Product Description SALSA® MLPA® probemix P093-C2 HHT/HPAH

To be used with the MLPA General Protocol.

Version C2. As compared to version C1, the length of several probes has been adjusted. For complete product history see page 10.

Catalogue numbers:
- **P093-025R:** SALSA® MLPA® probemix P093 HHT/HPAH, 25 reactions.
- **P093-050R:** SALSA® MLPA® probemix P093 HHT/HPAH, 50 reactions.
- **P093-100R:** SALSA® MLPA® probemix P093 HHT/HPAH, 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.

Intended use: The SALSA MLPA probemix P093 HHT/HPAH is an in vitro diagnostic (IVD) [1] or a research use only (RUO) assay for the detection of deletions or duplications in *ENG*, *ACVRL1* and *BMPR2* in order to (1) confirm a clinical diagnosis of Hereditary Hemorrhagic Telangiectasia (HHT) or Heritable Pulmonary Arterial Hypertension (HPAH) or to, (2) determine predisposition to HHT or HPAH of at-risk family members/individuals.

This assay is for use with human DNA extracted from peripheral blood, not for use with DNA extracted from formalin-fixed paraffin embedded or fresh tumour materials. Deletions or duplications detected with the P093 HHT/HPAH probemix must be verified by another technique. In particular, deletions or duplications detected by only a single probe always require validation by another method. Most defects in the *ENG*, *ACVRL1* and *BMPR2* genes are point mutations, the majority of which will not be detected by MLPA. It is therefore recommended to use this SALSA MLPA probemix in combination with sequence analysis of the *ENG*, *ACVRL1* and *BMPR2* genes. This assay is not intended to be used as a standalone assay for clinical decisions. The results of this test must be interpreted by a clinical molecular geneticist or equivalent.

[1]Please note that this probemix is for In Vitro Diagnostic use (IVD) in the countries specified at the end of this product description. In all other countries, the product is for Research Use Only (RUO).

Clinical background: Hereditary Hemorrhagic Telangiectasia (HHT) is a disease with an autosomal dominant inheritance pattern and is characterized by the presence of multiple arteriovenous malformations (AVM). In AVMs arteries connect directly to veins instead of through intervening capillaries, resulting in high blood pressure. AVMs occur on the skin, but also in the brain, lungs, liver and intestines. Depending on the location, rupture of these malformations can have catastrophic consequences for the patient. Diagnosis is based on the presence of multiple AVM in the skin, mucus membranes, or visceral organs. Recurrent nosebleeds are also a common finding in HHT patients. Molecular genetic testing is performed to confirm or establish a diagnosis in a proband. HHT is primarily caused by pathogenic variations in the genes endoglin (*ENG/HHT1*) and activing A receptor like type 1 (*ACVLR1/HHT2*). Both genes encode endothelial cell surface receptors that are part of a TGF-β/BMP signalling cascade, a pathway involved in angiogenesis, among multiple other developmental processes. Up to 10% of pathogenic variation consists of large deletions/duplications. More information is available at: https://www.ncbi.nlm.nih.gov/books/NBK1351/.

Heritable Pulmonary Arterial Hypertension (HPAH) is inherited in an autosomal dominant manner. This disease is caused by loss or obstruction of the smallest pulmonary arteries, resulting in high blood pressure

in the arteries of the lung. Diagnosis is based on the presence of pulmonary hypertension as confirmed through right heart catheterization, and subsequently by identification of a heterozygous pathogenic variant in a known associated gene (simplex cases) and/or confirmation of PAH in one or more of the proband's family members. Up to 75% of HPAH is caused by variation in the bone morphogenetic protein receptor type 2 (*BMPR2*) gene. Of this, 12-37% is caused by large duplications/deletions. Similar to *ENG* and *ACVLR1*, the *BMPR2* gene also encodes a cell surface receptor that is part of the TGF-β/BMP signalling pathway. Sporadically, PAH is observed as a symptom of HHT. The biological similarities between the causative genes suggests a similar aetiology between HPAH and HHT. This is supported by rare observations of mutations in *ACVLR1*, and even more infrequent in *ENG*, causing HPAH. In literature, a patient has been described with a combined PAH and HHT phenotype carrying a deletion of exons 6 and 7 in *BMPR2* (Handa et al. 2014). In very rare cases, HPAH can be caused by mutations in the *KCNK3*, *SMAD9* or *CAV1* gene. To the best of our knowledge, no HPAH causing deletions or duplications have been reported in these genes. More information is available at: https://www.ncbi.nlm.nih.gov/books/NBK1485/.

