Product Description SALSA® MLPA® Probemix P298-A1 BRAF-HRAS-KRAS-NRAS

To be used with the MLPA General Protocol.

Version A1.

This SALSA® MLPA® kit is for basic research and intended for experienced MLPA users only! This kit enables you to quantify genes or chromosomal regions in which the occurrence or relevance of copy number changes is not yet well-established. Hence, it will not always provide you with clear cut answers, and interpretation of results can be very complicated.

Catalogue numbers:

- **P298-025R:** SALSA MLPA Probemix P298 BRAF-HRAS-KRAS-NRAS, 25 reactions.
- **P298-050R:** SALSA MLPA Probemix P298 BRAF-HRAS-KRAS-NRAS, 50 reactions.
- **P298-100R:** SALSA MLPA Probemix P298 BRAF-HRAS-KRAS-NRAS, 100 reactions.

To be used in combination with a SALSA MLPA reagent kit, available for various number of reactions. MLPA reagent kits are either provided with FAM or Cy5.0 dye-labelled PCR primer, suitable for Applied Biosystems and Beckman capillary sequencers, respectively (see www.mlpa.com).

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

Precautions and warnings: For professional use only. Always consult the most recent product description AND the MLPA General Protocol before use: www.mlpa.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 P298 BRAF-HRAS-KRAS-NRAS is a **research use only (RUO)** assay for the detection of deletions or duplications in the *RAS* genes (*HRAS*, *KRAS* and *NRAS*) and the *BRAF* gene. *RAS* genes (*HRAS*, *KRAS*, *NRAS*) code for small guanine-nucleotide-binding proteins which are essential for signalling networks controlling cellular proliferation, differentiation and survival. In the normal Ras/Raf/MEK/ERK pathway these RAS proteins are regulated by growth factors through tyrosine kinase receptors, which mediate addition of an active GTP by GTPase activating proteins (GAPs) or an inactive GDP by GTP exchange factors (GEFs). Downstream proteins will only be activated by the active RAS-GTP, but not by the inactive RAS-GDP. However, when *RAS* is mutated to an oncogenic form (in about 15% of human cancers) this regulation is eliminated and, as a consequence, bypass the regulation of cell growth factors. BRAF plays an important role as intermediary in the Ras/Raf/MEK/ERK pathway. In a normal situation, BRAF is activated by RAS-GTP. When mutated, BRAF causes overactive downstream signalling via MEK and ERK, leading to excessive cell proliferation and survival independent of growth factors. The *BRAF* p.V600E (c.1799T>A) mutation is the most frequent mutation which allows BRAF to signal independently.

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: http://www.ncbi.nlm.nih.gov/sites/entrez?db=gene
For NM_ mRNA reference sequences: http://www.ncbi.nlm.nih.gov/sites/entrez?db=nucleotide
Locus Reference Genomic (LRG) database: http://www.lrg-sequence.org/

Probemix content: The SALSA MLPA Probemix P298-A1 BRAF-HRAS-KRAS-NRAS contains 57 MLPA probes with amplification products between 115 and 504 nucleotides (nt). This includes one probe specific for the *BRAF* p.V600E (c.1799T>A) mutation which will only generate a signal when the mutation is present, and two probes for *KRAS* c.34G and c.35G, both located in codon 12, which will only generate a signal when the wildtype allele is present. In addition, 15 reference probes are included which detect 15 different autosomal chromosomal locations. These target relatively copy number stable regions in various human tumours.

Complete probe sequences and the identity of the genes detected by the reference probes is available online (www.mlpa.com).

This probemix contains nine quality control fragments generating amplification products between 64 and 121 nt: four DNA Quantity fragments (Q-fragments), two DNA Denaturation fragments (D-fragments), one Benchmark fragment, and one chromosome X and one chromosome Y-specific fragment (see table below). More information on how to interpret observations on these control fragments can be found in the MLPA General Protocol and online at www.mlpa.com.

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

No DNA controls result in only five major peaks shorter than 121 nt: four Q-fragments at 64, 70, 76 and 82 nt, and one 19 nt peak corresponding to the unused portion of the fluorescent PCR primer. Non-specific peaks longer than 121 nt AND with a height >25% of the median of the four Q-fragments should not be observed. Note: peaks below this 25% threshold are not expected to affect MLPA reactions when sufficient amount of sample DNA (50-250 ng) is used.

