Product Description
SALSA® MLPA® Probemix P047-E3 RB1

To be used with the MLPA General Protocol and MS-MLPA General Protocol.

Version E3.
As compared to version E2, one reference probe has been replaced and several probes have changed in length but not in targeted sequence. For complete product history see page 14.

The predominant use of this probemix is the **copy number determination of the *RB1* gene** using the **MLPA General Protocol**. In addition to copy number determination, this probemix can also detect the **methylation status** of the *RB1* promoter region and *RB1* imprinted locus. When P047 is used for both ***RB1* copy number** and **methylation** determination the **MS-MLPA General Protocol** should be used. Both protocols can be found on www.mrcholland.com.

Catalogue numbers
- **P047-025R:** SALSA® MLPA® Probemix P047 RB1, 25 reactions
- **P047-050R:** SALSA® MLPA® Probemix P047 RB1, 50 reactions
- **P047-100R:** SALSA® MLPA® Probemix P047 RB1, 100 reactions

SALSA® MLPA® Probemix P047 RB1 (hereafter: P047 RB1) is to be used in combination with:

1. SALSA® MLPA® Reagent Kit (Cat. No: EK1-FAM, EK1-CY5, EK5-FAM, EK5-CY5, EK20-FAM),
2. Data analysis software Coffalyser.Net™ (Cat. No: n.a.)

Volumes and ingredients

Volumes			Ingredients
P047-025R	**P047-050R**	**P047-100R**	
40 µl	80 µl	160 µl	Synthetic oligonucleotides, oligonucleotides purified from bacteria, Tris-HCl, EDTA

The MLPA probemix 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).

Storage and handling

Recommended storage conditions		

A shelf life of until the expiry date is guaranteed, 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.

Certificate of Analysis
Information regarding 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 (MS-)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.

This probemix is NOT suitable for methylation determination in prenatal samples. See page 4 for more information.

General information

SALSA® MLPA® Probemix P047 RB1 is a **research use only (RUO)** assay for the detection of deletions or duplications in the *RB1* gene and of methylation status of the *RB1* gene promoter and imprinted locus in a DNA sample.

Retinoblastoma (RB; OMIM180200) is an embryonic neoplasm of retinal origin developing in early childhood and often bilaterally. The incidence of RB is estimated between 1:15000 and 1:20000 live births (Moll et al. 1997, Seregard et al. 2004). Retinoblastomas occur in two forms: hereditary and non-hereditary (sporadic), representing about 40 and 60% of all RB cases, respectively. In hereditary RB, germline mutations causing *RB1* gene loss or inactivation are inherited in autosomal dominant pattern and predispose to RB development with high penetrance. Over 80% of hereditary RB is caused by *de novo RB1* mutations arising during embryonic development. Bilateral and unilateral hereditary RB represent about 25-30% and 10-15% of all RB cases, respectively. None of the sporadic RBs is bilateral. Bilateral RB patients also have a predisposition for secondary cancers with the highest risk for osteosarcomas. In about 1.5% of unilateral RB cases somatic *MYCN* amplification (but no *RB1* pathogenic variants) was detected (Rushlow et al. 2013).

The *RB1* gene (27 exons) located on 13q14.2 spans about 180 kb of genomic DNA and is a well characterized tumour suppressor gene encoding a ubiquitously expressed nuclear protein involved in cell cycle regulation, cellular differentiation and survival. Point mutations, small and large deletions, as well as promoter methylation in the *RB1* gene affect the function of retinoblastoma-associated protein (pRB). Over 500 pathogenic germline variants were identified in the *RB1* gene (https://databases.lovd.nl/shared/variants/RB1/). Moreover, additional clinical features were described for deletions at 13q14 encompassing the *RB1* gene (Mitter et al. 2011), such as *RB1* gene deletions spanning to the *PCDH8* gene have been shown to play an important role in psychomotor delay in RB patients (Castera et al. 2013, Mitter et al. 2011). Contiguous loss of *MED4*, which is located centromeric to *RB1* is thought to contribute to synthetic lethality in cells with *RB1* homozygous loss (Dehainault et al. 2014).

pRB loss, predominantly via heterozygous deletion of *RB1* gene, is also a common abnormality for various cancer types, including breast cancer, lung cancer, prostate cancer and osteosarcoma, and is often associated with poor survival (reviewed in Mandigo et al. 2021).

RB1 inactivation by methylation of promoter region (CpG106) has been shown in 8-15% RB (Dommering et al. 2014, Greger et al. 1989, Price et al. 2014) and in other cancers (Sahi et al. 2014, Simpson et al. 2000, Nakamura et al. 2001). Moreover, an imprinted locus in intron 2 of the *RB1* gene (CpG85) – methylated at the maternal allele, has been identified (Kanber et al. 2009), and recently the importance of the methylation of this imprinted locus in hepatocellular carcinoma has been shown (Anwar et al. 2014).

More information is available at https://www.ncbi.nlm.nih.gov/books/NBK1452/.

This product is not CE/FDA registered for use in diagnostic procedures. The SALSA® MLPA® technique is covered by US patent 6,955,901 and corresponding patents outside the US. The purchase of this product includes a license to use only this amount of product solely for the purchaser's own use.

Gene structure and transcript variants:

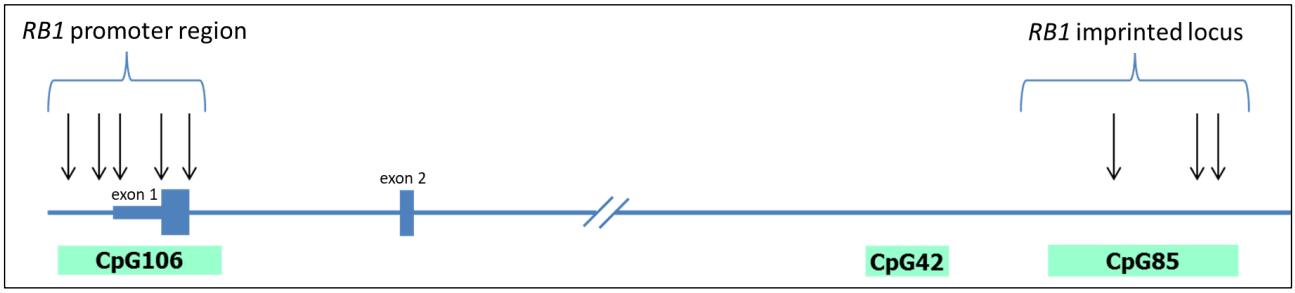
Entrez Gene shows transcript variants of each gene: https://www.ncbi.nlm.nih.gov/gene
For NM_ mRNA reference sequences: https://www.ncbi.nlm.nih.gov/nuccore?db=nucleotide
Matched Annotation from NCBI and EMBL-EBI (MANE): https://www.ncbi.nlm.nih.gov/refseq/MANE
Tark – Transcript Archive: https://tark.ensembl.org

