Product Description
SALSA® MLPA® Probemix P120-B2 PANK2/PLA2G6

To be used with the MLPA General Protocol.

Version B2
For complete product history see page 7.

Catalogue numbers:
- **P120-025R:** SALSA MLPA Probemix P120 PANK2/PLA2G6, 25 reactions.
- **P120-050R:** SALSA MLPA Probemix P120 PANK2/PLA2G6, 50 reactions.
- **P120-100R:** SALSA MLPA Probemix P120 PANK2/PLA2G6, 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 P120 PANK2/PLA2G6 is a **research use only (RUO)** assay for the detection of deletions or duplications in the *PANK2* and *PLA2G6* genes, which are associated with neurodegeneration with brain iron accumulation (NBIA).

NBIA is a rare autosomal recessive condition characterised by abnormal iron deposition in the basal ganglia and neuroaxonal dystrophy causing extrapyramidal features, dementia, and ocular abnormalities. The *PANK2* gene encodes the pantothenate kinase enzyme, which is expressed at high levels in the brain. Pantothenate kinase is an essential regulatory enzyme in the biosynthesis of coenzyme A, which is important in intermediary and fatty acid metabolism. Mutations in *PANK2* are associated with NBIA type 1, Harp syndrome, and pantothenate kinase-associated neurodegeneration (PKAN), formerly Hallervorden-Spatz syndrome. Mutations in *PLA2G6*, which encodes phospholipase A2, can cause NBIA type 2 that is characterised by abnormal iron accumulation.

More information is available at https://www.ncbi.nlm.nih.gov/books/NBK121988/.

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/

Exon numbering
The *PANK2* and *PLA2G6* exon numbering used in this P120-B2 PANK2/PLA2G6 product description is the exon numbering from the LRG_1016 and LRG_1015 sequences. The exon numbering of the NM_ sequence that was used for determining a probe's ligation site does not always correspond to the exon numbering obtained from the LRG sequences. 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.

Probemix content
The SALSA MLPA Probemix P120-B2 PANK2/PLA2G6 contains 41 MLPA probes with amplification products between 130 and 436 nucleotides (nt). This includes 12 probes for *PANK2* and 18 probes for *PLA2G6*. In addition, 11 reference probes are included that detect autosomal chromosomal locations. Complete probe sequences and the identity of the genes detected by the reference probes are available online (www.mrcholland.com).

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

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 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
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 unrelated individuals who are from families without a history of neurodegeneration with brain iron accumulation. 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. The quality of cell lines can change; therefore samples should be validated before use.

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 and the final ratio (FR) of each individual reference probe in the patient samples should be between 0.80 and 1.20. When these criteria are fulfilled, the following cut-off values for the FR of the probes can be used to interpret MLPA results for autosomal chromosomes or pseudo-autosomal regions:

Copy number status	Final ratio (FR)
Normal	0.80 < FR < 1.20
Homozygous deletion	FR = 0
Heterozygous deletion	0.40 < FR < 0.65
Heterozygous duplication	1.30 < FR < 1.65
Heterozygous triplication/homozygous duplication	1.75 < FR < 2.15
Ambiguous copy number	All other values

Note: The term "dosage quotient", used in older product description versions, has been replaced by "final ratio" to become consistent with the terminology of the Coffalyser.Net software. (Calculations, cut-offs and interpretation remain unchanged.) Please note that the Coffalyser.Net software also shows arbitrary borders as part of the statistical analysis of results obtained in an experiment. As such, arbitrary borders are different from the final ratio cut-off values shown here above.

- 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. Sequence changes (e.g. SNVs, point mutations) in the target sequence detected by a probe can be one cause. Incomplete DNA denaturation (e.g. due to salt contamination) can also lead to a decreased probe signal, in particular for probes located in or near a GC-rich region. The use of an additional purification step or an alternative DNA extraction method may resolve such cases. Additionally, contamination of DNA samples with cDNA or PCR amplicons of individual exons can lead to an increased probe signal (Varga et al. 2012). Analysis of an independently collected secondary DNA sample can exclude these kinds of contamination artefacts.
- Normal copy number variation in healthy individuals is described in the database of genomic variants: http://dgv.tcag.ca/dgv/app/home. Users should always consult the latest update of the database and scientific literature when interpreting their findings.
- Not all abnormalities detected by MLPA are pathogenic. In some genes, intragenic deletions are known that result in very mild or no disease (as described for *DMD* by Schwartz et al. 2007). For many genes, more than one transcript variant exists. Copy number changes of exons that are not present in all transcript variants may not have clinical significance. Duplications that include the first or last exon of a gene (e.g. exons 1-3) might not result in inactivation of that gene copy.
- Copy number changes detected by reference probes 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.

Limitations of the procedure

- In most populations, the major cause of genetic defects in *PANK2* and *PLA2G6* genes are small (point) mutations, none of which will be detected by using SALSA MLPA Probemix P120 PANK2/PLA2G6.
- 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.

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.

***PANK2* and *PLA2G6* mutation database**

https://databases.lovd.nl/shared/genes/PANK2 and https://databases.lovd.nl/shared/genes/PLA2G6. We strongly encourage users to deposit positive results in the Leiden Open Variation Database (LOVD). 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 SNVs and unusual results (e.g., a duplication of *PANK2* exons 5 and 7 but not exon 6) to MRC Holland: info@mrcholland.com.