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

Required specimens: Extracted DNA, which includes DNA derived from paraffin-embedded 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 derived DNA for MLPA can be found in Atanesyan et al. 2017.

Reference samples: All samples tested, including reference DNA samples, should be derived from the same tissue type, handled using the same procedure, and prepared using the same DNA extraction method when possible. Reference samples should be derived from healthy individuals. More information regarding the selection and use of reference samples can be found in the MLPA General Protocol.

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/home.html) have a diverse collection of biological resources which may be used as a positive control DNA sample in your MLPA experiments. Samples from the Coriell Institute have been tested at MRC Holland and can be used to detect a *KRAS* gain (NA07981), a *HRAS* gain (NA03435), a *BRAF* gain (NA12519 and NA01220) and a *BRAF* loss (NA07412 and NA08808). The quality of cell lines can change, therefore positive samples should be either be acquired from quality assessed biological sample repositories or be validated before use.

SALSA Binning DNA SD029: The SD029 Binning DNA provided with this probemix can be used as Binning DNA sample for binning of one mutation-specific probe (BRAF probe 08780-SP0039-L21281 for the p.V600E (c.1799T>A) mutation). SD029 Binning DNA is a mixture of genomic DNA from healthy individuals and plasmid DNA that contains the target sequence detected by the above mentioned probe. Inclusion of one reaction with 5 µl SD029 Binning DNA in initial MLPA experiments is essential as it can be used to aid in data binning of the peak pattern using Coffalyser.Net software. Furthermore, Binning DNA should be included in the experiment whenever changes have been applied to the set-up of the capillary electrophoresis device (e.g. when capillaries have been renewed). Binning DNA should never be used as a reference sample in the MLPA data analysis, neither should it be used in quantification of mutation signal(s), as for this purpose true mutation positive patient samples or cell lines should be used. It is strongly advised to use test DNA sample and reference DNA samples extracted with the same method and derived from the same source of tissue.

For further details, please consult the SD029 Binning DNA product description provided. **This product is for research use only (RUO).**

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.mlpa.com. Use of other non-proprietary software may lead to inconclusive or false results. For more details on MLPA quality control and data analysis, including normalisation, see the Coffalyser.Net Reference Manual.

Interpretation of results: The standard deviation of all probes in the reference samples should be ≤0.10. When this criterion is fulfilled, the following cut-off values for the dosage quotient DQ of the probes can be used to interpret MLPA results for autosomal or pseudo-autosomal regions:

Copy Number status	Dosage quotient
Normal	0.80 < DQ < 1.20
Homozygous deletion	DQ = 0
Heterozygous deletion	0.40 < DQ < 0.65
Heterozygous duplication	1.30 < DQ < 1.65
Heterozygous triplication / homozygous duplication	1.75 < DQ < 2.15
Ambiguous copy number	All other values

Please note that these above mentioned dosage quotients 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 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. Incomplete DNA denaturation (e.g. due to salt contamination) can 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 (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.

P298 specific note:

- 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 most genetic alterations in *BRAF*, *HRAS*, *KRAS* and *NRAS* genes are small (point) mutations, most of which will not be detected by using SALSA MLPA Probemix P298 BRAF-HRAS-KRAS-NRAS.
- 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. SNPs, point mutations, small indels) in the target sequence detected by a probe can cause false positive results. Mutations/SNPs (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.
- MLPA analysis on tumour samples provides information on the *average* situation in the cells from which the DNA sample was purified. Gains or losses of genomic regions or genes may not be detected if the percentage of tumour cells is low. In addition, subclonality of the aberration affects the final ratio of the corresponding probe. Furthermore, there is always a possibility that one or more reference probes *do* show a copy number alteration in a patient sample especially in solid tumours with more chaotic karyotypes.

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.

COSMIC mutation database: http://cancer.sanger.ac.uk/cosmic. We strongly encourage users to deposit positive results in the Catalogue of Somatic Mutations In Cancer (COSMIC). Recommendations for the nomenclature to describe deletions/duplications of one or more exons can be found on http://varnomen.hgvs.org/.