Gene structure: The *BMPR2* gene (13 exons) spans ~191 kb of genomic DNA and is located on chromosome 2q33.1-2, about 203 Mb from the p-telomere. The BMPR2 LRG_712 is identical to Genbank NG_009363.1 and is available at www.lrg-sequence.org. The *ACVRL1* (ALK1) gene (10 exons) spans ~16 kb of genomic DNA and is located on chromosome 12q13.3, about 51 Mb from the p-telomere. The ACVRL1 LRG_543 is identical to Genbank NG_009549.1 and is available at www.lrg-sequence.org. The *ENG* gene (14 exons) spans ~40 kb of genomic DNA and is located on chromosome 9q34.11, about 130 Mb from the p-telomere. The ENG LRG_589 is identical to Genbank NG_009551.1 and is available at www.lrg-sequence.org.

Transcript variants: One *BMPR2* transcript variant has been described: http://www.ncbi.nlm.nih.gov/gene/659. The sequence of *BMPR2* transcript variant 1 (NM_001204.6; 12086 nt; coding sequence 1149-4265) is a reference standard in the NCBI RefSeqGene project. In Table 2a, the ligation sites of the *BMPR2* MLPA probes are indicated according to this sequence which contains 13 exons. The ATG translation start site is located in exon 1 (1149-1151) and the stop codon is located in exon 13 (4263-4265).

Two *ACVLR1* transcript variants have been described: http://www.ncbi.nlm.nih.gov/gene/94. The sequence of *ACVRL1* transcript variant 1 (NM_000020.2; 4263 nt; coding sequence 284-1795) is a reference standard in the NCBI RefSeqGene project. In Table 2b, the ligation sites of the *ACVRL1* MLPA probes are indicated according to this sequence which contains 10 exons. The ATG translation start site is located in exon 1 (284-286) and the stop codon is located in exon 10 (1793-1795).

Three *ENG* transcript variants have been described: http://www.ncbi.nlm.nih.gov/gene/2022. The sequence of *ENG* transcript variant 2 (NM_000118.3; 3201 nt; coding sequence 419-2296) is a reference standard in the NCBI RefSeqGene project. In Table 2c, the ligation sites of the *ENG* MLPA probes are indicated according to this sequence which contains 14 exons. The ATG translation start site is located in exon 1 (419-421) and the stop codon is located in exon 14(b) (2294-2296).

Exon numbering: The exon numbering used for the *BMPR2*, *ACVRL1* and *ENG* genes in this P093-C2 HHT/PHAH product description and in the P093-C2 HTT/HPAH Coffalyser.Net analysis sheet is the exon numbering from the RefSeq transcripts NM_001204.6, NM_000020.2 and NM_000118.3, respectively. These sequences are identical to the LRG sequences.

P093-C2 probemix content: The P093-C2 HHT/HPAH probemix contains 51 probes with amplification products between 130 and 490 nt: 14 probes for the *BMPR2* gene (one probe for each exon and one additional probe for exon 1), 11 probes for the *ACVRL1* gene (one probe for each exon and one additional probe for exon 1) and 18 probes for the *ENG* gene (one probe for each exon, and one additional probe for exons 1 and 2, and two additional probes for exon 14b) and 8 reference probes. 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 105 nt: four DNA Quantity Fragments (Q-fragments), three DNA Denaturation Fragments (D-fragments), and one chromosome X and one chromosome Y-specific fragment (Table 1). The Q-fragments are only visible when less than 100 ng sample DNA is used. Low signal of the 88 or 96 nt fragment indicates incomplete

DNA denaturation. More information on how to interpret observations on these control fragments can be found in the MLPA General Protocol.

MLPA technique: The principles of the MLPA technique (Schouten et al. 2002) are described in the MLPA General Protocol (www.mlpa.com).

MLPA technique validation: Internal validation of the MLPA technique using 16 DNA samples from healthy individuals is required, in particular when using MLPA for the first time, or when changing the sample handling procedure, DNA extraction method or instruments used. This validation experiment should result in a standard deviation <0.10 for all probes over the experiment.

Required specimens: Purified DNA from peripheral blood, free from impurities known to affect MLPA reactions. For more information please refer to the section on DNA sample treatment found in the MLPA General Protocol.

Reference samples: Reference DNA samples should be derived from the same tissue type, handled using the same procedure, and prepared using the same DNA extraction method as the patient samples. Reference samples should be derived from unrelated individuals who are from families without a history of HHT or HPAH. 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 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. Sample ID numbers NA11213 (deletion BMPR2), NA01229 (duplication BMPR2) and NA10183 (duplication ENG) from the Coriell Institute have been tested at MRC-Holland and can be used as a positive control sample. The quality of cell lines can change, therefore samples should be validated before use.