Exon numbering

The *RB1* exon numbering used in this P047-E3 RB1 product description is the exon numbering derived from the MANE project (release version 1.5) based on MANE Select transcript NM_000321.3. 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. Please note that exon numbering for the same gene might be different in other MRC Holland product descriptions, where other resources used for exon numbering are indicated.

Probemix content

P047-E3 RB1 contains 57 MLPA probes with amplification products between 129 and 500 nucleotides (nt). This includes 35 probes for *RB1* covering all exons, except exon 15 which is located at a close distance to the adjacent exons, of which five methylation-specific probes target the *RB1* promoter region (CpG106) and three methylation-specific probes target the imprinted CpG island in intron 2 (CpG85). These methylation-specific probes contain an HhaI recognition site and provide information on the methylation status of selected GCGC sites in CpG106 and CpG85. In the figure below the schematic structure of the *RB1* promoter and the imprinted CpG island is presented. Methylation-specific probe locations are indicated by arrows:



In addition, 13 reference probes are included that are not affected by HhaI digestion and target relatively copy number stable regions in various cancer types. Also, one digestion control probe is included in this probemix indicating whether or not restriction endonuclease digestion in the MS-MLPA reaction was complete. Partial probe sequences 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 (MS-)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)

(MS-)MLPA technique

The principles of MLPA (Schouten et al. 2002) and MS-MLPA technique (Nygren et al. 2005) are described in the MLPA General Protocol and the MS-MLPA General Protocol, respectively (www.mrcholland.com). More information on the use of MLPA in tumour applications can be found in Hömig-Hölzel and Savola (2012).

(MS-)MLPA technique validation

Internal validation using 16 different DNA samples from healthy individuals is required, in particular when using (MS-)MLPA for the first time, or when changing the sample type or 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.

Results of MS-MLPA are highly dependent on the HhaI enzyme used. HhaI enzymes that are resistant to heat inactivation are NOT compatible with the MS-MLPA technique and will give aberrant results. These include, but may not be limited to, Thermo Fisher Scientific enzymes HhaI, ANZA 59 HhaI, and FastDigest HhaI. We recommend using SALSA HhaI enzyme (SMR50) as this restriction enzyme has been validated for use with MS-MLPA by MRC Holland.

Required specimens

Extracted DNA, which includes DNA derived from formalin-fixed, paraffin-embedded (FFPE) tissues, free from impurities known to affect MLPA reactions. MRC Holland has tested and can recommend the following extraction methods:

- QIAGEN Autopure LS (automated) and QIAamp DNA mini/midi/maxi kit (manual)
- Promega Wizard Genomic DNA Purification Kit (manual)
- Salting out (manual)

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. For more information please refer to the section on DNA sample treatment found in the (MS-)MLPA General Protocol. More information on the use of FFPE tissue samples for MLPA can be found in Atanesyan et al. (2017).

Prenatal samples

MRC Holland has not validated the P047 probemix for both copy number analysis and methylation analysis on prenatal samples. Therefore, it is not recommended to use such samples with this probemix. The results of methylation-specific probes tested on chorionic villi samples (CVS) and amniotic fluid samples might not reflect the actual epigenetic constitution of the fetus (Beygo et al. 2023, Eggerman et al. 2016 and Paganini et al. 2015). Furthermore, Paganini et al. demonstrated that the rate at which the imprinting is set may differ between, and even within, loci. Paganini et al. also showed that methylation profiles in CVS can vary after culturing in a locus-specific manner. In addition, Gede et al. showed that methylation ratios in uncultured amniotic fluid are different and more variable than ratios obtained in blood samples. Furthermore, the uncertainty regarding timing of abnormal methylation and the mosaic nature of the syndromes complicates the interpretation of the results obtained on prenatal samples (Gede et al. 2016).

Reference samples

A sufficient number (≥3) of different reference samples from unrelated individuals should be included in each (MS-)MLPA experiment for data normalisation. Reference samples should be derived from heathy individuals without a history of cancer. More information regarding the selection and use of reference samples can be found in the (MS-)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 (MS-)MLPA experiments. Sample ID numbers NA03330, NA12606, NA13721, and NA14164 from the Coriell Institute, ACC-009 (U-266), ACC-163 (NCI-H929), ACC-277 (DK-MG), ACC-410 (MFE-28), ACC-427 (DU-4475) from Leibniz Institute DSMZ and Human Methylated DNA from Zymo Research (cat. no. D5014) have been tested with this P047-E3 probemix at MRC Holland and can be used as a positive control samples to detect copy number and methylation status of target genes/regions as described in the table below. The quality of cell lines can change; therefore deviations to the indicated copy number alteration (CNA) and methylation findings might occur.