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

Table 1. SALSA MLPA Probemix P298-A1 BRAF-HRAS-KRAS-NRAS

Length (nt)	SALSA MLPA probe	Chromosomal position (hg18)				
		References	*BRAF*	*HRAS*	*KRAS*	*NRAS*
64-105	Control fragments – see table in probemix content section for more information					
115	Reference probe S0973-L26704	4p13				
121	Reference probe S0864-L27364	21q22				
124	**KRAS probe** 20117-L27312				Exon 6	
131	Reference probe 11622-L23542	10q25				
137 «	**HRAS probe** 19301-L28332			Exon 6		
142 «	**HRAS probe** 19302-L25529			Exon 2		
148	**BRAF probe** 19303-L25530		Exon 1			
154 «	**HRAS probe** 19305-L26063			Exon 5		
160	**BRAF probe** 04259-L03624		Exon 5			
166	Reference probe 08726-L08737	9q21				
174	**BRAF probe** 10498-L25559		Exon 2			
178	**KRAS probe** 20379-L28333				Exon 2	
186	Reference probe 20361-L28315	3q24				
192 ∞ Ж	**KRAS probe** 19306-SP0797-L25533				**wild type** c.35G (Exon 2)	
198	**NRAS probe** 10525-L13934					Exon 7
205	**NRAS probe** 19307-L28439					Exon 1
211	Reference probe 20540-L17529	18p11				
222 ∞ Ж	**KRAS probe** 19308-SP0798-L25535				**wild type** c.34G (Exon 2)	
229 § Ж	**BRAF probe** 08780-SP0039-L21281		**p.V600E** (c.1799T>A; Exon 15)			
233	**NRAS probe** 19309-L28334					Exon 4
237 «	**HRAS probe** 10511-L11064			Exon 1		
244	**NRAS probe** 19332-L11079					Exon 6
250	Reference probe 10716-L26062	6p12				
256	**BRAF probe** 19310-L25537		Exon 3			
262	**KRAS probe** 19311-L25538				Exon 1	
269	**BRAF probe** 19312-L25539		Exon 18			
274	**BRAF probe** 10504-L11057		Exon 11			
281	**BRAF probe** 19313-L25540		Exon 16			
287	Reference probe 17874-L23697	19q13				
292	**NRAS probe** 10521-L26065					Exon 3
299	**BRAF probe** 19314-L26066		Exon 17			
309	**BRAF probe** 19315-L25542		Exon 7			
315	**BRAF probe** 20380-L27792		Exon 15			
322 «	**HRAS probe** 16999-L28446			Exon 4		
328 «	**HRAS probe** 19317-L28445			Exon 3		
337	**BRAF probe** 19318-L28444		Exon 10			
344	Reference probe 12785-L27941	2q13				
355	**BRAF probe** 19319-L28443		Exon 6			
362	**KRAS probe** 19321-L25548				Exon 5	
373	Reference probe 08141-L28316	14q11				
379	**BRAF probe** 19322-L25549		Exon 4			
386 Ж	**KRAS probe** 17605-SP0543-L21602				Exon 4	
391	Reference probe 09631-L09916	17q11				
402	**KRAS probe** 19323-L25550				Exon 3	
409	**BRAF probe** 19324-L25551		Exon 12			
417 «	**HRAS probe** 20381-L27793			Exon 4		
426	**NRAS probe** 19326-L25553					Exon 2
432	Reference probe 12456-L28335	22q12				
438 Ø	**KRAS probe** 19327-L25554				Intron 2	
447	**BRAF probe** 19328-L25555		Exon 14			
454	**BRAF probe** 19329-L25556		Exon 9			

Length (nt)	SALSA MLPA probe	Chromosomal position (hg18)				
		References	*BRAF*	*HRAS*	*KRAS*	*NRAS*
463	Reference probe 14741-L24649	4q22				
472	**BRAF probe** 19330-L28336		Exon 13			
481	**NRAS probe** 20382-L28337					Exon 5
489	**BRAF probe** 19331-L25558		Exon 8			
497	Reference probe 15203-L22591	3p12				
504	Reference probe 09870-L19465	2p15				

§ Mutation-specific probe. This probe will only generate a signal when the *BRAF* p.V600E (c.1799T>A) mutation is present. It has been tested on artificial DNA **but not on positive human samples!**
∞ Wild type sequence detected. The presence of the *KRAS* codon 12 point mutations by substitution at nucleotide position 34 or 35 will result in a decreased probe signal at 222 nt or 192 nt respectively.
« Probe located in or near a GC-rich region. A low signal can be caused by salt contamination in the DNA sample leading to incomplete DNA denaturation, especially of GC-rich regions.
Ж This probe consists of three parts and has two ligation sites. A low signal of this probe can be due to depurination of the sample DNA, e.g. due to insufficient buffering capacity or a prolonged denaturation time.
Ø Intron probe. Only included to help determine the extent of a deletion/duplication. Copy number alterations of a single intron probe are unlikely to be related to the condition tested.