Performance characteristics: The majority of HHT patients have pathogenic variants that are easily detected by sequence analysis. It is estimated that ~5% of all HHT patients have large rearrangements in the *ACVRL1* or *ENG* gene, either involving part of the gene or the whole gene. When MLPA is used in addition to sequence analysis of the *ACVRL1* and *ENG* genes, the detection rate generally increases by ~10%.

The majority of HPAH patients have pathogenic variants that are easily detected by sequence analysis. It is estimated that approximately ~12% of all HHT patients have large rearrangements in the *BMPR2* gene, either involving part of the gene or the whole gene. When MLPA is used in addition to sequence analysis of the *BMPR2* gene, the detection rate generally increases by ~20%.

The analytical sensitivity and specificity (based on a 2005-2016 literature review) for the detection of deletions in *BMPR2, ACVRL1* and *ENG* is very high and can be considered >99%.
Analytical performance can be compromised by: SNPs or other polymorphisms (e.g. indels) in the DNA target sequence, impurities in the DNA sample, incomplete DNA denaturation, the use of insufficient or too much sample DNA, the use of insufficient or unsuitable reference samples, problems with capillary electrophoresis or a poor data normalisation procedure and other technical errors. The MLPA General Protocol contains technical guidelines and information on data evaluation/normalisation.

Data analysis: Coffalyser.Net software must be used for data analysis in combination with the appropriate lot-specific MLPA Coffalyser sheet. For both, the latest version should be used which are 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, see the Coffalyser.Net Manual.

Interpretation of results: The expected results for the *BMPR2*, *ACVRL1* and *ENG* probes detecting autosomal sequences are allele copy numbers of 2 (normal), 1 (heterozygous deletion), or 3 (heterozygous duplication).

The standard deviation of all probes in the reference samples should be <0.10 and the dosage quotient (DQ) of the reference probes in the patient samples should be between 0.80 and 1.20. When these criteria are fulfilled, the following cut-off values for the DQ of the probes can be used to interpret MLPA results:

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

- Arranging probes according to chromosomal location facilitates interpretation of the results and may reveal more subtle changes such as those observed in mosaic cases. Analysis of parental samples may be necessary for correct interpretation of complex results.
- False positive results: Please note that abnormalities detected by a single probe (or multiple consecutive probes) still have a considerable chance of being a false positive result. Incomplete DNA denaturation (e.g. due to salt contamination) can lead to a decreased probe signal, in particular for probes located within a CpG island in or near the *BMPR2* gene. The use of an additional purification step or an alternative DNA extraction method may resolve such cases.
- False positive duplication results: Contamination of DNA samples with cDNA or PCR amplicons of individual exons can lead to false positive duplication results (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 in some cases 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.

Limitations of the procedure:

- In most populations, the major cause of genetic defects in the *BMPR2, ACVRL1 and ENG* genes are small (point) mutations, most of which will not be detected by using SALSA® MLPA® probemix P093 HHT/HPAH.
- 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.

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 one or 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 database: BMPR2 mutation database https://databases.lovd.nl/shared/genes/BMPR2; ACVRL1 mutation database https://databases.lovd.nl/shared/genes/ACVRL1 and ENG mutation database https://databases.lovd.nl/shared/genes/ENG.
We strongly encourage users to deposit positive results in these respective databases. Recommendations for the nomenclature to describe deletions/duplications of one or more exons can be found on www.hgvs.org/mutnomen/. Please report copy number changes detected by the reference probes, false positive results due to SNPs and unusual results (e.g., a duplication of *BMPR2* exons 6 and 8 but not exon 7) to MRC-Holland: info@mlpa.com.

