# Product Description
# SALSA® MLPA® Probemix P298-A2
# BRAF-HRAS-KRAS-NRAS

To be used with the MLPA General Protocol.

**Version A2**
For complete product history see page 11.

This SALSA MLPA probemix is for basic research and intended for experienced MLPA users only! This probemix enables you to quantify genes or chromosomal regions in which the occurrence of copy number changes is not yet well-established and the relationship between genotype and phenotype is not yet clear. Since it will not provide you with clear cut answers, interpretation of results can be complicated. MRC Holland recommends thoroughly screening any available literature. Suggestions from specialists for improvement of this product or product description are highly appreciated.

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

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

**Precautions and warnings**
For professional use only. Always consult the most recent product description AND the MLPA General Protocol before use: www.mrcholland.com. It is the responsibility of the user to be aware of the latest scientific knowledge of the application before drawing any conclusions from findings generated with this product.

**General information**
The SALSA MLPA Probemix P298 BRAF-HRAS-KRAS-NRAS is a **research use only (RUO)** assay for the detection of deletions or amplifications in the *RAS* genes (*HRAS*, *KRAS* and *NRAS*) and the *BRAF* gene, all core components of the Ras/Raf/MEK/ERK pathway. *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 Ras/Raf/MEK/ERK pathway, RAS proteins are regulated by receptor tyrosine kinases (RTKs) which are activated upon growth factor binding. These RTKs mediate addition of an active GTP by GTPase activating proteins (GAPs) or an inactive GDP by GTP exchange factors (GEFs) (Simanshu et al., 2017). Downstream proteins will only be activated by the active RAS-GTP, but not by the inactive RAS-GDP. Oncogenic mutations in *RAS* occur in about 15% of human cancers, predominantly affecting the G12 residue of the protein. These oncogenic mutations result in the constitutive activation of RAS by keeping the protein in its GTP-bound state, thereby bypassing the need for growth factors to activate the pathway (Simanshu et al., 2017; Braicu et al. 2019).

BRAF is activated by RAS-GTP as a result of ligand-induced RTK activation in healthy cells. Mutations in the activating kinase domain of BRAF cause 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 confers oncogenic BRAF function (Davies et al., 2002; Braicu et al. 2019).

**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
Matched Annotation from NCBI and EMBL-EBI (MANE): https://www.ncbi.nlm.nih.gov/refseq/MANE/ and http://tark.ensembl.org/
Locus Reference Genomic (LRG) database: http://www.lrg-sequence.org/

**Exon numbering**
The *BRAF*, *HRAS, KRAS* and *NRAS* exon numbering in this P298 BRAF-HRAS-KRAS-NRAS product description is the exon numbering derived from MANE project (release version 1.0) based on MANE Select transcripts NM_004333.6, NM_005343.4, NM_04985.5 and NM_002524.5, respectively. The *KRAS* exon numbering has changed; the exon numbering used in previous versions of this product description was from transcript NM_033360.4 which can be found in between brackets in Tables 1 and 2 (if different from the MANE project). 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 from literature and in other MRC Holland product descriptions, where other resources used for exon numbering are indicated.

**Probemix content**
The SALSA MLPA Probemix P298 BRAF-HRAS-KRAS-NRAS contains 57 MLPA probes with amplification products between 115 and 504 nucleotides (nt). This includes 19, seven, nine and seven probes for the *BRAF, HRAS, KRAS* and *NRAS* genes respectively. These 42 probes include 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 that detect autosomal chromosomal locations and target relatively copy number stable regions in various cancer types. Complete probe sequences and the identity of the genes detected by the reference probes are available online (www.mrcholland.com) and in Table 3.

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

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

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

**MLPA technique validation**
Internal validation of the MLPA technique using 16 DNA samples from healthy individuals is required, in particular when using MLPA for the first time, or when changing the sample handling procedure, DNA

extraction method or instruments used. This validation experiment should result in a standard deviation ≤0.10 for all probes over the experiment.