Note: The *BRAF, HRAS, KRAS* and *NRAS* exon numbering used in this P298-A1 BRAF-HRAS-KRAS-NRAS product description is the exon numbering from the RefSeq transcripts NM_004333 (*BRAF;* identical to LRG_299), NM_005343 (*HRAS;* identical to LRG_506), NM_004985 and NM_033360 (*KRAS;* identical to LRG_344), and NM_002524 (*NRAS;* identical to LRG_92). The exon numbering and NM_ sequence used have been retrieved on 05/2019. As changes to the NCBI database can occur after release of this product description, exon numbering may not be up-to-date.

Table 2. P298-A1 probes arranged according to chromosomal location

Table 2a. P298-A1 target probes

Length (nt)	SALSA MLPA probe	Gene/ Exon	Ligation site	Partial sequence (24 nt adjacent to ligation site)	Distance to next probe
***NRAS*, at 1p13.2.** Indicated ligation sites are in NM_002524.5.					
205	19307-L28439	Exon 1	106-107	CAGAGGCAGTGG-AGCTTGAGGTAA	0.6 kb
		start codon	*132-134 (exon 2)*		
426	19326-L25553	Exon 2	20 nt after exon 2	CAGTGGTAGCCC-GCTGACCTGATC	2.2 kb
292	10521-L26065	Exon 3	420-421	TTAACCTCTACA-GGTACTAGGAGC	4.2 kb
233	19309-L28334	Exon 4	535-536	CGAACTGGCCAA-GAGTTACGGGAT	1.0 kb
481	20382-L28337	Exon 5	676-675 reverse	ATGGCAATCCCA-TACAACCCTGAG	0.4 kb
		stop codon	*699-701 (exon 5)*		
244	19332-L11079	Exon 6	2 nt after exon 6	CACTTTCAAGGT-AGGACAAGTTTG	0.1 kb
198	10525-L13934	Exon 7	787-788	GAGGAGAAGTAT-TCCTGTTGCTGT	-
***BRAF*, at 7q34.** Indicated ligation sites are in NM_004333.6.					
		start codon	*227-229 (exon 1)*		
148	19303-L25530	Exon 1	174 nt after exon 1 reverse	TTTACGTAGGAA-GGCGCTGCATGA	74.3 kb
174	10498-L25559	Exon 2	458-459	CACCATCAATAT-ATCTGGAGGTAA	15.3 kb
256	19310-L25537	Exon 3	473-474	TATAGGCCTATG-AAGAATACACCA	25.9 kb
379 #	19322-L25549	Exon 4	758-759	GAGTTACAGTCC-GAGACAGTCTAA	0.9 kb
160	04259-L03624	Exon 5	864-865	TTGGGACACTGA-TATTTCCTGGCT	6.6 kb
355	19319-L28443	Exon 6	1071-1072	GATGTGTGTTAA-TTATGACCAACT	1.0 kb
309	19315-L25542	Exon 7	1183-1184	TCATCCCCTTCC-GCACCCGCCTCG	6.1 kb
489	19331-L25558	Exon 8	1 nt after exon 8	TCAATATTGATG-TAAGTATCCAGC	6.7 kb
454	19329-L25556	Exon 9	6 nt before exon 9 reverse	CAAGTCCTACAA-ATAAATAGTAAT	4.6 kb
337	19318-L28444	Exon 10	1530-1531	CTCAGAAGACAG-GAATCGAATGGT	1.5 kb
274	10504-L11057	Exon 11	8 nt after exon 11	CATGGTAAGTAT-GTAATGTGGTGA	3.5 kb
409 #	19324-L25551	Exon 12	1688-1689	TGTTGAATGTGA-CAGCACCTACAC	1.1 kb
472	19330-L28336	Exon 13	1912-1913	CAGACTGCACAG-GGCATGGAGTAA	22.7 kb
447	19328-L25555	Exon 14	1964-1965	ACCTCAAGAGTA-ATAGTATCCTTC	0.9 kb
229 § Ж	08780-SP0039-L21281	**p.V600E** (c.1799T>A; Exon 15)	1985-1986 and 2025-2026	TTCTTCATGAAG-ACCTCACAGTAA AAATAGGTGATTTTGGTCTAGCTACA G**A**-GAAATCTCGATG	0.1 kb
315	20380-L27792	Exon 15	57 nt after exon 15 reverse	CTTTCTAGTAAC-TCAGCAGCATCT	3.9 kb
281	19313-L25540	Exon 16	6 nt after exon 16	GACCAGGTAAAT-ATTTACCACGTC	9.5 kb
299	19314-L26066	Exon 17	2349-2350	ACCACTCTTTCC-CCAAGTAAGTAA	5.5 kb
		stop codon	*2525-2527 (exon 18)*		
269 #	19312-L25539	Exon 18	2824-2825	TATTTTAAGTAG-TAAACTTCAGTT	-
***HRAS*, at 11p15.5.** Indicated ligation sites are in NM_005343.4.					
237 «	10511-L11064	Exon 1	158-159	TCGCGCCTGTGA-ACGGTGAGTGCG	1.1 kb
142 «	19302-L25529	Exon 2	168-169	GGCAGGTGGGGC-AGGAGACCCTGT	0.4 kb
		start codon	*215-217 (exon 2)*		
328 «	19317-L28445	Exon 3	333-334	GCAGGATTCCTA-CCGGAAGCAGGT	0.5 kb
417 «	20381-L27793	Exon 4	664-663 reverse	AGCTGCCTCACC-TGCCGGGTCTTG	0.1 kb
322 «	16999-L28446	Exon 4	86 nt after exon 4	GACCCTCTCCCT-TGACACAGGGCA	0.7 kb
154 «	19305-L26063	Exon 5	728-729	TGCGGAAGCTGA-ACCCTCCTGATG	0.4 kb
		stop codon	*782-784 (exon 5)*		
137 «	19301-L28332	Exon 6	1059-1060	AGTAAATTATTG-GATGGTCTTGAT	-
***KRAS*, at 12p12.1.** Indicated ligation sites are in NM_033360.4.					
262	19311-L25538	Exon 1	170-171	GCTCCCAGGTGC-GGGAGAGAGGTA	5.4 kb
		start codon	*191-193 (exon 2)*		
192 ∞ Ж	19306-SP0797-L25533	**wild type** c.35G (Exon 2)	225-224 and 184-183 reverse	TGCCTACGCCA**C**-41 nt spanning oligo-AGGCCTTATAAT	0.1 kb
222 ∞ Ж	19308-SP0798-L25535	**wild type** c.34G (Exon 2)	224-225 and 288-289	TAGTTGGAGCT**G**-64 nt spanning oligo-TCCAACAATAGA	0.2 kb
178	20379-L28333	Exon 2	181 nt after exon 2 reverse	ACATACTCCCAA-GGAAAGTAAAGT	10.7 kb
438 Ø	19327-L25554	Intron 2	7 kb before exon 3	AGTAGTATAATG-AACCTCATGTGT	7.0 kb