Table 1. SALSA® MLPA® probemix P093-C2 HHT/HPAH

Length (nt)	SALSA MLPA probe	Chromosomal position[a]			
		reference	BMPR2	ENG	ACVRL1
64-70-76-82	Q-fragments: DNA quantity; only visible with less than 100 ng sample DNA				
88-92-96	D-fragments: Low signal of 88 or 96 nt fragment indicates incomplete denaturation				
100	X-fragment: Specific for the X chromosome				
105	Y-fragment: Specific for the Y chromosome				
130	Reference probe 00797-L00463	5q31			
137	**BMPR2 probe** 04004-L03427		**Exon 1**		
142	**ENG probe** 05235-L04615			**Exon 5**	
148	**BMPR2 probe** 04010-L03433		**Exon 7**		
154	**BMPR2 probe** 08198-L11404		**Exon 1**		
160	**ACVRL1 probe** 03790-L03965				**Exon 10**
166	**ENG probe** 03005-L02445			**Exon 1**	
172 «	**BMPR2 probe** 04011-L03434		**Exon 8**		
178	**ACVRL1 probe** 05229-L04609				**Exon 1**
184	**ENG probe** 03006-L02446			**Exon 2**	
190	**BMPR2 probe** 04005-L03428		**Exon 2**		
197	Reference probe 10688-L11270	6p12			
203	**ENG probe** 03007-L03614			**Exon 3**	
209	**ACVRL1 probe** 05230-L05738				**Exon 3**
214	**ACVRL1 probe** 03789-L05739				**Exon 1**
220 «	**BMPR2 probe** 04012-L03435		**Exon 9**		
226	**ACVRL1 probe** 05231-L04611				**Exon 5**
232	**ENG probe** 03008-L05740			**Exon 4**	
238	Reference probe 08858-L08980	3q22			
244	**ACVRL1 probe** 03016-L20477				**Exon 2**
250	Reference probe 02869-L20478	1p21			
256	**ENG probe** 03009-L02449			**Exon 6**	
265	**ACVRL1 probe** 03017-L02457				**Exon 4**
270 ±	**BMPR2 probe** 04338-L03966		**Exon 3**		
276	**ACVRL1 probe** 08196-L11406				**Exon 7**
283	**ENG probe** 07680-L07402			**Exon 8**	
290	Reference probe 15677-L17643	9p21			
297	**ACVRL1 probe** 03018-L20479				**Exon 6**
304	**BMPR2 probe** 04013-L20480		**Exon 10**		
312	**ENG probe** 03011-L20481			**Exon 9**	
319	**ACVRL1 probe** 03019-L02459				**Exon 8**
328	**BMPR2 probe** 04007-L03430		**Exon 4**		
337	**ENG probe** 03583-L03013			**Exon 11**	
346 ¥	**ACVRL1 probe** 03020-L28916				**Exon 9**
355	**BMPR2 probe** 04014-L03437		**Exon 11**		
364	**ENG probe** 03013-L02453			**Exon 13**	
376 ¥	**ENG probe** 05237-L28917			**Exon 10**	
385 ¥	Reference probe 16932-L28918	4q12			
393 ¥	**ENG probe** 05236-L28919			**Exon 7**	
400 ¥	**ENG probe** 03788-L28920			**Exon 14b**	
409 ¥	**BMPR2 probe** 04008-L28921		**Exon 5**		
417 ¥	**BMPR2 probe** 04015-L28922		**Exon 12**		
426 ¥	Reference probe 16449-L28923	18q21			
433 ¥	**BMPR2 probe** 04009-L28924		**Exon 6**		
441 ¥	**BMPR2 probe** 04016-L28925		**Exon 13**		
449 ¥	**ENG probe** 08501-L28926			**Exon 14b**	
457 ¥	**ENG probe** 05234-L28927			**Exon 2**	
465 ¥	**ENG probe** 05233-L28928			**Exon 1**	
472 ¥	**ENG probe** 08502-L28929			**Exon 14b**	
481 ¥	**ENG probe** 08500-L28930			**Exon 12**	
490 ¥	Reference probe 09966-L28931	17q12			

(a) The exon numbering used for the *BMPR2*, *ACVRL1* and *ENG* genes in this P093-C2 HHT/PHAH product description and in the P093-C2 HTT/HPAH Coffalyser.Net analysis sheet is the exon numbering from the RefSeq transcripts NM_001204.6, NM_000020.2 and NM_000118.3, respectively. These sequences are identical to the LRG sequences.

¥ Changed in version C2 (from lot C2-1015 onwards). Change in length, no change in sequence detected.
« Probe located within, or near a CpG island. A low signal can be caused by salt contamination in the DNA sample leading to incomplete DNA denaturation, especially of CG rich regions.
± SNP rs863223425 could influence the probe signal. In case of apparent deletions, it is recommended to sequence the region targeted by this probe.

