Product Description
SALSA® MLPA® Probemix P016-C2 VHL

To be used with the MLPA General Protocol.

Version C2
For complete product history see page 9.

Catalogue numbers:
- **P016-025R:** SALSA MLPA Probemix P016 VHL, 25 reactions.
- **P016-050R:** SALSA MLPA Probemix P016 VHL, 50 reactions.
- **P016-100R:** SALSA MLPA Probemix P016 VHL, 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 P016 VHL is an in vitro diagnostic (IVD)[1] or research use only (RUO) semi-quantitative assay[2] for the detection of deletions in the *VHL* gene in genomic DNA isolated from human peripheral whole blood specimens. P016 VHL is intended to confirm a potential cause for and clinical diagnosis of Von Hippel-Lindau (VHL) disease and for molecular genetic testing of at-risk family members.

Copy number variations (CNVs) detected with P016 VHL should be confirmed with a different technique. In particular, CNVs detected by only a single probe always require confirmation by another method. Most defects in the *VHL* 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 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 and Coffalyser.Net analysis software.

Clinical background
Von Hippel-Lindau (VHL) disease is a dominantly inherited familial cancer syndrome predisposing to a variety of malignant and benign neoplasms, most frequently retinal, cerebellar and spinal hemangioblastoma, renal cell carcinoma, pheochromocytoma, and pancreatic tumours. The basis of familial inheritance of VHL disease is a germline mutation in the *VHL* tumour suppressor gene, located in chromosomal region 3p25.3. Approximately 80% of individuals with VHL disease inherit a mutation, whereas 20% of individuals have a de

novo mutation (http://www.ncbi.nlm.nih.gov/books/NBK1463/). Of the mutations in the *VHL* gene, 30-60% are missense mutations, 20–40% are large intragenic deletions (0.5–250 kb), 12–20% are microdeletions or insertions and 7–11% are nonsense mutations (Decker et al. 2014).

Interestingly, loss of the nearby *BRK1* gene in combination with loss of (part of) the *VHL* gene can be associated with a reduced risk of renal cell carcinoma as compared to defects in the *VHL* gene only (Escobar et al. 2010, McNeill et al. 2009). This probemix contains two probes that target the *BRK1* gene.

Gene structure
The *VHL* gene spans ~10 kilobases (kb) on chromosome 3p25.3 and contains 3 exons. The *VHL* LRG_322 is available at www.lrg-sequence.org and is identical to GenBank NG_008212.3.

Transcript variants
For *VHL*, three transcript variants have been described (https://www.ncbi.nlm.nih.gov/gene/7428). Transcript variant 1 encodes the longest isoform (NM_000551.4; 4414 nt; coding sequence 71-712). Transcript variant 2 (NM_198156.3) is a shorter variant that lacks an in-frame coding exon, and transcript variant 3 (NM_001354723.2) lacks an alternate in-frame exon and contains another alternate exon compared to transcript variant 1.

Exon numbering
The *VHL* exon numbering used in this P016-C2 VHL product description is the exon numbering from the LRG_322 sequence. The exon numbering of the NM_ sequence 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 P016-C2 VHL contains 29 MLPA probes with amplification products between 166 and 427 nucleotides (nt). This includes nine probes for the *VHL* gene (two or more probes for each exon), six probes for genes located close to *VHL* (*FANCD2*, *BRK1*, *IRAK2* and *GHRL*), and two probes for genes on 3p located further telomeric or centromeric from *VHL* (*CNTN6* and *MLH1*). In addition, 12 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.

Required specimens
Extracted DNA from human peripheral whole blood specimens, 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 VHL disease. 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 NA10985, NA13249, NA13250 and NA13256 from the Coriell Institute have been tested with this P016-C2 probemix at MRC Holland and can be used as a positive control samples to detect *VHL* (exon) deletions. The quality of cell lines can change; therefore samples should be validated before use.