**Required specimens**
Extracted DNA, 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 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 from healthy 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 NA12519, NA07412, NA08808, NA01220, NA03435 and NA07981 from the Coriell Institute have been tested with this P298 BRAF-HRAS-KRAS-NRAS probemix at MRC Holland and can be used as a positive control sample(s) to detect copy number alterations. The quality of cell lines can change; therefore samples should be validated before use.

| Sample name | Chromosomal position of CNA (hg18)* | Altered target genes in P298-A2 | Expected copy number alteration |
|---|---|---|---|
| NA12519 | 7q34 | *BRAF* | Homozygous duplication/Heterozygous triplication |
| NA07412 | 7q34 | *BRAF* | Heterozygous deletion |
| NA08808 | 7q34 | *BRAF* | Heterozygous deletion |
| NA01220 | 7q34 | *BRAF* | Heterozygous duplication |
| NA03435 | 11p15.5 | *HRAS* | Heterozygous duplication |
| NA07981 | 12p12.1 | *KRAS* | Homozygous duplication/Heterozygous triplication |

* Indicated chromosomal bands accommodate genes targeted by MLPA probes, however, the whole extent of copy number alteration (CNA) present in these cell lines cannot be determined by this P298 BRAF-HRAS-KRAS-NRAS probemix.

**SALSA Binning DNA SD029**
The SD029 Binning DNA provided with this probemix can be used for binning of all probes including the 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). It is strongly advised that all samples tested are extracted with the same method and derived from the same source of tissue. For further details, please consult the SD029 Binning DNA product description, available online: www.mrcholland.com. **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.mrcholland.com. Use of other non-proprietary software may lead to inconclusive or false results. For more details on MLPA quality control and data analysis, including normalisation, see the Coffalyser.Net Reference Manual.

**Interpretation of results**

The standard deviation of each individual probe over all the reference samples should be ≤0.10. When this criterion is 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 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) can also lead to a decreased probe signal, in particular for probes located in or near a GC-rich region or in or near the *HRAS* gene. The use of an additional purification step or an alternative DNA extraction method may resolve such cases. Additionally, contamination of DNA samples with cDNA or PCR amplicons of individual exons can lead to an increased probe signal (Varga et al. 2012). Analysis of an independently collected secondary DNA sample can exclude these kinds of contamination artefacts.
- Normal copy number variation in healthy individuals is described in the database of genomic variants: http://dgv.tcag.ca/dgv/app/home. Users should always consult the latest update of the database and scientific literature when interpreting their findings.
- Not all abnormalities detected by MLPA are pathogenic. In some genes, intragenic deletions are known that result in very mild or no disease (as described for *DMD* by Schwartz et al. 2007). For many genes, more than one transcript variant exists. Copy number changes of exons that are not present in all transcript variants may not have clinical significance. Duplications that include the first or last exon of a gene (e.g. exons 1-3) might not result in inactivation of that gene copy.
- Copy number changes detected by reference probes or flanking probes are unlikely to have any relation to the condition tested for.
- False results can be obtained if one or more peaks are off-scale. For example, a duplication of one or more exons can be obscured when peaks are off-scale, resulting in a false negative result. The risk on off-scale peaks is higher when probemixes are used that contain a relatively low number of probes. Coffalyser.Net software warns for off-scale peaks while other software does not. If one or more peaks are off-scale, rerun

the PCR products using either: a lower injection voltage or a shorter injection time, or a reduced amount of sample by diluting PCR products.

**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 major cause of genetic defects in cancer 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. 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.
- 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.

**Mutation databases**

LOVD mutation database: LOVD - An Open Source DNA variation database system; COSMIC mutation database**:** https://cancer.sanger.ac.uk/cosmic. We strongly encourage users to deposit positive results in the COSMIC Database. Recommendations for the nomenclature to describe deletions/duplications of one or more exons can be found on http://varnomen.hgvs.org/.

Please report false positive results due to SNVs and unusual results (e.g., a duplication of *NRAS* exons 2 and 4 but not exon 3) to MRC Holland: info@mrcholland.com.