Table 2. P093 probes arranged according to chromosomal location

Table 2a. BMPR2 gene

Length (nt)	SALSA MLPA probe	BMPR2 exon[(a)]	Ligation site[(b)] NM_001204.6	Partial sequence[(e)] (24 nt adjacent to ligation site)	Distance to next probe
		start codon	*1149-1151 (ex 1)*		
154	08198-L11404	Exon 1	573-574	TTGATCCAGTCA-AGGAAGAGGATT	0.5 kb
137	04004-L03427	Exon 1	1119-1120	ATTTCTTTTCTT-TGCCCTCCTGAT	87.4 kb
190	04005-L03428	Exon 2	1256-1257	CTATGTGCGTTT-AAAGATCCGTAT	2.7 kb
270 ±	04338-L03966	Exon 3	1443-1444	ACTATGAAGAAT-GTGTAGTAACTA	46.2 kb
328	04007-L03430	Exon 4	1600-1601	AGATGAGACAAT-AATCATTGCTTT	1.2 kb
409	04008-L28921	Exon 5	1706-1707	CTTCACAGTATG-AACATGATGGAG	4.1 kb
433	04009-L28924	Exon 6	1930-1931	TGCCCGCTTTAT-AGTTGGAGATGA	1.2 kb
148	04010-L03433	Exon 7	2069-2070	CTTGCTCATTCT-GTTACTAGAGGA	10.7 kb
172 «	04011-L03434	Exon 8	2225-2226	ATGAGGCTGACT-GGAAATAGACTG	1.7 kb
220 «	04012-L03435	Exon 9	2308-2309	GGCACCAGAAGT-GCTAGAAGGAGC	9.8 kb
304	04013-L20480	Exon 10	2496-2497	TTGAGGATATGC-AGGTTCTCGTGT	10.4 kb
355	04014-L03437	Exon 11	2646-2647	GTGCTGAGGAAA-GGATGGCTGAAC	2.7 kb
417	04015-L28922	Exon 12	2938-2939	AGCACAAGCTCG-AATCCCCAGCCC	4.4 kb
441	04016-L28925	Exon 13	4154-4155	GAAGTCAACAAT-AATGGCAGTAAC	
		stop codon	*4263-4265 (ex 13)*		

± SNP rs863223425 could influence the probe signal. In case of apparent deletions, it is recommended to sequence the region targeted by this probe.
« Probe located within, or near a CpG island. A low signal can be caused by salt contamination in the DNA sample leading to incomplete DNA denaturation, especially of CG rich regions.

Table 2b. ACVRL1 gene

Length (nt)	SALSA MLPA probe	ACVRL1 exon[(a)]	Ligation site[(c)] NM_000020.2	Partial sequence[(e)] (24 nt adjacent to ligation site)	Distance to next probe
		start codon	*284-286 (ex 2)*		
214	03789-L05739	Exon 1	14-15	GAAACGGTTTAT-TAGGAGGGAGTG	>0.1 kb
178	05229-L04609	Exon 1	64-63 reverse	AGCAAAAATGTT-TCTTATTCCAGC	5.0 kb
244	03016-L20477	Exon 2	315-316	GAAAGGCCTTCT-GATGCTGCTGAT	0.8 kb
209	05230-L05738	Exon 3	557-558	ACTGCTGCGACA-GCCACCTCTGCA	0.4 kb
265	03017-L02457	Exon 4	759-760	CGAGCTGGGAGA-GTCCAGTCTCAT	0.3 kb
226	05231-L04611	Exon 5	897-898	GGTTGCCTTGGT-GGAGTGTGTGGG	0.4 kb
297	03018-L20479	Exon 6	960-961	CGGTGAGAGTGT-GGCCGTCAAGAT	0.7 kb
276	08196-L11406	Exon 7	1061-1062	ACACAGGCTTCA-TCGCCTCAGACA	0.8 kb
319	03019-L02459	Exon 8	1370-1371	GCGATTACCTGG-ACATCGGCAACA	2.9 kb
346	03020-L28916	Exon 9	1556-1557	ATAGACCACCCT-TCTATGATGTGG	1.9 kb
160	03790-L03965	Exon 10	1792-1793	AAAGTGATTCAA-TAGCCCAGGAGC	
		stop codon	*1793-1795 (ex 10)*		

Table 2c. ENG gene

Length (nt)	SALSA MLPA probe	ENG exon[a]	Ligation site[d] NM_000118.3	Partial sequence[e] (24 nt adjacent to ligation site)	Distance to next probe
		start codon	*419-421 (ex 1)*		
465	05233-L28928	Exon 1	311 nt before ex 1 reverse	GAGATCACTGGT-TCAAGAAGATGG	0.8 kb
166	03005-L02445	Exon 1	459-460	TGCCCTGCTGCT-GGCCAGCTGCAG	11.1 kb
457	05234-L28927	Exon 2	566-565 reverse	GCAGCCCTTCGA-GACCTGGCTAGT	0.0 kb
184	03006-L02446	Exon 2	595-596	CAGGCCCCCAAT-GCCATCCTTGAA	13.4 kb
203	03007-L03614	Exon 3	708-709	AGAGGTGCTTCT-GGTCCTCAGTGT	3.2 kb
232	03008-L05740	Exon 4	903-904	TGCTGCTGAGCT-GAATGACCCCCA	0.7 kb
142	05235-L04615	Exon 5	965-966	TGTCCTTCTGCA-TGCTGGAAGCCA	0.6 kb
256	03009-L02449	Exon 6	1176-1177	CGTCCTCATCCT-GCAGGGTCCCCC	0.4 kb
393	05236-L28919	Exon 7	1355-1356	GCATTGTGGCAT-CCTTCGTGGAGC	0.4 kb
283	07680-L07402	Exon 8	1414-1415	CCCACAGGTGGT-AGGCTGCAGACC	4.5 kb
312	03011-L20481	Exon 9 (9a)	1652-1653	CTTACTCCAGCT-GTGGCATGCAGG	0.3 kb
376	05237-L28917	Exon 10 (9b)	1717-1718	CTGTCGAGCTCA-TCACCACAGCGG	0.8 kb
337	03583-L03013	Exon 11 (10)	1770-1771	CCTCTCTTTCCA-GCTGGGCCTCTA	0.6 kb
481	08500-L28930	Exon 12 (11)	2028-2029	CCTCCACTTCTA-CACAGTACCCAT	1.0 kb
364	03013-L02453	Exon 13 (12)	2119-2120	GTCCATAGGACT-GTCTTCATGCGC	1.2 kb
449	08501-L28926	Exon 14b (13)	2258-2259	TCTGGTACATCT-ACTCGCACACGC	0.2 kb
472	08502-L28929	Exon 14b (13)	2476-2477	CAGCAGCACCAA-CCACAGCATCGG	0.4 kb
400	03788-L28920	Exon 14b (13)	2877-2878	GGAGAACTTGAA-ACAGATTCAGGC	
		stop codon	*2294-2296 (ex 14b)*		