Sample name	Source	Copy number alteration of genes*	Expected copy number variation
NA10985	Coriell Institute	*VHL, FANCD2, BRK1, IRAK2, GHRL, CNTN6*	Heterozygous deletion
NA13249	Coriell Institute	*VHL* exon 2 and 3, *IRAK2*	Heterozygous deletion
NA13250	Coriell Institute	*VHL* exon 1 and 2	Heterozygous deletion
NA13256	Coriell Institute	*VHL, FANCD2, BRK1*	Heterozygous deletion

* Indicated genes targeted by MLPA probes, however, the whole extent of the CNV present in this cell line cannot be determined by this P016-C2 VHL probemix.

Performance characteristics
The expected number of pathogenic mutations in the *VHL* gene that can be detected with this MLPA probemix is 20-40%, as (partial) deletions account for 20-40% of all mutations in VHL patients (Decker et al. 2014). The analytical sensitivity and specificity for the detection of deletions in *VHL* is very high and can be considered >99% (based on a 2005 -2020 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 *VHL*-specific MLPA probes are allele copy numbers of 2 (normal), 0 (homozygous deletion), 1 (heterozygous deletion), 3 (heterozygous duplication), and 4 (heterozygous triplication/ homozygous duplication).

The standard deviation of each individual probe 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 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.

- 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. 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.

P016 specific note:

- The P016 VHL probemix has been used in a research setting to detect *VHL* deletions in tumour tissue in order to identify loss of heterozygosity (LOH) in VHL patients and to study phenotype-genotype correlation (Banks et al. 2006, Patard et al. 2009, Young et al. 2009). **Importantly, the diagnostic use of P016 VHL with DNA extracted from tumour tissue has not been validated**.

Limitations of the procedure

- In most populations, the major cause of genetic defects in the *VHL* gene are small (point) mutations, none of which will be detected by using SALSA MLPA Probemix P016-C2 VHL.
- 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.
- LOH is often detected at the *VHL* locus in DNA isolated from VHL-associated tumours. Please note that deletion of one copy of the *VHL* gene, which can be detected by MLPA, is only one of the possible causes of LOH. LOH caused by non-disjunction or somatic recombination does not result in a change in copy number of the *VHL* gene and cannot be detected by MLPA.
- MLPA analysis on tumour samples provides information on the *average* situation in the cells from which the DNA sample was purified. Gains or losses of genomic regions or genes may not be detected if the percentage of tumour cells is low. In addition, subclonality of the aberration affects the final ratio of the corresponding probe. Furthermore, there is always a possibility that one or more reference probes *do* show a copy number alteration in a patient sample, especially in solid tumours with more chaotic karyotypes.
- MLPA may not be able to identify deletions of *VHL* in cases in which mosaicism is considered.

Confirmation of results

Copy number changes detected by only a single probe always require confirmation by another method. An apparent deletion detected by a single 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.

***VHL* mutation database**

https://databases.lovd.nl/shared/genes/VHL. We strongly encourage users to deposit positive results in the *VHL* mutation database. 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 deletion of *VHL* exons 1 and 3 but not exon 2) to MRC Holland: info@mrcholland.com.