Sample name	Source	Copy number status			Expected methylation status	
		Chromosomal position of CNA*	Altered (target) genes in P047-E3	Expected copy number alteration	*RB1* promoter (CpG106 probes)	*RB1* imprinted locus (CpG85 probes)
NA03330	Coriell	13q14.11-q21.1	*ENOX1, MED4, ITM2B,* ***RB1****, RCBTB2, DLEU1, PCDH8*	Heterozygous duplication	not methylated	all copies methylated
NA12606	Coriell	13q14.11-q21.1	*ENOX1, MED4, ITM2B,* ***RB1****, RCBTB2, DLEU1, PCDH8*	Heterozygous duplication	not methylated	two copies methylated
NA13721	Coriell	13q14.11-q21.1	*ENOX1, MED4, ITM2B,* ***RB1****, RCBTB2, DLEU1, PCDH8*	Heterozygous deletion	not methylated	remaining copy *not* methylated
NA14164	Coriell	13q14.2-q21.1	*MED4, ITM2B,* ***RB1****, RCBTB2, DLEU1, PCDH8*	Heterozygous deletion	not methylated	remaining copy methylated
ACC-009◊ (U-266)	DSMZ	13q14.11-q21.1	*ENOX1, MED4, ITM2B,* ***RB1****, RCBTB2, DLEU1, PCDH8*	Heterozygous deletion	not methylated	remaining copy methylated
ACC-163◊ (NCI-H929)	DSMZ	13q14.11-q21.1	*ENOX1, MED4, ITM2B,* ***RB1****, RCBTB2, DLEU1, PCDH8*	Heterozygous deletion	not methylated	remaining copy methylated
ACC-277◊ (DK-MG)	DSMZ	13q14.11-q21.1	*ENOX1, MED4, ITM2B,* ***RB1****, RCBTB2, DLEU1, PCDH8*	Heterozygous deletion	not methylated	remaining copy methylated
ACC-410◊ (MFE-28)	DSMZ	13q14.2	***RB1****, RCBTB2*	Homozygous deletion	No methylation data can be derived as both copies of *RB1* gene including the CpG106 and CpG85 are deleted in these samples.	
		13q14.11 & 13q14.2 & 13q14.3-q21.1	*ENOX1 & ITM2B & DLEU1, PCDH8*	Heterozygous deletion		
ACC-427 (DU-4475)	DSMZ	13q14.2	***RB1****, RCBTB2*	Homozygous deletion		
		13q14.2 & 13q14.3	*MED4, ITM2B & DLEU1*	Heterozygous deletion		
Human HCT116 DKO Methylated DNA π	Zymo Research	none	none	-	all targets methylated	

* Indicated chromosomal bands accommodate genes targeted by MLPA probes, however, the whole extent of CNA present in this cell line cannot be determined by P047-E3 RB1.

◊ In this cell line DNA sample some of the reference probes are affected by CNAs.

π Digestion control probe at 132 nt is not digested in this methylated control sample. The HhaI digestion of this probe is NOT dependent on the methylation state of the target DNA, as the GCGC site is located in the probe's stuffer sequence.

Data analysis

Coffalyser.Net should be used for data analysis in combination with the appropriate lot-specific Coffalyser sheet. For both, the latest version should be used. Coffalyser.Net 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

Copy number results

The standard deviation of each individual probe over all the reference samples should be ≤0.10 and in germline sample analysis 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/gain	1.30 < FR < 1.65
Heterozygous triplication/homozygous duplication/gain	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 Coffalyser.Net (Calculations, cut-offs and interpretation remain unchanged.) Please note that Coffalyser.Net 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.

Please note that these above mentioned final ratios are only valid for germline testing. Final ratios are affected both by percentage of tumour cells and by possible subclonality.

Methylation results

This probemix is intended for determining if the DNA sequences targeted by the methylation-specific probes show differential methylation as compared to the reference samples. This requires the determination of a "baseline" level of methylation, which can be used to determine if the methylation level in a test sample is significantly different from the reference samples.

The baseline methylation level must be determined for every individual methylation-specific probe, and is applicable for one particular experiment. This is important because the level of methylation in samples from healthy individuals depends on the probe's target sequence and its location in the CpG island, the tissue type and, in certain cases, even on the age of the individual. The detection of methylation can also be influenced by impurities in the DNA sample that alter the activity of the HhaI enzyme. The presence of such impurities may differ between tissue types and DNA extraction methods.

To determine the baseline methylation level, it is required to test a sufficient number (≥3) of reference samples from healthy individuals. These samples should be derived from the same tissue type, handled using the same procedure (e.g. FFPE vs. fresh frozen), and prepared using the same DNA extraction method.

The baseline methylation level is then calculated by taking the average value of final ratios of the reference samples per probe and adding two times the standard deviation. The table below contains a hypothetical example. Note that each individual methylation-specific probe should have a separate baseline methylation level and those values should not be averaged between the probes.

Probe	Reference sample 1	Reference sample 2	Reference sample 3	Average	Standard deviation	Baseline level (mean+2×stdev)
Methylation-specific probe 1	0.08	0.00	0.06	0.047	0.042	0.13
Methylation-specific probe 2	0.05	0.07	0.03	0.050	0.020	0.09
Methylation-specific probe 3	0.02	0.02	0.02	0.020	0	0.02

To determine if a test sample has a significantly increased methylation level for a particular probe, compare the methylation ratio of the probe with the baseline level.

- Methylation ratio of a probe in test sample > baseline: methylation is increased.
- Methylation ratio of a probe in test sample ≤ baseline: methylation is *not* increased.

Interpretation of methylation positive samples is dependent on the application used.

NOTE: In case the digestion control probe is not fully digested (final ratio >0.10), please contact info@mrcholland.com for more information.

- Arranging probes according to chromosomal location facilitates interpretation of the results and may reveal more subtle changes such as those observed in mosaic or subclonal cases.
- 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 in the DNA sample) 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: https://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.
- Digestion Control Probes. The target sequences of the digestion control probes are unmethylated in most blood-derived DNA samples. The signals of the digestion control probes should be gone upon complete digestion by HhaI.
- mRNA levels. We have no data showing that methylation detected by a particular probe indeed influences the corresponding mRNA levels.

P047 RB1-specific notes:

- Three methylation-specific probes are present for the imprinted CpG island CpG85 in intron 2 and provide information about the methylation status of this region. As these three probes target an imprinted locus, one allele is expected to be methylated (maternal) and the other is unmethylated (paternal) in healthy individuals. As compared to reference probes that do not contain a HhaI site, the signal of the methylation-specific probes in the imprinted locus is reduced by approximately 50% upon HhaI digestion in DNA samples from normal individuals.
- Please note that two probes have multiple HhaI restriction sites. All of these sites need to be methylated in order to not be digested!
- In samples from tumour tissues, reference probes are more prone to have deviating copy number results as compared to blood derived germline samples. When regions targeted by reference probes are affected by copy number alterations, it can help to turn the slope correction off in Coffalyser.Net analysis to get the correct copy number interpretation on the target region.