(a) The exon numbering used for the *BMPR2*, *ACVRL1* and *ENG* genes in this P093-C2 HHT/PHAH product description and in the P093-C2 HTT/HPAH Coffalyser.Net analysis sheet is the exon numbering from the RefSeq transcripts NM_001204.6, NM_000020.2 and NM_000118.3, respectively. These sequences are identical to the LRG sequences.

(b) Ligation sites for the *BMPR2* gene of the P093 HHT/HPAH MLPA probes are indicated according to Refseq sequence NM_001204.6 containing 13 exons.

(c) Ligation sites for the *ACVRL1* gene of the P093 HHT/HPAH MLPA probes are indicated according to Refseq sequence NM_000020.2 containing 10 exons.

(d) Ligation sites for the *ENG* gene of the P093 HHT/HPAH MLPA probes are indicated according to Refseq sequence NM_000118.3 containing 14 exons.

(e) Only partial probe sequences are shown. Complete probe sequences are available at www.mlpa.com. Please notify us of any mistakes: info@mlpa.com.

Note: Exon numbering used here may differ from literature! We have used the ENG exon numbering that is present in the NCBI NM_ reference sequences. Between brackets is the exon numbering that is often used in the literature. Complete probe sequences are available on request: info@mlpa.com. Please notify us of any mistakes: info@mlpa.com.

Related SALSA® MLPA® probemixes

- P158 Juvenile Polyposis Syndrome: Contains probes for the SMAD4, BMPR1A and PTEN genes.

References

- Schouten JP et al. (2002). Relative quantification of 40 nucleic acid sequences by multiplex ligation-dependent probe amplification. *Nucleic Acids Res.* 30:e57.
- Handa T et al. (2014). BMPR2 gene mutation in pulmonary arteriovenous malformation and pulmonary hypertension: A case report. Respiratory investigation. 52:195-198.
- 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.
- Schwartz M et al. (2007). Deletion of exon 16 of the dystrophin gene is not associated with disease. *Hum Mutat.* **28**:205.

