Product Description
SALSA® MLPA® Probemix P047-E2 RB1

To be used with the (MS-)MLPA General Protocol.

Version E2
As compared to version E1, two flanking probes for *MED4* have been added, and several probes have change in their length but not in the sequence targeted. For complete product history see page 13.

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 using the **MS-MLPA General Protocol**. 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.

To be used in combination with a SALSA MLPA reagent kit, SALSA HhaI (SMR50; for MS-MLPA General protocol) 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 (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.

General information
The 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). 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).

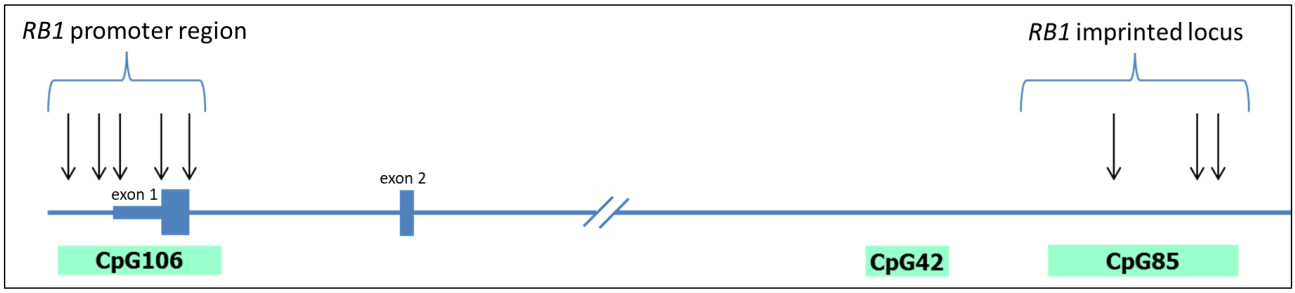
More information is available at https://www.ncbi.nlm.nih.gov/books/NBK1452/

This SALSA MLPA probemix is not CE/FDA registered for use in diagnostic procedures. Purchase of this product includes a limited license for research purposes.

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-E2 RB1 product description is the exon numbering derived from the MANE project (release version 1.4) 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
The SALSA MLPA Probemix P047-E2 RB1 contains 57 (MS-)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 MS-MLPA probes target the *RB1* promoter region (CpG106) and three MS-MLPA probes target the imprinted CpG island in intron 2 (CpG85). These MS-MLPA 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. MS-MLPA 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. Complete 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 the MLPA technique (Schouten et al. 2002) and MS-MLPA technique (Nygren et al. 2005) are described in the MLPA General Protocol and 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. (Homig-Holzel and Savola 2012).

(MS-)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.

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

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 individuals without a history of cancer. 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 NA03330, NA12606, NA13721 and NA14164 from the Coriell Institute, and ACC-009 (U-266), ACC-163 (NCI-H929), ACC-277 (DK-MG), ACC-410 (MFE-28) and ACC-427 (DU-4475) from Leibniz Institute DSMZ have been tested with this P047-E2 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 samples should be validated before use.

Sample name	Copy number status			Expected methylation status	
	Chromosomal position of copy number alteration*	Altered (target) genes in P047-E2	Expected copy number alteration	*RB1* promoter (CpG106 probes)	*RB1* imprinted locus (CpG85 probes)
NA03330	13q14.11-q21.1	*ENOX1, MED4, ITM2B,* ***RB1****, RCBTB2, DLEU1, PCDH8*	Heterozygous duplication	not methylated	all copies methylated
NA12606	13q14.11-q21.1	*ENOX1, MED4, ITM2B,* ***RB1****, RCBTB2, DLEU1, PCDH8*	Heterozygous duplication	not methylated	two copies methylated
NA13721	13q14.11-q21.1	*ENOX1, MED4, ITM2B,* ***RB1****, RCBTB2, DLEU1, PCDH8*	Heterozygous deletion	not methylated	remaining copy *not* methylated
NA14164	13q14.2-q21.1	*MED4, ITM2B,* ***RB1****, RCBTB2, DLEU1, PCDH8*	Heterozygous deletion	not methylated	remaining copy methylated
ACC-009◊ (U-266)	13q14.11-q21.1	*ENOX1, MED4, ITM2B,* ***RB1****, RCBTB2, DLEU1, PCDH8*	Heterozygous deletion	not methylated	remaining copy methylated
ACC-163◊ (NCI-H929)	13q14.11-q21.1	*ENOX1, MED4, ITM2B,* ***RB1****, RCBTB2, DLEU1, PCDH8*	Heterozygous deletion	not methylated	remaining copy methylated
ACC-277◊ (DK-MG)	13q14.11-q21.1	*ENOX1, MED4, ITM2B,* ***RB1****, RCBTB2, DLEU1, PCDH8*	Heterozygous deletion	not methylated	remaining copy methylated
ACC-410◊ (MFE-28)	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)	13q14.2	***RB1****, RCBTB2*	*Homozygous* deletion		
	13q14.2 & 13q14.3	*MED4, ITM2B & DLEU1*	Heterozygous deletion		

* Indicated chromosomal bands accommodate genes targeted by MLPA probes, however, the whole extent of copy number alteration present in this cell line cannot be determined by this P047-E2 RB1 probemix.
◊ In this cell line DNA sample some of the reference probes are affected by CNAs.