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

| Length (nt) | SALSA MLPA probe | Chromosomal position (hg18)[a] | | | | |
|---|---|---|---|---|---|---|
| | | Reference | *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 5 (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 |
| 238 ¥ « | **HRAS** probe 10511-L32903 | | | 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 | | | |
| 308 | **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 Ø | **KRAS** probe 19321-L25548 | | | | Intron 4 (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 | |

| Length (nt) | SALSA MLPA probe | Chromosomal position (hg18)[a] | | | | |
|---|---|---|---|---|---|---|
| | | Reference | *BRAF* | *HRAS* | *KRAS* | *NRAS* |
| 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 | | | |
| 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 | | | | |

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

¥ Changed in version A2. Minor alteration, no change in sequence detected.

§ Mutation-specific probe. This probe will only generate a signal when the BRAF p.V600E mutation is present. It has been tested on artificial DNA **but not on positive human samples!**

∞ Wild type sequence detected. A lowered probe signal can be due to *KRAS* codon 12 point mutations by substitution at nucleotide position 34 or 35. Other variants near the ligation site can also cause a lowered signal. A positive result must be confirmed by another method.

« 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. When this occurs in reference samples, it can look like an increased signal in the test samples.

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.

## Table 2. P298-A2 probes arranged according to chromosomal location

| Length (nt) | SALSA MLPA probe | Exon[a] | Ligation site | Partial sequence[b] (24 nt adjacent to ligation site) | Distance to next probe |
|---|---|---|---|---|---|
| ***NRAS***, at 1p13.2. Indicated ligation sites are in NM_002524.5. | | | | | |
| 198 | 10525-L13934 | Exon 7 | 787-788 | GAGGAGAAGTAT-TCCTGTTGCTGT | 0.1 kb |
| 244 | 19332-L11079 | Exon 6 | 2 nt after exon 6 | CACTTTCAAGGT-AGGACAAGTTTG | 0.4 kb |
| | | *stop codon* | *699-701 (exon 5)* | | |
| 481 | 20382-L28337 | Exon 5 | 676-675, reverse | ATGGCAATCCCA-TACAACCCTGAG | 1.0 kb |
| 233 | 19309-L28334 | Exon 4 | 535-536 | CGAACTGGCCAA-GAGTTACGGGAT | 4.2 kb |
| 292 | 10521-L26065 | Exon 3 | 420-421 | TTAACCTCTACA-GGTACTAGGAGC | 2.2 kb |
| 426 | 19326-L25553 | Exon 2 | 20 nt after exon 2 | CAGTGGTAGCCC-GCTGACCTGATC | 0.6 kb |
| | | *start codon* | *132-134 (exon 2)* | | |
| 205 | 19307-L28439 | Exon 1 | 106-107 | CAGAGGCAGTGG-AGCTTGAGGTAA | |
| ***BRAF***, at 7q34. Indicated ligation sites are in NM_004333.6. | | | | | |