Selected publications using SALSA® MLPA® Probemix P093 HHT/HPAH

- Aimi Y et al. (2013). A novel break point of the BMPR2 gene exonic deletion in a patient with pulmonary arterial hypertension. *J Hum Genet*. 58:815-818.
- Aldred MA et al. (2010). Somatic chromosome abnormalities in the lungs of patients with pulmonary arterial hypertension. *American journal of respiratory and critical care medicine*. 182:1153-1160.
- Aldred MA et al. (2006). BMPR2 gene rearrangements account for a significant proportion of mutations in familial and idiopathic pulmonary arterial hypertension. *Hum Mutat*. 27:212-213.
- Asosingh K et al. (2008). Circulating angiogenic precursors in idiopathic pulmonary arterial hypertension. *The American Journal of Pathology*. 172:615-627.
- Boeri L et al. (2013). Hereditary Hemorrhagic Telangiectasia: Breakpoint Characterization of a Novel Large Deletion in ACVRL1 Suggests the Causing Mechanism. *Mol Syndromol*. 4:119-124.
- Canzonieri C et al. (2013). Endoscopic evaluation of gastrointestinal tract in patients with hereditary hemorrhagic telangiectasia and correlation with their genotypes. *Genet Med*. 16:3-10.
- Chen YJ et al. (2013). Clinical and genetic characteristics of Chinese patients with hereditary haemorrhagic telangiectasia–associated pulmonary hypertension. *Eur J Clin Invest*. 43:1016-1024.
- Chida A et al. (2012). Missense mutations of the BMPR1B (ALK6) gene in childhood idiopathic pulmonary arterial hypertension. *Circ J*. 76:1501-1508.
- Chida A et al. (2012). Outcomes of childhood pulmonary arterial hypertension in BMPR2 and ALK1 mutation carriers. *The American journal of cardiology*. 110:586-593.
- Cogan JD et al. (2006). High frequency of BMPR2 exonic deletions/duplications in familial pulmonary arterial hypertension. *Am J Respir Crit Care Med*. 174:590-598.
- De Gottardi A et al. (2012). Bone morphogenetic protein receptor 2 in patients with idiopathic portal hypertension. *J Cell Mol Med*. 16:2017-2021.
- Federici C et al. (2015). Increased mutagen sensitivity and DNA damage in pulmonary arterial hypertension. *Am J Respir Crit Care Med*. 192:219-228.
- Fontalba A et al. (2008). Mutation study of Spanish patients with hereditary hemorrhagic telangiectasia. *BMC Med Genet*. 9:1.
- Girerd B et al. (2010). Clinical outcomes of pulmonary arterial hypertension in patients carrying an ACVRL1 (ALK1) mutation. *Am J Respir Crit Care Med*. 181:851-861.
- Girerd B et al. (2015). Genetic counselling in a national referral centre for pulmonary hypertension. Eur *Respir J*. ERJ-00717-02015.
- Hinderhofer K et al. (2014). Identification of a new intronic BMPR2-mutation and early diagnosis of heritable pulmonary arterial hypertension in a large family with mean clinical follow-up of 12 years. *PLoS One*. 9:e91374.
- Kataoka M et al. (2013). Alu-mediated nonallelic homologous and nonhomologous recombination in the BMPR2 gene in heritable pulmonary arterial hypertension. *Genet Med*. 15:941-947.
- Kerstjens M, Dixons J, Westermann C & van Amstel JP Multiplex Ligation-dependent Probe Amplification analysis identifies ENG and ACVRL1 deletions/duplicati ons in hereditary hemorrhagic telangiectasia. *Hereditary Hemorrhagic Telangiectasia Clinical and Molecular Genetics*. 45.
- Kjeldsen A, Tørring P, Nissen H & Andersen P (2014). Cerebral abscesses among Danish patients with hereditary haemorrhagic telangiectasia. *Acta Neurologica Scandinavica.* 129:192-197.
- Lee ST et al. (2009). Clinical features and mutations in the ENG, ACVRL1, and SMAD4 genes in Korean patients with hereditary hemorrhagic telangiectasia. *J Korean Med Sci*. 24:69-76.
- Liu D et al. (2012). Molecular genetics and clinical features of Chinese idiopathic and heritable pulmonary arterial hypertension patients. *Eur Respir J*. 39:597-603.
- Liu D et al. (2012). BMPR2 mutations influence phenotype more obviously in male patients with pulmonary arterial hypertension. *Circ Cardiovasc Genet*. 5:511-518.
- McDonald J et al. (2011). Molecular diagnosis in hereditary hemorrhagic telangiectasia: findings in a series tested simultaneously by sequencing and deletion/duplication analysis. *Clin Genet*. 79:335-344.
- Möller T et al. (2010). A novel BMPR2 gene mutation associated with exercise-induced pulmonary hypertension in septal defects. *Scand Cardiovasc J*. 44:331-336.
- Momose Y et al. (2015). De novo mutations in the BMPR2 gene in patients with heritable pulmonary arterial hypertension. *Ann Hum Genet*. 79:85-91.
- Montani D et al. (2011). Pulmonary hypertension in patients with neurofibromatosis type I. *Medicine*. 90:201-211.
- Pfarr N et al. (2011). Hemodynamic and clinical onset in patients with hereditary pulmonary arterial hypertension and BMPR2 mutations. *Respir Res*. 12:1.