Limitations of the procedure

- In most populations, the major cause of germline genetic defects in the *RB1* gene are small (point) mutations, none of which will be detected by using P047 RB1. Likewise, in many tumour samples genetic alterations in the *RB1* gene are small (point) mutations none of which will be detected by using P047 RB1.
- 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.
- (MS-)MLPA analysis on tumour samples provides information on the *average* situation in the cells from which the DNA sample was purified. Changes in methylation status, 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.
- Methylation-specific probes target specific HhaI sites in a CpG island; if methylation is absent for a particular CpG-site, this does not necessarily mean that the whole CpG island is unmethylated!
- Rare cases are known in which apparent methylation as detected by a methylation-specific probe proved to be due to a sequence change in or very near the HhaI site.
- No discrimination between uniparental disomy and imprinting defects can be made with this probemix. For this, it is necessary to perform microsatellite analysis in the patient and parents.

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 in sequence data 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.

***RB1* mutation databases**

Cosmic *RB1* mutation database https://cancer.sanger.ac.uk/cosmic/gene/analysis?ln=RB1 and the LOVD *RB1* mutation database https://databases.lovd.nl/shared/genes/RB1. We strongly encourage users to deposit positive results in the Cosmic and LOVD databases. 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 in germline samples, false positive results due to SNVs and unusual results (e.g., a duplication of *RB1* exons 21 and 23 but not exon 22) to MRC Holland: info@mrcholland.com.

Table 1. P047-E3 RB1

Length (nt)	MLPA probe	Chromosomal position (hg18)[a]		*for methylation analysis*	
		Reference	***RB1*** and flanking	**HhaI site**	% expected signal reduction[b]
64-105	Control fragments – see table in probemix content section for more information				
129	Reference probe 18709-L26847	5q31		-	
132 π	Digestion control probe S0750-L25688	2q12		+	100
136	Reference probe 15794-L17854	19q13		-	
142 Ø ‡	**RB1 probe** 15264-L25114		**Intron 2**	+	45 (CpG85; imprinted)
146 ¬	ENOX1 probe 19220-L25288		13q14.11	-	
151 Ø ‡	**RB1 probe** 15265-L25148		**Intron 2**	+	45 (CpG85; imprinted)
157	Reference probe 03797-L26004	21q22		-	
163 ¬	MED4 probe 23110-L32699		13q14.2	-	
169	**RB1 probe** 21733-L17333		**Exon 5**	-	
175	**RB1 probe** 15136-L32610		**Exon 11**	-	
179	**RB1 probe** 01779-L17334		**Exon 2**	-	
184	Reference probe 16424-L26003	18q21		-	
191 +	**RB1 probe** 15137-L16906		**Exon 16**	+	0
196 ¬	ITM2B probe 19221-L25907		13q14.2	-	
204	**RB1 probe** 01781-L17719		**Exon 4**	-	
210	Reference probe 12424-L26001	22q12		-	
216	**RB1 probe** 19222-L26556		**Exon 18**	-	
223	**RB1 probe** 19549-L26919		**Exon 13**	-	
230	**RB1 probe** 01782-L26611		**Exon 6**	-	
235 Ø	**RB1 probe** 19147-L17021		**Intron 2**	+	45 (CpG85; imprinted)
241	**RB1 probe** 19148-L25910		**Exon 22**	-	
246	Reference probe 08715-L30393	9q21		-	
253	**RB1 probe** 19149-L16909		**Intron 23**	-	
258	**RB1 probe** 19223-L25911		**Exon 12**	-	
265 Δ	**RB1 probe** 07944-L25912		**Exon 1**	+	100 (CpG106; promoter)
270	**RB1 probe** 19181-L17341		**Intron 11**	-	
277	**RB1 probe** 15135-L17339		**Exon 10**	-	
283 *	Reference probe 18166-L22686	3p21		-	
289 ¬	RCBTB2 probe 19224-L25292		13q14.2	-	
297	**RB1 probe** 19144-L25955		**Exon 1**	+	100 (CpG106; promoter)
305	**RB1 probe** 19225-L27630		**Exon 20**	-	
311	**RB1 probe** 19226-L25294		**Exon 14**	-	
319	**RB1 probe** 19146-L26559		**Upstream**	+	100 (CpG106; promoter)
325	Reference probe 08048-L25770	5p15		-	
331	**RB1 probe** 02734-L25117		**Upstream**	+	100 (CpG106; promoter)
339	**RB1 probe** 01790-L25914		**Exon 17**	-	
347 ¬	PCDH8 probe 19111-L25915		13q21.1	-	
355 ¥ ¬	MED4 probe 23435-L16944		13q14.2	-	
359 ¥	**RB1 probe** 19150-L33277		**Exon 19**	-	
363	**RB1 probe** 19550-L26088		**Exon 1**	+	100 (CpG106; promoter)
373	Reference probe 14012-L15214	15q11		-	
379	**RB1 probe** 19227-L25920		**Exon 27**	-	
386	**RB1 probe** 01794-L25919		**Intron 20**	-	
394 ¬	PCDH8 probe 19112-L25918		13q21.1	-	
403	**RB1 probe** 19228-L25296		**Exon 25**	-	
409 ¬	DLEU1 probe 00801-L27246		13q14.3	-	
418	Reference probe 20130-L30396	10p11		-	
426	**RB1 probe** 01796-L27326		**Exon 23**	-	
431	**RB1 probe** 19152-L27250		**Exon 8**	-	
442	**RB1 probe** 19153-L27249		**Exon 26**	-	
448	**RB1 probe** 19230-L25298		**Exon 7**	-	
456	Reference probe 17129-L21829	11p11		-	
463 ¥	**RB1 probe** 23429-L33235		**Exon 27**	-	

Length (nt)	MLPA probe	Chromosomal position (hg18)[a]		*for methylation analysis*	
		Reference	***RB1*** and flanking	**HhaI site**	% expected signal reduction[b]
472 ¥	**RB1 probe** 19182-L25151		**Exon 9**	-	
487	**RB1 probe** 19180-L27253		**Exon 3**	-	
494	Reference probe 15318-L27254	2q33		-	
500	Reference probe 06676-L21510	11p15		-	

[a] See section Exon numbering on page 3 for more information.
[b] Expected signal reduction on blood DNA derived samples. On other tissue or tumour derived samples these percentages can be different.