| 269 # | 19312-L25539 | Exon 18 | 2824-2825 | TATTTTAAGTAG-TAAACTTCAGTT | 5.5 kb |
| | | *stop codon* | *2525-2527 (exon 18)* | | |
| 299 | 19314-L26066 | Exon 17 | 2349-2350 | ACCACTCTTTCC-CCAAGTAAGTAA | 9.5 kb |
| 281 | 19313-L25540 | Exon 16 | 6 nt after exon 16 | GACCAGGTAAAT-ATTTACCACGTC | 3.9 kb |
| 315 | 20380-L27792 | Exon 15 | 57 nt after exon 15, reverse | CTTTCTAGTAAC-TCAGCAGCATCT | 0.1 kb |
| 229 § Ж | 08780-SP0039-L21281 | **p.V600E** (c.1799T>A; Exon 15) | 1985-1986 and 2025-2026 | TTCTTCATGAAG-ACCTCACAGTAA AAATAGGTGATTTTGGTCTAGCTAC AG**A**-GAAATCTCGATG | 0.9 kb |
| 447 | 19328-L25555 | Exon 14 | 1964-1965 | ACCTCAAGAGTA-ATAGTATCCTTC | 22.7 kb |
| 472 | 19330-L28336 | Exon 13 | 1912-1913 | CAGACTGCACAG-GGCATGGAGTAA | 1.1 kb |
| 409 # | 19324-L25551 | Exon 12 | 1688-1689 | TGTTGAATGTGA-CAGCACCTACAC | 3.5 kb |
| 274 | 10504-L11057 | Exon 11 | 8 nt after exon 11 | CATGGTAAGTAT-GTAATGTGGTGA | 1.5 kb |
| 337 | 19318-L28444 | Exon 10 | 1530-1531 | CTCAGAAGACAG-GAATCGAATGGT | 4.6 kb |
| 454 | 19329-L25556 | Exon 9 | 6 nt before exon 9, reverse | CAAGTCCTACAA-ATAAATAGTAAT | 6.7 kb |
| 489 | 19331-L25558 | Exon 8 | 1 nt after exon 8 | TCAATATTGATG-TAAGTATCCAGC | 6.1 kb |
| 308 | 19315-L25542 | Exon 7 | 1183-1184 | TCATCCCCTTCC-GCACCCGCCTCG | 1.0 kb |
| 355 | 19319-L28443 | Exon 6 | 1071-1072 | GATGTGTGTTAA-TTATGACCAACT | 6.6 kb |
| 160 | 04259-L03624 | Exon 5 | 864-865 | TTGGGACACTGA-TATTTCCTGGCT | 0.9 kb |
| 379 # | 19322-L25549 | Exon 4 | 758-759 | GAGTTACAGTCC-GAGACAGTCTAA | 25.9 kb |
| 256 | 19310-L25537 | Exon 3 | 473-474 | TATAGGCCTATG-AAGAATACACCA | 15.3 kb |
| 174 | 10498-L25559 | Exon 2 | 458-459 | CACCATCAATAT-ATCTGGAGGTAA | 74.3 kb |
| 148 | 19303-L25530 | Exon 1 | 174 nt after exon 1, reverse | TTTACGTAGGAA-GGCGCTGCATGA | |
| | | *start codon* | *227-229 (exon 1)* | | |
| ***HRAS***, at 11p15.5. Indicated ligation sites are in NM_005343.4. | | | | | |
| 137 « | 19301-L28332 | Exon 6 | 1059-1060 | AGTAAATTATTG-GATGGTCTTGAT | 0.4 kb |
| | | *stop codon* | *782-784 (exon 5)* | | |
| 154 « | 19305-L26063 | Exon 5 | 728-729 | TGCGGAAGCTGA-ACCCTCCTGATG | 0.7 kb |
| 322 « | 16999-L28446 | Exon 4 | 86 nt after exon 4 | GACCCTCTCCCT-TGACACAGGGCA | 0.1 kb |
| 417 « | 20381-L27793 | Exon 4 | 664-663, reverse | AGCTGCCTCACC-TGCCGGGTCTTG | 0.5 kb |
| 328 « | 19317-L28445 | Exon 3 | 333-334 | GCAGGATTCCTA-CCGGAAGCAGGT | 0.4 kb |
| | | *start codon* | *215-217 (exon 2)* | | |
| 142 « | 19302-L25529 | Exon 2 | 168-169 | GGCAGGTGGGGC-AGGAGACCCTGT | 1.1 kb |
| 238 ¥ « | 10511-L32903 | Exon 1 | 158-159 | TCGCGCCTGTGA-ACGGTGAGTGCG | |
| ***KRAS***, at 12p12.1. Indicated ligation sites are in NM_004985.5 unless otherwise specified. | | | | | |
| 124 | 20117-L27312 | Exon 5 (6) | 2697-2698 | GAGTCACATCAG-AAATGCCCTACA | 7.7 kb |
| | | *stop codon* | *758-760 (exon 5)* | | |