Length (nt)	SALSA MLPA probe	Gene/ Exon	Ligation site	Partial sequence (24 nt adjacent to ligation site)	Distance to next probe
402	19323-L25550	Exon 3	308-307 reverse	TTGCTTCCTGTA-GGAATCCTGAGA	1.7 kb
386 Ж	17605-SP0543-L21602	Exon 4	532-533 and 574-575	ATGGTCCTAGTA-42 nt spanning oligo-CAGGCTCAGGAC	10.1 kb
362	19321-L25548	Exon 5	3 nt before exon 5	TGTTTTACAATG-CAGAGAGTGGAG	7.7 kb
		stop codon	*758-760 (exon 5)*		
124	20117-L27312	Exon 6	2821-2822	GAGTCACATCAG-AAATGCCCTACA	-

§ Mutation-specific probe. This probe will only generate a signal when the *BRAF* p.V600E (c.1799T>A) mutation is present. It has been tested on artificial DNA **but not on positive human samples!**
∞ Wild type sequence detected. The presence of the *KRAS* codon 12 point mutations by substitution at nucleotide position 34 or 35 will result in a decreased probe signal at 222 nt or 192 nt respectively.
« Probe located in or near a GC-rich region. A low signal can be caused by salt contamination in the DNA sample leading to incomplete DNA denaturation, especially of GC-rich regions.
Ж This probe consists of three parts and has two ligation sites. A low signal of this probe can be due to depurination of the sample DNA, e.g. due to insufficient buffering capacity or a prolonged denaturation time.
Ø Intron probe. Only included to help determine the extent of a deletion/duplication. Copy number alterations of a single intron probe are unlikely to be related to the condition tested.
This probe's specificity relies on a single nucleotide difference compared to a related gene or pseudogene. As a result, an apparent duplication of only this probe can be the result of a non-significant single nucleotide sequence change in the related gene or pseudogene.