* New in version E3.
¥ Changed in version E3. Minor alteration, no change in sequence detected.
¬ 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.
π Digestion control: warns for insufficient digestion. Upon digestion, this probe should not give a signal.
Ø Intron probe. Only included to help determine the extent of a deletion/duplication. Copy number alterations of only this probe are of unknown clinical significance.
‡ This probe contains two GCGC motifs for HhaI. Loss of probe peak signal (indicative of absence of methylation) will occur not only if both sites are unmethylated but also if one of the sites is unmethylated and the other methylated.
Δ More variable. This probe may be sensitive to certain experimental variations. Aberrant results should be treated with caution.
+ This probe is not located in a CpG island; it has a HhaI site and in our tests it has been always 100% methylated on blood-derived DNA.

The probe lengths in the table above may vary slightly depending on the capillary electrophoresis machine settings. Please see the most up-to-date Coffalyser sheet for exact probe lengths obtained at MRC Holland.

SNVs located in the target sequence of a probe can influence probe hybridisation and/or probe ligation. Single probe aberration(s) must be confirmed by another method.

Table 2. P047-E3 probes arranged according to chromosomal location

Table 2a. RB1 and flanking probes

Length (nt)	MLPA probe	Gene/ Exon[a]	Ligation site[b]/ location	**Partial sequence** (copy number probes; 24 nt adjacent to ligation site) / **Complete sequence** (methylation-specific probes)[c]	**Distance to next probe**
Centromeric flanking probes					
146 ¬	19220-L25288	*ENOX1*	13q14.11	CTGCCCCTCTGG-ACACTCATAATT	4,7 **M**b
163 ¬	23110-L32699	*MED4*	13q14.2	AGCTCCATCCCA-AGGCCGATACTC	7,8 kb
355 ¬	23435-L16944	*MED4*	13q14.2	AATTCACCGAGA-TGGGGAATTTCA	172,5 kb
196 ¬	19221-L25907	*ITM2B*	13q14.2	ACAAACTGCAAC-GCAGAGAAACTA	44,5 kb
***RB1* gene at 13q14.2.** Ligation sites are indicated according to NM_000321.3.					
		start codon	*163-165 (exon 1)*		
331	02734-L25117	Upstream	358 nt before exon 1 (CpG106; promoter)	CGCCCAAGGAGGGAGAGTGGCGCTC-CCGCCGAGGGTGCACTAGCCAGATATTCCCTGCG	0,2 kb
319	19146-L26559	Upstream	162 nt before exon 1 (CpG106; promoter)	GCCGGATGCCTCCTGGAAGGCGCCTG-GACCCACGCC AGGTTTCCCAGTTTAATTCCTCATGACTTAGCGTCCC	0,2 kb
363	19550-L26088	Exon 1	12 nt before exon 1 (CpG106; promoter)	GCGGAAGTGACGTTTTCCCGCGGTTG-GACGCGGCGCTCAGTTGCCGGGCGGGGGAGG	0,1 kb
265 Δ	07944-L25912	Exon 1	136-137 (CpG106; promoter)	CGTCGTCCTCCCCGGCGCTCCTCCACAGCTC-GCTGGCTCCCGCCGCGGAAAGGCGTCATGCC	0,2 kb
297	19144-L25955	Exon 1	25 nt after exon 1 (CpG106; promoter)	TCGTCAGGTGAGCGAGCAGAGCCGCCGTCGCC-TCACGCGGGAAGGGCGCCCCGGGTGTGCGTAG	3,3 kb
179	01779-L17334	Exon 2	388-389	AGAGAGCTTGGT-TAACTTGGGAGA	11,7 kb
142 Ø‡	15264-L25114	Intron 2	11.6 kb after exon 2 (CpG85; imprinted)	CGGCAGGGTAGTCTTGGAAATGCCCAA-GATTG CTTCCGCGCGCGTCAGTTCAGCGGACGTGTCT	0,5 kb
151 Ø‡	15265-L25148	Intron 2	12.1 kb after exon 2 reverse (CpG85; imprinted)	CCAGCAGCCCCCTGCAGCCCTTCAGAAGCAC-CACAGAATAAAAGCGGGGTCAAGAGGAGCGCTGGCGC CCTGAG	0,1 kb