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 copy number results

The standard deviation of each individual probe over all the reference samples should be ≤0.10 and, in addition, in germline 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	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.

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.

- 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. For germline, 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 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.

Interpretation of methylation results on blood and tissue derived DNA samples:
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 an 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.

P047 specific notes:

- Three MS-MLPA 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 MS-MLPA probes in the imprinted locus is reduced by approximately 50% upon HhaI digestion in DNA samples from normal individuals.
- Please note that several 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 genetic defects in the *RB1* are small (point) mutations, most of which will not be detected by using SALSA MLPA Probemix 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.
- MS-MLPA 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 an MS-MLPA 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 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. Confirmation of methylation status can be performed with another technique, such as MSP (methylation-specific PCR), pyrosequencing, digestion-based PCR assays, etc.

***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. SALSA (MS-)MLPA Probemix P047-E2 RB1

Length (nt)	SALSA (MS-)MLPA probe	Chromosomal position (hg18)		*for methylation analysis*	
		reference	***RB1***[a] 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; promotor)
270	**RB1 probe** 19181-L17341		**Intron 11**	-	
277	**RB1 probe** 15135-L17339		**Exon 10**	-	
283	Reference probe 15289-L07048	12q13		-	
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	-	
357	**RB1 probe** 19150-L25916		**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		-	
462 ¥	**RB1 probe** 21732-L32609		**Exon 27**	-	
472 * ¬	MED4 probe 23019-L16944		13q14.2	-	
480	**RB1 probe** 19182-L27252		**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 2 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 E2.
¥ Changed in version E2. Minor alteration, no change in sequence detected.
Δ More variable. This probe may be sensitive to certain experimental variations. Aberrant results should be treated with caution.
¬ 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.
Ø Intron probe. Only included to help determine the methylated status of imprinted locus (Kanber et al. 2009). Copy number alterations of only this probe are of unknown clinical significance.
+ 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.
‡ 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.
π Digestion control: warns for insufficient digestion. Upon digestion, this probe should not give a signal.

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

Table 2. P047-E2 probes arranged according to chromosomal location

Table 2a. RB1 and flanking probes

Length (nt)	SALSA (MS-)MLPA probe	Gene/ Exon[a]	Ligation site / location	**Partial sequence**[b] (copy number probes; 24 nt adjacent to ligation site) / **Complete sequence** (MS-MLPA probes)	Distance to next probe
Centromeric flanking probes					
146 ¬	19220-L25288	*ENOX1*	13q14.11	CTGCCCCTCTGG-ACACTCATAATT	4665.7 kb
163 ¬	23110-L32699	*MED4*	13q14.2	AGCTCCATCCCA-AGGCCGATACTC	7.8 kb
472 ¬	23019-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, in CpG106	CGCCCAAGGAGGGAGAGTGGCGCTC-CCGCCGAGGGTGCACTAGCCAGATATTCCCTGCG	0.2 kb
319	19146-L26559	upstream	162 nt before exon 1, CpG106	GCCGGATGCCTCCTGGAAGGCGCCTG-GACCCACGC CAGGTTTCCCAGTTTAATTCCTCATGACTTAGCGTCCC	0.2 kb
363	19550-L26088	Exon 1	12 nt before exon 1, in CpG106	GCGGAAGTGACGTTTTCCCGCGGTTG-GACGCGGCGCTCAGTTGCCGGGCGGGGGAGG	0.1 kb
265 Δ	07944-L25912	Exon 1	136-137, in CpG106	CGTCGTCCTCCCCGGCGCTCCTCCACAGCTC-GCTGGCTCCCGCCGCGGAAAGGCGTCATGCC	0.2 kb
297	19144-L25955	Exon 1	25 nt after exon 1, in CpG106	TCGTCAGGTGAGCGAGCAGAGCCGCCGTCGCC-TCACGCGGGAAGGGCGCCCGGGTGTGCGTAG	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, in CpG85	CGGCAGGGTAGTCTTGGAAATGCCCAA-GATTGC TTCCGCGCGCGTCAGTTCAGCGGACGTGTCT	0.5 kb
151 Ø ‡	15265-L25148	Intron 2	12.1 kb after exon 2, reverse, in CpG85	CCAGCAGCCCCCTGCAGCCCTTCAGAAGCAC-CACAGAATAAAAGCGGGGTCAAGAGGAGCGCTG GCGCCCTGAG	0.1 kb
235 Ø	19147-L17021	Intron 2	12.2 kb after exon 2, in CpG85	CGTTCGTCTTTGCTAACCGGGGAGGTTTGCGA-AAG GCGAACTCTTTATGGGCGCCCTTCAGACCCTGCCG	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

