	1656-1657	TTTTGAAGTACT-TCGGGTGAGAAC	0.6 kb
337	08597-L23694	Exon 11	11 nt before exon 11	CTGAGTCCTGCT-GTCCTCCTCAGA	0.1 kb
188 § ±	08598-L31913	Exon 11	1769-1768, reverse	AGGAGAGCACGT-GGGGGGGATCTG	-
195 § ±	08598-L31789	Exon 11	1769-1768, reverse	GAGAGCACGTGG-GGGGGGGATCTG	1.2 kb
172	08599-L08602	Exon 12	1878-1879	CAAGTACGAGTT-TGTGGTGACCAG	0.2 kb
265	08600-L08603	Exon 13	2007-2008	CGTCTGCCTCAA-GGAGGAGTGGAT	1.2 kb
301	08601-L08604	Exon 14	2085-2086	GGACACACAGAA-GCTGCTGAGCAT	
		stop codon	*2222-2224 (exon 14)*		

[a] See section Exon numbering on page 2 for more information.
[b] Only partial probe sequences are shown. Complete probe sequences are available at www.mrcholland.com. Please notify us of any mistakes: info@mrcholland.com.

§ Mutation-specific probe. The probes at 188 nt and 195 nt will only generate a signal when the c.1285delC or c.1285dupC mutations are present, respectively. Clear signal (at least 20% of the median peak height of all reference probes in that sample) for one of these probes indicates that the mutation is present.
± SNP rs41459448 could increase the background signal on wildtype DNA for both 188 nt and 195 nt mutation specific probes. In case of an apparent mutation, it is recommended to sequence the region targeted by this probe.
+ The ligation site of this probe is located in an alternative exon present in alternative transcript NM_001353229.1 (324-323 reverse). The significance of deletions/duplications of only this alternative exon is not clear as this exon is non-coding and alternative transcript variants using other transcription start sites are known.

Complete probe sequences are available at www.mrcholland.com.

Related SALSA MLPA probemixes

P369 Smith-Magenis	Contains probes for the 17p11.2 region, including several probes for the *FLCN* gene. This region is associated with Smith-Magenis syndrome (SMS).
P225 PTEN	Contains probes for the *PTEN* gene. This gene is associated with Cowden syndrome, which is a disease related to BHDS.

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P256 product history	
Version	*Modification*
C1	A second probe for *FLCN* exon 1 has been included, the downstream flanking probe has been removed, and four target probes have been adjusted in length but not in sequence detected. The background signal of the c.1285delC mutation-specific probe has been reduced to 0%. In addition, seven reference probes have been replaced and one new reference probe has been added.
B4	Three reference probes have been replaced.
B3	One reference probe has been replaced and one has been added.
B2	Three reference probes have been replaced and the control fragments have been adjusted (QDX2).
B1	Three new reference probes have been added and one flanking probe has been removed.
A1	First release.

Implemented changes in the product description
Version C1-02 – 17 August 2021 (04P) - Product description adapted to a new template. - The intended purpose was rewritten and adapted to a new template. - Performance characteristics section and References section updated. - P256 specific notes section added. - Ligation sites of the probes targeting the *FLCN* gene updated according to new version of the NM_ reference sequence. - Warning for SNP (188 nt probe and 195 nt probe) was added under the table 1 and 2. - Warning for SNP (366 nt probe) was removed under the table 1 and 2. - Throughout document several statement are included/rephrased emphasizing that only the presence of the two common mutations (c.1285delC and c.1285dupC) can be detected and not the zygosity. - References and Selected publications were curated and new literature was included. - UK added to the list of countries in Europe that accept the CE mark. *Version C1-01 — 12 December 2019 (02P)* - Product description rewritten and adapted to a new template. - P256-C1 is now CE marked. - Product description adapted to a new product version (version number changed, changes in Table 1 and Table 2). - Interpretations of results for mutation-specific probes has been described in more detail. - P225 has been added to related probemixes. *Version B4-01 – 23 October 2018 (01P)* - Product description restructured and adapted to a new template. - Product description adapted to a new product version (version number changed, changes in Table 1 and Table 2). *Version 11 – 12 December 2016 (55)* - Description of background signal for the mutation-specific probes clarified.

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IVD	EUROPE* CE
RUO	ALL OTHER COUNTRIES

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