Length (nt)	MLPA probe	Gene/ Exon[a]	Ligation site[b]/ location	**Partial sequence** (copy number probes; 24 nt adjacent to ligation site) / **Complete sequence** (methylation-specific probes)[c]	Distance to next probe
235 Ø	19147-L17021	Intron 2	12.2 kb after exon 2 (CpG85; imprinted)	CGTTCGTCTTTGCTAACCGGGGAGGTTTGCGA-AA GGCGAACTCTTTATGGGCGCCCTTCAGACCCTGCCG	23,0 kb
487	19180-L27253	Exon 3	483-484	TTTATTGCAGCA-GTTGACCTAGAT	2,5 kb
204	01781-L17719	Exon 4	611-612	TGCTATGTCAAG-ACTGTTGAAGAA	2,7 kb
169	21733-L17333	Exon 5	689-688 reverse	AACTGCTGGGTT-GTGTCAAATATA	1,3 kb
230	01782-L26611	Exon 6	83 nt after exon 6	ATTCCCCAATTT-TTATTGAGTAAT	11,0 kb
448	19230-L25298	Exon 7	832-833	TATGTGTCCTTG-ACTATTTTATTA	2,8 kb
431	19152-L27250	Exon 8	935-936	GCGAGGTCAGAA-CAGGAGTGCACG	2,0 kb
472	19182-L25151	Exon 9	1073-1072 reverse	ATGTTACAAGTC-CAAGAGAATTCA	2,6 kb
277	15135-L17339	Exon 10	1134-1135	TACGAAGAAATT-TATCTTAAAAAT	1,0 kb
175	15136-L32610	Exon 11	1269-1268 reverse	GTGTGTGGAGGA-ATTACATTCACC	4,7 kb
270	19181-L17341	Intron 11	114 nt before exon 12	ATGTAGAGACAA-GTGGGAGGCAGT	0,2 kb
258	19223-L25911	Exon 12	1367-1368	AAATCTGATTTC-CTATTTTAACGT	3,5 kb
223	19549-L26919	Exon 13	1473-1472 reverse	ATTTCGACACAA-CCCTGTCCCACA	2,6 kb
311	19226-L25294	Exon 14	1536-1537	CGAGTAATGGAA-TCCATGCTTAAA	0,6 kb
191 +	15137-L16906	Exon 16	1615-1616	TTTTTCATATGT-CTTTATTGGCGT	1,2 kb
339	01790-L25914	Exon 17	1805-1806	AGAAATGATAAA-ACATTTAGAACG	71,6 kb
216	19222-L26556	Exon 18	1905-1906	GACCGAGAAGGA-CCAACTGATCAC	3,2 kb
359	19150-L33277	Exon 19	2041-2042	ATTCTACTGCAA-ATGCAGAGACAC	3,5 kb
305	19225-L27630	Exon 20	2159-2160	AAATACACTTTG-TGAACGCCTTCT	3,8 kb
386	01794-L25919	Intron 20	176 nt before exon 21	TTTTGTTCTTTA-AACACACTTTGG	1,5 kb
241	19148-L25910	Exon 22	2463-2464	CTGAAAACAAAT-ATTTTGCAGTAT	0,1 kb
426	01796-L27326	Exon 23	2507-2508	CTTGTCACCAAT-ACCTCACATTCC	7,9 kb
253	19149-L16909	Intron 23	265 nt before exon 24	GAAACTTGCCTT-TGCCCTCCCTAA	3,7 kb
403	19228-L25296	Exon 25	2775-2776	CCTCCTAAACCA-CTGAAAAAACTA	0,6 kb
442	19153-L27249	Exon 26	2847-2848	CCAGGAGAGTCC-AAATTTCAGCAG	2,6 kb
463	23429-L33235	Exon 27	2898-2899	ACACGAATGCAA-AAGCAGAAAATG	1,2 kb
379	19227-L25920	Exon 27	4110-4111	CCACATTATTTC-TAGTCCAAAATT	30,8 kb
		stop codon	*2947-2949 (exon 27)*		
Telomeric flanking probes					
289 ¬	19224-L25292	*RCBTB2*	13q14.2	AGTAGTTGTGAC-CATAGCATGTGG	1,6 **M**b
409 ¬	00801-L27246	*DLEU1*	13q14.3	GAAGAACAGAAC-CTTCAGGAATTG	2,7 **M**b
347 ¬	19111-L25915	*PCDH8*	13q21.1	AAGTTATTGACC-GGTTTCAGTGTT	1,0 kb
394 ¬	19112-L25918	*PCDH8*	13q21.1	GCAAAGACAGCG-GTAAAGGGGACA	-

Table 2b. Digestion control and reference probes

Length (nt)	MLPA probe	Gene	Chr. position (hg18)	Location (hg18) in kb	**Partial** sequence[c] (24 nt adjacent to ligation site)
132 π	S0750-L25688	*SLC9A2*	2q12	02-102.641	GGACACTTTGGA-ATTCCATTGGCA
494	15318-L27254	*SATB2*	2q33	02-199.897	TGCCATTTATGA-CGAGATCCAACA
283	18166-L22686	*COL7A1*	3p21	03-048.595	ATACAGGCTCTA-ACCCTCAGCCCC
325	08048-L25770	*DNAH5*	5p15	05-013.847	AGATGACTGTTT-TTATTGATGATG
129	18709-L26847	*IL4*	5q31	05-132.038	ATCGACACCTAT-TAATGGGTCTCA
246	08715-L30393	*PCSK5*	9q21	09-077.964	AAGCTGAGACCT-AGTTCCAGAGGG
418	20130-L30396	*ZNF25*	10p11	10-038.301	CAGGTGATTCCT-GGGGCTGCCAGC
500	06676-L21510	*SMPD1*	11p15	11-006.369	CTGCTGAAGATA-GCACCACCTGCC
456	17129-L21829	*MYBPC3*	11p11	11-047.311	CACCCAACTATA-AGGCCCTGGACT
373	14012-L15214	*UBE3A*	15q11	15-023.172	AGAAAGGAGCAA-GCTCAGCTTACC
184	16424-L26003	*MYO5B*	18q21	18-045.765	AAGTGTCCTCCA-GTGCCTCATGCC
136	15794-L17854	*ATP1A3*	19q13	19-047.165	CCTGCAGACATA-CGAGCAGAGGAA
157	03797-L26004	*KCNJ6*	21q22	21-037.920	CTCGAAGCTCCT-ACATCACCAGTG
210	12424-L26001	*LARGE1*	22q12	22-032.064	CAGTGAGGCTGA-TGTCAACAGTGA

[a] See section Exon numbering on page 3 for more information.
[b] Ligation sites are relative to the start of the NM_ sequence, and not relative to the coding sequence.
[c] Please notify us of any mistakes: info@mrcholland.com.

¬ 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.
π Digestion control: warns for insufficient digestion. Upon digestion, this probe should not give a signal.
Ø Intron probe. Only included to help determine the extent of a deletion/duplication. Copy number alterations of only this probe are of unknown clinical significance.
‡ This probe contains two GCGC motifs for HhaI. Loss of probe peak signal (indicative of absence of methylation) will occur not only if both sites are unmethylated but also if one of the sites is unmethylated and the other methylated.
Δ More variable. This probe may be sensitive to certain experimental variations. Aberrant results should be treated with caution.
+ This probe is not located in a CpG island; it has a HhaI site and in our tests it has been always 100% methylated on blood-derived DNA.

SNVs located in the target sequence of a probe can influence probe hybridisation and/or probe ligation. Single probe aberration(s) must be confirmed by another method.