Table 1. SALSA MLPA Probemix P016-C2 VHL

Length (nt)	SALSA MLPA probe	Chromosomal position (hg18)[a]		
		Reference	*VHL*	Other
64-105	Control fragments – see table in probemix content section for more information			
166	Reference probe 09890-L10303	16p		
175	**VHL probe** 13624-L16363		**Exon 2**	
184 ¬	CNTN6 probe 06307-L05830			3p26.3
193 ¤	BRK1 probe 14676-L16328			3p25.3
201 ±	**VHL probe** 02390-L16140		**Exon 2**	
211	Reference probe 13450-L14905	5q		
219 ±	**VHL probe** 01626-L01211		**Exon 1**	
229 ¬	IRAK2 probe 02264-L01750			3p25.3
238 ¬	MLH1 probe 00892-L00480			3p22.2
247	**VHL probe** 01162-L00718		**Exon 3**	
257	Reference probe 01055-L00628	17q		
265	Reference probe 02454-L01898	15q		
274	**VHL probe** 01628-L01213		**Exon 1**	
283 ¬	FANCD2 probe 02138-L01631			3p25.3
301 ¬	GHRL probe 02266-L01752			3p25.3
310 ¬	FANCD2 probe 02140-L01633			3p25.3
319	Reference probe 05981-L05406	20p		
328	**VHL probe** 13625-L15079		**Exon 1**	
337	Reference probe 01082-L00660	22q		
346 «	Reference probe 01335-L00879	7q		
355	Reference probe 00547-L00116	11q		
364 ¤	BRK1 probe 14675-L16327			3p25.3
373	**VHL probe** 01158-L13266		**Exon 1**	
382	Reference probe 00973-L00560	10q		
391	**VHL probe** 13322-L14735		**Exon 2**	
400	Reference probe 00801-L00639	13q		
409 ± ◎	Reference probe 00669-L00373	11p		
418 ±	**VHL probe** 01161-L00717		**Exon 3**	
427	Reference probe 00680-L00121	7q		

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

¤ Please note that loss of the *BRK1* gene in combination with loss of (part of) the *VHL* gene can be associated with a reduced risk of renal cell carcinoma as compared to defects in the *VHL* gene only (Escobar et al. 2010, McNeill et al. 2009).

± SNP rs115744107 and SNP rs185858030 could influence the 201 nt probe signal; SNP rs3087462 could influence the 219 nt probe signal; SNP rs143428254 could influence the 409 nt probe signal; SNP rs5030820 and a SNP at +3 from the ligation site could influence the 418 nt probe signal. In case of apparent deletions, it is recommended to sequence the region targeted by this probe.

« Probe located in or near a GC-rich region. A low signal can be caused by salt contamination in the DNA sample leading to incomplete DNA denaturation, especially of GC-rich regions.

¬ Flanking probe. Included to help determine the extent of a deletion/duplication. Copy number alterations of only the flanking or reference probes are unlikely to be related to the condition tested.

◎ We have been informed that a duplication of this sequence has been observed in healthy individuals. Normal copy number variation in healthy individuals is described in the database of genomic variants: http://dgv.tcag.ca/dgv/app/home.

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. P016-C2 probes arranged according to chromosomal location

Length (nt)	SALSA MLPA probe	Gene exon[a]	Ligation site NM_000551.4	Partial sequence[b] (24 nt adjacent to ligation site	Distance to next probe
184	06307-L05830	CNTN6	1110 kb from p-telomere	CAGCTGGGAAAA-AGAGTCCCATTA	8995.5 kb
283	02138-L01631	FANCD2		TGGGGATAAAGA-GAAGAGCAACAT	10.3 kb
310	02140-L01633	FANCD2		TATCTCTACAAA-ACCCACCAGAGT	26.8 kb
364 ¤	14675-L16327	BRK1		GCAACACTAAAC-GAGAAATTGACA	0.6 kb
193 ¤	14676-L16328	BRK1		AAAAGGTGAGAC-ACTCACCTAGAA	15.5 kb
	VHL gene	*start codon*	*71-73 (Exon 1)*		
219 ±	01626-L01211	**Exon 1**	9 nt before exon 1	GGTCGACTCGGG-AGCGCGCACGCA	0.1 kb
274	01628-L01213	**Exon 1**	107-108	ACGAGGCCGAGG-TAGGCGCGGAGG	0.2 kb
373	01158-L13266	**Exon 1**	296-297	CCCAGGTCATCT-TCTGCAATCGCA	0.2 kb
328	13625-L15079	**Exon 1**	116 nt after exon 1	TGAGGCAGGACA-CATCCAGGGTGA	3.8 kb
175	13624-L16363	**Exon 2**	398 nt before exon 2, reverse	TGACCTACACAA-AAAGCTTACCTC	0.4 kb
201 ±	02390-L16140	**Exon 2**	34 nt before exon 2	CCAGCCACCGGT-GTGGCTCTTTAA	0.1 kb
391	13322-L14735	**Exon 2**	489-488, reverse	CGTCAACATTGA-GAGATGGCACAA	3.3 kb
418 ±	01161-L00717	**Exon 3**	586-587	CTAGTCAAGCCT-GAGAATTACAGG	0.1 kb
247	01162-L00718	**Exon 3**	655-656	CCAAATGTGCAG-AAAGACCTGGAG	28.0 kb
		stop codon	*710-712 (Exon 3)*		
229	02264-L01750	IRAK2		GCGGAAGATCAA-GTCCATGGAGCG	107.9 kb
301	02266-L01752	GHRL		GGCTTTTCGCTT-GCTTCTGCAGCA	26725.9 kb
238	00892-L00480	MLH1		ACACAATGCAGG-CATTAGTTTCTC	