| Length (nt) | SALSA MLPA probe | Exon[a] | Ligation site | Partial sequence[b] (24 nt adjacent to ligation site) | Distance to next probe |
|---|---|---|---|---|---|
| 362 Ø | 19321-L25548 | Intron 4 (Exon 5) | 5.7 kb before exon 5; NM_033360.4: 3 nt before alternative exon 5 | TGTTTTACAATG-CAGAGAGTGGAG | 10.1 kb |
| 386 Ж | 17605-SP0543-L21602 | Exon 4 | 532-533 and 574-575 | ATGGTCCTAGTA-42 nt spanning oligo-CAGGCTCAGGAC | 1.7 kb |
| 402 | 19323-L25550 | Exon 3 | 308-307, reverse | TTGCTTCCTGTA-GGAATCCTGAGA | 7.0 kb |
| 438 Ø | 19327-L25554 | Intron 2 | 7 kb before exon 3 | AGTAGTATAATG-AACCTCATGTGT | 10.7 kb |
| 178 | 20379-L28333 | Exon 2 | 181 nt after exon 2, reverse | ACATACTCCCAA-GGAAAGTAAAGT | 0.2 kb |
| 222 ∞ Ж | 19308-SP0798-L25535 | **wild type** c.34G; Exon 2 | 224-225 and 288-289 | AGTTGGAGCT**G**-64 nt spanning oligo-TCCAACAATAGA | 0.1 kb |
| 192 ∞ Ж | 19306-SP0797-L25533 | **wild type** c.35G; Exon 2 | 225-224 and 184-183, reverse | TGCCTACGCCA**C**-41 nt spanning oligo-AGGCCTTATAAT | 5.4 kb |
| | | *start codon* | *191-193 (exon 2)* | | |
| 262 | 19311-L25538 | Exon 1 | 170-171 | GCTCCCAGGTGC-GGGAGAGAGGTA | |

[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.

¥ Changed in version A2. Minor alteration, no change in sequence detected.

§ Mutation-specific probe. This probe will only generate a signal when the *BRAF* p.V600E mutation is present. It has been tested on artificial DNA **but not on positive human samples!**

∞ Wild type sequence detected. A lowered probe signal can be due to *KRAS* codon 12 point mutations by substitution at nucleotide position 34 or 35. Other variants near the ligation site can also cause a lowered signal. A positive result must be confirmed by another method.

« 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. When this occurs in reference samples, it can look like an increased signal in the test samples.

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'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.

## Table 3. Reference probes arranged according to chromosomal location.

| Length (nt) | SALSA MLPA probe | Gene | Chromosomal band (hg18) | 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 |

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

## 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.
- Braicu C et al. (2019) A Comprehensive Review on MAPK: A Promising Therapeutic Target in Cancer. *Cancers (Basel)*. 11:1618.
- Davies H et al. (2002). Mutations of the BRAF gene in human cancer. *Nature*. 417:949-54.
- 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.
- Simanshu DK et al. (2017) RAS Proteins and Their Regulators in Human Disease. *Cell*. 170:17-33.
- 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


- Modena P et al .(2012) Predictors of outcome in an AIEOP series of childhood ependymomas: a multifactorial analysis. *Neuro Oncol.*14:1346-56.
- 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* |
| A2 | One probe has a small change in length, but no change in the sequence detected |
| A1 | First release. |

**Implemented changes in the product description**

Version A2-02 – 13 July 2023 (04P)
- Positive sample information on NA01220 Coriell sample has been corrected in table on page 3 to be heterozygous duplication.

Version A2-01 – 20 June 2023 (04P)
- Product description adapted to new template.
- List of references updated.
- Exon numbering of the *KRAS* gene has been changed.
- Small changes of probe lengths in Table 1 and 2 in order to better reflect the true lengths of the amplification products.

Version A1-02 – 10 January 2023 (01P)
- Information about possible small signal for BRAF V600E mutation probe on a sample with the V600K mutation added to the P298 specific notes section and Tables 1 and 2.

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.

| **More information: www.mrcholland.com; www.mrcholland.eu** | |
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