Related products

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References

- Anwar SL et al. (2014). Deregulation of RB1 expression by loss of imprinting in human hepatocellular carcinoma. *J Pathol.* 233:392-401.
- Atanesyan L et al. (2017). Optimal Fixation Conditions and DNA Extraction Methods for MLPA Analysis on FFPE Tissue-Derived DNA. *Am J Clin Pathol.* 147:60-8.
- Castera L et al. (2013). Fine mapping of whole RB1 gene deletions in retinoblastoma patients confirms PCDH8 as a candidate gene for psychomotor delay. *Eur J Hum Genet.* 21:460-4.
- Beygo J et al. (2023). Prenatal testing for imprinting disorders: A laboratory perspective. *Prenat Diagn*. 43(8):973-982.
- Dehainault C et al. (2014). The survival gene MED4 explains low penetrance retinoblastoma in patients with large RB1 deletion. *Hum Mol Genet.* 23:5243-50.
- Dommering CJ et al. (2014). RB1 mutation spectrum in a comprehensive nationwide cohort of retinoblastoma patients. *J Med Genet.* 51:366-74.
- Eggermann T et al. (2016). Prenatal molecular testing for Beckwith-Wiedemann and Silver-Russell syndromes: a challenge for molecular analysis and genetic counseling. *Eur J Hum Genet*. 24(6):784-793.
- Gede LB et al. (2016). Feasibility study on the use of methylation-specific MLPA for the 11p15 region on prenatal samples. *Prenat Diagn*. 36:100-3.
- Greger V et al. (1989). Epigenetic changes may contribute to the formation and spontaneous regression of retinoblastoma. *Hum Genet.* 83:155-8.
- Hömig-Hölzel C and Savola S. (2012). Multiplex ligation-dependent probe amplification (MLPA) in tumor diagnostics and prognostics. *Diagn Mol Pathol.* 21:189-206.
- Kanber D et al. (2009). The human retinoblastoma gene is imprinted. *PLoS Genet.* 5:e1000790.
- Mandigo AC et al. (2021). Relevance of pRB Loss in Human Malignancies. *Clin Cancer Res.*
- Mitter D et al. (2011). Genotype-phenotype correlations in patients with retinoblastoma and interstitial 13q deletions. *Eur J Hum Genet.* 19:947-58.
- Moll AC et al. (1997). Incidence and survival of retinoblastoma in The Netherlands: a register based study 1862-1995. *Br J Ophthalmol.* 81:559-62.
- Nakamura M et al. (2001). Promoter hypermethylation of the RB1 gene in glioblastomas. *Lab Invest.* 81:77-82.
- Nygren AO et al. (2005). Methylation-specific MLPA (MS-MLPA): simultaneous detection of CpG methylation and copy number changes of up to 40 sequences. *Nucleic Acids Res.* 33:e128.
- Paganini L et al. (2015). Beckwith-Wiedemann syndrome prenatal diagnosis by methylation analysis in chorionic villi. *Epigenetics*. 10:643-649.
- Price EA et al. (2014). Spectrum of RB1 mutations identified in 403 retinoblastoma patients. *J Med Genet.* 51:208-14.
- Rushlow DE et al. (2013). Characterisation of retinoblastomas without RB1 mutations: genomic, gene expression, and clinical studies. *Lancet Oncol.* 14:327-34.

- Sahi H et al. (2014). RB1 gene in Merkel cell carcinoma: hypermethylation in all tumors and concurrent heterozygous deletions in the polyomavirus-negative subgroup. *APMIS.* 122:1157-66.
- Simpson DJ et al. (2000). Loss of pRb expression in pituitary adenomas is associated with methylation of the RB1 CpG island. Cancer Res. 60:1211-6.
- Schouten JP et al. (2002). Relative quantification of 40 nucleic acid sequences by multiplex ligation-dependent probe amplification. *Nucleic Acids Res.* 30:e57.
- Schwartz M et al. (2007). Deletion of exon 16 of the dystrophin gene is not associated with disease. *Hum Mutat.* 28:205.
- Seregard S et al. (2004). Incidence of retinoblastoma from 1958 to 1998 in Northern Europe: advantages of birth cohort analysis. *Ophthalmology.* 111:1228-32.
- Varga RE et al. (2012). MLPA-based evidence for sequence gain: pitfalls in confirmation and necessity for exclusion of false positives. *Anal Biochem.* 421:799-801.

Selected publications using P047 RB1

- Ahani A et al. (2013). Screening for large rearrangements of the RB1 gene in Iranian patients with retinoblastoma using multiplex ligation-dependent probe amplification. *Mol Vis.* 19:454-62.
- Busch M et al. (2018). Characterization of etoposide- and cisplatin-chemoresistant retinoblastoma cell lines. *Oncol Rep.* 39:160-72.
- Creytens D et al. (2014). Atypical spindle cell lipoma: a clinicopathologic, immunohistochemical, and molecular study emphasizing its relationship to classical spindle cell lipoma. *Virchows Arch.* 465:97-108.
- Dalay N (2018). Detection of RB1 Gene Copy Number Variations Using a Multiplex Ligation-Dependent Probe Amplification Method. *Methods Mol Biol.* 1726:7-18.
- Devarajan B et al. (2015). Targeted next generation sequencing of RB1 gene for the molecular diagnosis of Retinoblastoma. *BMC Cancer.* 15:320.
- Dittner-Moormann S et al. (2020). 13q deletion syndrome resulting from balanced chromosomal rearrangement in father: the significance of parental karyotyping. *Mol Cytogenet.* 13:31.
- Dommering CJ et al. (2014). RB1 mutation spectrum in a comprehensive nationwide cohort of retinoblastoma patients. *J Med Genet.* 51:366-74.
- Escudero A et al. (2021). Genetic predisposition to fetal and neonatal cancer. *Clin Transl Oncol.* 23:1179-84.
- Faranoush M et al. (2025). Delving Into Retinoblastoma Genetics: Discovery of Novel Mutations and Their Clinical Impact: Retrospective Cohort Study. *Cancer Med*. 14:e70922.
- Frenkel S et al. (2016). Genotype-phenotype correlation in the presentation of retinoblastoma among 149 patients. *Exp Eye Res.* 146:313-7.
- Gelli E et al. (2019). Evidence of predisposing epimutation in retinoblastoma. *Hum Mutat.* 40:201-6.
- Gomez-Mariano G et al. (2025). Mosaicism and intronic variants in RB1 gene revealed by next generation sequencing in a cohort of Spanish retinoblastoma patients. *Exp Eye Res*. 251:110233.
- Gupta H et al. (2021). Retinoblastoma genetics screening and clinical management. *BMC Med Genomics.* 14:188.
- Kiet NC et al. (2019). Spectrum of mutations in the RB1 gene in Vietnamese patients with retinoblastoma. *Mol Vis.* 25:215-21.
- Kim Y et al. (2025). High-resolution Chromosomal Microarray with Diagnostic Potential for Detecting Exon-level Copy Number Variations Using Targeted and Non-targeted Approaches. *Ann Lab Med*. 46:190-9.
- Lan X et al. (2020). Spectrum of RB1 Germline Mutations and Clinical Features in Unrelated Chinese Patients With Retinoblastoma. *Front Genet.* 11:142.
- Lee YJ et al. (2024). A comprehensive genotype-phenotype study in 203 individuals with retinoblastoma. *Exp Eye Res*. 248:110102.
- Mitter D et al. (2011). Genotype-phenotype correlations in patients with retinoblastoma and interstitial 13q deletions. *Eur J Hum Genet.* 19:947-58.
- Ono M et al. (2021). The mechanisms involved in the resistance of estrogen receptor-positive breast cancer cells to palbociclib are multiple and change over time. *J Cancer Res Clin Oncol.* Epub ahead of print. PMID: 34244855.
- Parma D et al. (2017). RB1 gene mutations in Argentine retinoblastoma patients. Implications for genetic counseling. *PLoS One.* 12:e0189736.
- Perez-Becerra JJ et al. (2026). Spectrum of Germline Cytogenomic Alterations in RB1 in Mexican Patients With Retinoblastoma. *Pediatr Blood Cancer*. 73:e70267.
- Price EA et al. (2014). Spectrum of RB1 mutations identified in 403 retinoblastoma patients. *J Med Genet.* 51:208-14.