- Rajkumar R et al. (2010). Genomewide RNA expression profiling in lung identifies distinct signatures in idiopathic pulmonary arterial hypertension and secondary pulmonary hypertension. *American Journal of Physiology-Heart and Circulatory Physiology*. 298:H1235-H1248.
- Roofthooft M et al. Pulmonary Arterial Hypertension associated with Isolated Atrial Septal Defect: the role of BMPR2-mutations and outcome after defect closure.
- Rosenzweig EB et al. (2008). Clinical implications of determining BMPR2 mutation status in a large cohort of children and adults with pulmonary arterial hypertension. *The Journal of Heart and Lung Transplantation.* 27:668-674.
- Shoukier M, Teske U, Weise A, Engel W & Argyriou L (2008). Characterization of five novel large deletions causing hereditary haemorrhagic telangiectasia. *Clin Genet*. 73:320-330.
- Staropoli JF, Xin W & Sims KB (2010). Co-segregation of Norrie disease and idiopathic pulmonary hypertension in a family with a microdeletion of the NDP region at Xp11.3-p11.4. *J Med Genet*. 47:786-790.
- Sztrymf B et al. (2008). Clinical outcomes of pulmonary arterial hypertension in carriers of BMPR2 mutation. *Am J Respir Crit Care Med*. 177:1377-1383.
- Tørring PM, Brusgaard K, Ousager LB, Andersen PE & Kjeldsen AD (2014). National mutation study among Danish patients with hereditary haemorrhagic telangiectasia. *Clin Genet*. 86:123-133.
- Tørring PM et al. (2016). Germline mutations in BMP9 are not identified in a series of Danish and French patients with hereditary hemorrhagic telangiectasia. *Gene Reports*. 5:30-33.
- Tørring PM, Kjeldsen AD, Ousager LB, Brasch-Andersen C & Brusgaard K (2012). Allelic dropout in the ENG gene, affecting the results of genetic testing in hereditary hemorrhagic telangiectasia. *Genetic testing and molecular biomarkers*. 16:1419-1423.
- Van Der Bruggen CE et al. (2016). Bone Morphogenetic Protein Receptor Type 2 Mutation in Pulmonary Arterial Hypertension: A View on the Right Ventricle. *Circulation*. 115.020696.
- van Hattem WA et al. (2008). Large genomic deletions of SMAD4, BMPR1A and PTEN in juvenile polyposis. *Gut*. 57:623-627.
- Viales RR et al. (2015). Mutation in BMPR2 promoter: a 'second hit' for manifestation of pulmonary arterial hypertension? *PLoS One*. 10:e0133042.
- Wooderchak W et al. (2010). Hereditary hemorrhagic telangiectasia: two distinct ENG deletions in one family. *Clin Genet*. 78:484-489.
- Wooderchak-Donahue W et al. (2011). Verification of multiplex ligation-dependent probe amplification probes in the absence of positive samples. *Genetic testing and molecular biomarkers*. 15:793-799.
- Xi Q, Liu Z, Zhao Z, Luo Q & Huang Z (2016). High Frequency of Pulmonary Hypertension-Causing Gene Mutation in Chinese Patients with Chronic Thromboembolic Pulmonary Hypertension. *PLoS One*. 11:e0147396.

P093 Product history	
Version	*Modification*
C2	Length of several probes has been adjusted.
C1	Five reference probes have been replaced and two new reference probes have been added. The length of 9 probes have been changed without change in the sequence. Finally, new control fragments at 88, 96, 100 and 105 nt have been added.
B2	Two extra control fragments at 100 and 105 nt have been added. One probe has a small change in length (277 nt).
B1	Four target probes have been replaced (BMPR2: 1, ACVRL1: 1 and ENG: 2), 1 reference probe has been replaced, 1 reference probe has been added, 3 ENG target probes have been added and 1 ACVRL1 exon 10 probe has been removed.
(A)	First release

Implemented changes in the product description
Version C2-01 - 19 December 2017 (03) - Product name changed from P093 HHT/PPH1 to P093 HHT/HPAH - Product description restructured and adapted to a new template. *Version 21 – 15 January 2015 (55)* - Product description adapted to a new product version (version number changed, lot number added, small changes in Table 1 and Table 2, new picture included). - Exon numbering and ligation sites of the ENG gene have been changed in Table 1 and Table 2b according to NM_000118.3. - Minor textual changes. *Version 20 – 22 July 2015 (54)* - Figure based on the use of old MLPA buffer (replaced in December 2012) removed. - Various minor textual changes throughout the document. *Version 19 (48)* - Electropherogram pictures using the new MLPA buffer (introduced in December 2012) added. *Version 18 (48)* - Various minor textual changes. *Version 17 (46)* - Product description adapted to a new product version (version number changed, lot number added, small changes in Table 1 and Table 2, new picture included). - Various minor textual changes on p.1. - Warning added below table 1 for salt sensitive probes. - Warning added below table 1 for probes 184 nt (03006-L02446) and 250 nt (05234-L11407) regarding SNPs. - Various minor layout changes. *Version 16 (46)* - Ligation sites of the probes targeting the BMPR2 gene updated according to new version of the NM_reference sequence. - Old exon numbering of the ENF gene added between brackets in Table 2. Various minor textual changes on page 1.

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***EUROPE:** CE IVD

OUTSIDE EUROPE: RUO

*comprising EU member states, EU member state candidates and members of the European Free Trade Association (EFTA).
The product is for RUO in all other countries within Europe