Length (nt)	SALSA (MS-)MLPA probe	Gene/ Exon[a]	Ligation site / location	Partial sequence[b] (copy number probes; 24 nt adjacent to ligation site) / Complete sequence (MS-MLPA probes)	Distance to next probe
480	19182-L27252	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
357	19150-L25916	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, reverse	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
462	21732-L32609	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	1592.7 kb
409 ¬	00801-L27246	*DLEU1*	13q14.3	GAAGAACAGAAC-CTTCAGGAATTG	2739.7 kb
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)	SALSA MLPA probe	Gene	Location	Partial sequence[b]
132 π	S0750-L25688	*SLC9A2*	2q12	GGACACTTTGGA-ATTCCATTGGCA
494	15318-L27254	*SATB2*	2q33	TGCCATTTATGA-CGAGATCCAACA
325	08048-L25770	*DNAH5*	5p15	AGATGACTGTTT-TTATTGATGATG
129	18709-L26847	*IL4*	5q31	ATCGACACCTAT-TAATGGGTCTCA
246	08715-L30393	*PCSK5*	9q21	AAGCTGAGACCT-AGTTCCAGAGGG
418	20130-L30396	*ZNF25*	10p11	CAGGTGATTCCT-GGGGCTGCCAGC
500	06676-L21510	*SMPD1*	11p15	CTGCTGAAGATA-GCACCACCTGCC
456	17129-L21829	*MYBPC3*	11p11	CACCCAACTATA-AGGCCCTGGACT
283	15289-L07048	*COL2A1*	12q13	CTGCTGGGCATT-AGGATCCTAGCC
373	14012-L15214	*UBE3A*	15q11	AGAAAGGAGCAA-GCTCAGCTTACC
184	16424-L26003	*MYO5B*	18q21	AAGTGTCCTCCA-GTGCCTCATGCC
136	15794-L17854	*ATP1A3*	19q13	CCTGCAGACATA-CGAGCAGAGGAA
157	03797-L26004	*KCNJ6*	21q22	CTCGAAGCTCCT-ACATCACCAGTG
210	12424-L26001	*LARGE1*	22q12	CAGTGAGGCTGA-TGTCAACAGTGA

[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.

Δ More variable. This probe may be sensitive to certain experimental variations. Aberrant results should be treated with caution.

¬ 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.

Ø Intron probe. Only included to help determine the methylated status of imprinted locus (Kanber et al. 2009). Copy number alterations of only this probe are of unknown clinical significance.
+ 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.
‡ 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.
π Digestion control: warns for insufficient digestion. Upon digestion, this probe should not give a signal.

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

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

Related SALSA (MS-)MLPA probemixes

P037 CLL-1, **P038 CLL-2** and **P040 CLL**: contain one (exon 21), two (exon 17 and 27) and two (exon 1 and 17) RB1 probes, respectively, in the context of 13q14 deletion detection in chronic lymphocytic leukaemia.
P175 Tumour Gain: contains two probes for *MYCN* gene found amplified in RB, as well as probes targeting frequently gained genes and chromosomal regions in various cancers, and one probe specific for *BRAF* p.V600E mutation.
P252 NB mix 2: contains two probes for *MYCN* gene found amplified in RB, as well as probes targeting frequently deleted or gained genes and chromosomal regions in neuroblastomas.
P335 ALL-IKZF1: contains five RB1 probes (exon 6, 14, 19, 24 and 26), as well as multiple probes for the detection of deletions of the *IKZF1* gene and copy number determination of *PAX5*, *ETV6*, *BTG1*, *EBF1*, *CDKN2A*, *CDKN2B* genes and PAR1 region in acute lymphoblastic leukemia.
P377 Hematologic Malignancies: contains two RB1 probes (exon 23 and 27), as well as probes for several genes and chromosomal regions known to have a significant diagnostic or prognostic role in hematologic malignancies, and one probe specific for *JAK2* p.V617F mutation.
P425 Multiple Myeloma: contains two RB1 probes (exon 8 and 26) in the context of 13q14-q22 deletion detection in multiple myeloma.

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P047 product history	
Version	*Modification*
E2	Two flanking probes have been added (*MED4* gene).
E1	One MS-MLPA 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 MS-MLPA 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 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.

Version 22 – 04 December 2019 (55, T08, 16)
- Added a text block on page 1 clarifying the possibility of using the probemix with both MLPA and MS-MLPA General Protocols.

Version 21 – 26 September 2019 (55, T08, 16)
- Updated MS-MLPA compatible enzyme information from Promega's HhaI (R6441) to SALSA HhaI (SMR50).

Version 20 – 13 December 2017 (55, T08, 16)
- Updated information about RB incidence on page 1.
- Added references for P047 probemix on page 2.

- Added footnotes about two variable probes in Table 1 and Table 2a.
- Updated ligation site information for CpG106 probes in Table 2a.
- Updated location/chromosome band information for PCDH8 probes according to hg18.
- Removed mapview location column from Table 2b.
- Added information about the digestion control probe in Table 2b.
- Small changes of probe lengths in Table 1 and 2 in order to better reflect the true lengths of the amplification products.
- Various minor textual and layout changes.

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