[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.mlpa.com. Please notify us of any mistakes: info@mrcholland.com.

¤ Please note that loss of the *BRK1* gene in combination with loss of (part of) the *VHL* gene can be associated with a reduced risk of renal cell carcinoma as compared to defects in the *VHL* gene only (Escobar et al. 2010, McNeill et al. 2009).

± SNP rs115744107 and SNP rs185858030 could influence the 201 nt probe signal; SNP rs3087462 could influence the 219 nt probe signal; SNP rs5030820 and a SNP at +3 from the ligation site could influence the 418 nt probe signal. In case of apparent deletions, it is recommended to sequence the region targeted by this probe.

Related SALSA MLPA probemixes

P226 SDH	Contains probes for *SDHB*, *SDHC*, *SDHD*, *SDHAF1* and *SDHAF2*; involved in pheochromocytomas and paragangliomas.
P429 SDHA-MAX	Contains probes for *SDHA* and *MAX*; involved in pheochromocytomas and paragangliomas.

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P016 product history	
Version	*Modification*
C2	The 88 and 96 nt DNA Denaturation fragments have been replaced by the QDX2 control fragments.
C1	Three VHL probes and six reference probes have been replaced. Two probes for the flanking *BRK1* (*C3orf10/HSPC300*) gene have been included.
B2	Four extra control probes at 88, 96, 100 and 105 nt have been added.
B	First release.

Implemented changes in the product description

Version C2-05 – 05 December 2023 (04P)
- Morocco removed from the list of countries in which the product is IVD registered.

Version C2-04 – 29 June 2021 (04P)
- Product description rewritten and adapted to a new template.
- Intended purpose updated.
- Transcript variants section and link to mutation database updated.
- Positive Coriell samples added in the section positive control DNA samples.
- Ligation sites of the probes targeting the *VHL* gene updated according to new version of the NM_ reference sequence.
- Warnings for SNPs (201 nt probe, 219 nt probe and 409 nt probe) and a GC-rich region (346 nt probe) added under Table 1 and 2.
- Warning symbols under Table 1 and 2 updated.
- Selected publications section updated.
- UK added to the list of countries in Europe that accept the CE mark.

Version C2-03 – 18 April 2019 (04)
- Positive control DNA samples section updated; new positive control NA10985.
- Gene structure and transcript variants section updated; information on transcript variant 3 was added.
- Warning regarding Reference probe 00669-L00373 (409nt) was placed under table 1.
- Warnings (¥ and ‡) under table 1 and 2 were updated.
- Product is now registered for IVD use in Morocco and Israel.
- Product description adapted to a new template.

More information: www.mrcholland.com; www.mrcholland.eu	
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IVD	EUROPE* CE ISRAEL
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.