- Rodriguez-Martin C et al. (2020). Frequency of low-level and high-level mosaicism in sporadic retinoblastoma: genotype-phenotype relationships. *J Hum Genet.* 65:165-74.
- Rojanaporn D et al. (2018). Spectrum of germline RB1 mutations and clinical manifestations in retinoblastoma patients from Thailand. *Mol Vis.* 24:778-88.
- Sagi M et al. (2015). Genetic screening in patients with Retinoblastoma in Israel. *Fam Cancer.* 14:471-80.
- Schwermer M et al. (2019). Comprehensive characterization of RB1 mutant and MYCN amplified retinoblastoma cell lines. *Exp Cell Res.* 375:92-9.
- Sellner LN et al. (2006). Screening for RB1 mutations in tumor tissue using denaturing high performance liquid chromatography, multiplex ligation-dependent probe amplification, and loss of heterozygosity analysis. *Pediatr Dev Pathol.* 9:31-7.
- Shahraki K et al. (2017). Genetic screening in Iranian patients with retinoblastoma. *Eye (Lond).* 31:620-7.
- Singh J et al. (2016). Next-generation sequencing-based method shows increased mutation detection sensitivity in an Indian retinoblastoma cohort. *Mol Vis.* 22:1036-47.
- Thirumalairaj K et al. (2015). A stepwise strategy for rapid and cost-effective RB1 screening in Indian retinoblastoma patients. *J Hum Genet.* 60:547-52.
- Tomar S et al. (2017). Mutation spectrum of RB1 mutations in retinoblastoma cases from Singapore with implications for genetic management and counselling. *PLoS One.* 12:e0178776.
- van Rensburg I et al. (2026) Expanding the RB1 variant landscape of heritable retinoblastoma: unlocking precision oncology potential in Southern Africa. *BMC Cancer*. 26:341.
- Wicher D et al. (2025). Analysis of cytogenetic germline changes in Polish patients with retinoblastoma. *Mol Vis*. 31:267-74.
- Zou Y et al. (2021). Spectrum of germline mutations in RB1 in Chinese patients with retinoblastoma: Application of targeted next-generation sequencing. *Mol Vis.* 27:1-16.

P047 product history	
Version	*Modification*
E3	One reference probe is replaced and several probes have a change in length but not in targeted sequence.
E2	Two flanking probes have been added (*MED4* gene).
E1	One methylation-specific probe in the *RB1* intron 2 imprinted locus has been removed. In addition, three reference probes have been replaced.
D1	Probemix made suitable also for MS-MLPA analysis. Nine RB1 probes (including methylation-specific probes), two flanking probes and 11 reference probes have been replaced. In addition, five new RB1 probes, three flanking probes and one digestion control probe have been included.
C1	One RB1 probe and two reference probes have been replaced. In addition, the 88 and 96 nt control fragments have been replaced (QDX2).
B1	Three RB1 probes and several reference probes have been replaced. In addition, seven RB1 probes and four extra control fragments at 88, 96, 100 and 105 nt have been included.
A1	First release.

Implemented changes in the product description

Version E3-01– 22 May 2026 (05P)
- Product description adapted to a new template and to a new product version (version number changed, changes in Table 1 and Table 2).
- Various minor textual or layout changes.
- New references added.

Version E2-03 – 15 September 2025 (04P)
- Clarified the use of General MS-MLPA protocol when P047 is used for both *RB1* copy number and methylation determination.
- Added precaution regarding the use of P047 on prenatal samples on page 1 and page 3, section 'Prenatal samples'.
- Added new references on page 11-12 from 'Prenatal samples' section.

Version E2-02 – 25 February 2025 (04P)
- Modified 'Gene structure and transcript variants' and 'Exon numbering' sections on page 2 to include information related to use of MANE database for exon numbering.
- Probe location modified for 319, 331 probes (exon 1 to upstream) and for 270, 386 and 253 nt probes (exon to intron) in Table 1 and 2.
- Removed ME002 and P294 probemixes from the ' Related SALSA (MS-)MLPA probemixes' section.

Version E2-01 – 5 November 2021 (04P)
- Product description rewritten and adapted to a new template and to a new product version (version number changed, changes in Table 1 and Table 2).
- Removed the note about variability of RB1 probe 19144-L25955 at 297 nt in Table 1 and Table 2a.
- Various minor textual or layout changes.
- Added information about positive control DNA samples on pages 3 and 4.
- Added a note on page 6 regarding the expected values for CpG85 probes in the digested reactions.
- Ligation sites of the probes targeting the *RB1* are updated according to new version of the NM_ reference sequence.
- Small changes of probe lengths in Table 1 and 2 in order to better reflect the true lengths of the amplification products.
- For uniformity, the chromosomal locations and bands in this document are now all based on hg18 (NCBI36).
- List of selected publications using SALSA MLPA Probemix P047 RB1 has been updated.

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