Note: The *BRAF, HRAS, KRAS* and *NRAS* exon numbering used in this P298-A1 BRAF-HRAS-KRAS-NRAS product description is the exon numbering from the RefSeq transcripts NM_004333 (*BRAF;* identical to LRG_299), NM_005343 (*HRAS;* identical to LRG_506), NM_004985 and NM_033360 (*KRAS;* identical to LRG_344), and NM_002524 (*NRAS;* identical to LRG_92). The exon numbering and NM_ sequence used have been retrieved on 06/2019. As changes to the NCBI database can occur after release of this product description, exon numbering may not be up-to-date.

Table 2b. Reference probes arranged according to chromosomal location.

Length (nt)	SALSA MLPA probe	Gene	Location	Partial sequence (24 nt adjacent to ligation site)	Location (hg18) in kb
504	09870-L19465	PEX13	2p15	TGAGGATGACCA-TGTAGTTGCCAG	02-061.126
344	12785-L27941	EDAR	2q13	CCCAGAACTGGA-TGGTACCTGACT	02-108.972
497	15203-L22591	GBE1	3p12	GACCTAGAGGGA-CTCATGATCTTT	03-081.775
186	20361-L28315	ZIC4	3q24	AAGGTTTGGTCT-ACAACACAGTGA	03-148.587
115	S0973-L26704	ATP8A1	4p13	CAGATTCTTCTT-CGAGGAGCTCAG	04-042.278
463	14741-L24649	PKD2	4q22	TGTGCATCTGTA-AGTAGAATATTT	04-089.160
250	10716-L26062	PKHD1	6p12	GCCATCCTTGTT-TCTGATGGTGGA	06-051.907
166	08726-L08737	PCSK5	9q21	ACACCTGCCAGA-GATGCCAAGGAA	09-078,038
131	11622-L23542	NRAP	10q25	ACAGCTCCCAGA-TGGAGCACGCCA	10-115,376
373	08141-L28316	RPGRIP1	14q11	TATTCCTTCTAT-GACTTTGAAACC	14-020.863
391	09631-L09916	MYO1D	17q11	CTGATGCCATGA-AAGTCATTGGCT	17-028.119
211	20540-L17529	RNMT	18p11	TACAATGAACTT-CAGGAAGTTGGT	18-013.724
287	17874-L23697	SLC7A9	19q13	AAAGTGCTTTCT-TACATCAGCGTC	19-038.027
121	S0864-L27364	KCNJ6	21q22	AGCTCCTACATC-ACCAGTGAGATC	21-037.920
432	12456-L28335	LARGE1	22q12	CCTGCCAGGTCA-TTCACTGGAACT	22-032.064

Related SALSA MLPA probemixes

- P294 Tumour Loss: Contains probes for tumour suppressor genes.
- P175 Tumour Gain: Contains probes for frequently gained or amplified genes in various tumour types.

References

- 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.
- Hömig-Hölzel C and Savola S. (2012). Multiplex ligation-dependent probe amplification (MLPA) in tumor diagnostics and prognostics. *Diagn Mol Pathol*. 21:189-206.
- 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.
- 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 SALSA MLPA Probemix P298 BRAF-HRAS-KRAS-NRAS

- Schubert S et al. (2019) The identification of pathogenic variants in BRCA1/2 negative, high risk, hereditary breast and/or ovarian cancer patients: High frequency of FANCM pathogenic variants. *Int J Cancer*. 144:2683-94.

P298 Product history	
Version	*Modification*
A1	First release.

Implemented changes in the product description
Version A1-01 –28 June 2019 (01P) - Product description restructured and adapted to a new template. - Ligation sites of the probes targeting the *BRAF*, *HRAS*, *KRAS* and *NRAS* genes updated according to new version of the NM_reference sequences. - Warning added to Table 2a for probe specificity relying on a single nucleotide difference between target gene and related gene or pseudogene. - For uniformity, the chromosomal positions and bands for target probes in this document are now all based on hg18 (NCBI36). - Small changes of probe lengths in Table 1 and 2 in order to better reflect the true lengths of the amplification products. *Version 01 (T08)- 02 AUG 2016* - Not applicable, new document.

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