Table 1. SALSA MLPA Probemix P120-B2 PANK2/PLA2G6

Length (nt)	SALSA MLPA probe	Chromosomal position (hg18)[a]		
		Reference	*PANK2*	*PLA2G6*
64-105	Control fragments – see table in probemix content section for more information			
130	Reference probe 00797-L19287	5q		
136	**PANK2 probe** 16540-L19030		**Exon 1**	
142	**PLA2G6 probe** 09575-L19386			**Exon 10**
148	**PANK2 probe** 12570-L13620		**Exon 1**	
154	**PANK2 probe** 09562-L19080		**Exon 5**	
160	**PLA2G6 probe** 09569-L19081			**Exon 5**
166	**PLA2G6 probe** 12571-L13621			**Exon 17**
171	Reference probe 07294-L17071	6q		
176	**PLA2G6 probe** 12644-L13936			**Exon 7**
184	Reference probe 09724-L10074	12q		
190	**PANK2 probe** 09561-L19082		**Exon 4**	
196	**PLA2G6 probe** 09580-L19083			**Exon 14**
203	Reference probe 07820-L19291	1q		
208	**PLA2G6 probe** 12572-L13622			**Exon 3**
214	**PLA2G6 probe** 09582-L14062			**Exon 16**
220	**PLA2G6 probe** 09570-L10024			**Exon 5**
226	Reference probe 14471-L16191	4q		
232	**PLA2G6 probe** 09573-L19084			**Exon 8**
241	Reference probe 15025-L19290	11p		
249	**PLA2G6 probe** 09577-L10031			**Exon 12**
259	**PANK2 probe** 20618-L28849		**Exon 3**	
265	Reference probe 14385-L11820	13q		
274	**PLA2G6 probe** 09568-L18996			**Exon 4**
283	**PLA2G6 probe** 09574-L18997			**Exon 9**
292	**PANK2 probe** 10183-L10633		**Exon 2**	
303	**PANK2 probe** 09564-L10018		**Exon 7**	
310	**PLA2G6 probe** 20619-L18798			**Exon 15**
319	**PLA2G6 probe** 20620-L18797			**Exon 1**
328	**PLA2G6 probe** 09571-L10025			**Exon 6**
337	Reference probe 09073-L09242	19p		
346	**PANK2 probe** 12573-L18799		**Exon 6**	
355	**PANK2 probe** 12574-L13624		**Exon 4**	
364	Reference probe 04736-L04153	7q		
373	**PANK2 probe** 16541-L19385		**Exon 1**	
382	Reference probe 13329-L14755	18q		
391	**PLA2G6 probe** 09576-L10030			**Exon 11**
400	**PANK2 probe** 09557-L19288		**Exon 1**	
409	**PLA2G6 probe** 09579-L10034			**Exon 13**
418	**PLA2G6 probe** 09566-L10020			**Exon 2**
427	**PANK2 probe** 12576-L13626		**Exon 2**	
436	Reference probe 10103-L10527	8q		

[a] See section Exon numbering on page 1 for more information.

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

Table 2. P120-B2 probes arranged according to chromosomal location

Table 2a. *PANK2*

Length (nt)	SALSA MLPA probe	*PANK2* exon[a]	Ligation site NM_153638.4	Partial sequence[b] (24 nt adjacent to ligation site)	Distance to next probe
		start codon	*179-181 (Exon 1)*		
136	16540-L19030	Exon 1	85 nt before exon 1	GCTTAGCCCAAA-CATGCTGGGGGA	0.1 kb
373	16541-L19385	Exon 1	14 nt before exon 1	AGGCTAAGGTCA-GCCGCGGTTCAA	0.4 kb
400	09557-L19288	Exon 1	329-330	GCCTCTCATTGG-ACGGAGGCACGG	0.4 kb
148	12570-L13620	Exon 1	749-750	TGGGCTCTTACA-GCGGCCCCACCT	18.4 kb
427	12576-L13626	Exon 2	961-962	TATGGGTCTACA-GGCATTCGGGAC	0.1 kb
292	10183-L10633	Exon 2	1029-1030	CAATCTGCACTT-TATACGCTTTCC	2.5 kb
259	20618-L28849	Exon 3	1270-1271	TATTACTTTGAA-AACCCTGCTGAT	1.8 kb
355	12574-L13624	Exon 4	1463-1464	TTACTGGCTGTA-CCACTTTTGAAG	0.1 kb
190	09561-L19082	Exon 4	1564-1565	GAGAGGTTTGGA-CTGCCAGGCTGG	4.4 kb
154	09562-L19080	Exon 5	1694-1695	GCTCAATAGCAA-GAATGTGTGCCC	1.7 kb
346	12573-L18799	Exon 6	1732-1733	AACCAGGTGGTA-TTTGTTGGAAAT	4.9 kb
303	09564-L10018	Exon 7	2228-2229	AGTTGACTGGTT-TTGTGTCCTGTT	
		stop codon	*1889-1891 (Exon 7)*		

Table 2b. *PLA2G6*

Length (nt)	SALSA MLPA probe	*PLA2G6* exon[a]	Ligation site NM_003560.4	Partial sequence[b] (24 nt adjacent to ligation site)	Distance to next probe
		start codon	*213-215 (Exon 2)*		
319	20620-L18797	Exon 1	162-163	GTGTCTCCGATT-CTGAGGCATGTC	12.4 kb
418	09566-L10020	Exon 2	346-347	AGGGCAGCTGAT-TCTGTTCCAGAA	23.8 kb
208	12572-L13622	Exon 3	558-559	ACCTCATCCGTA-ACCACCCCAGCT	2.4 kb
274	09568-L18996	Exon 4	785-786	ACCGTCTTCCAT-TATGCTGTCCAG	3.0 kb
160	09569-L19081	Exon 5	7 nt before exon 5	TCCCTTCCTCTT-TCATCAGCTCCT	0.1 kb
220	09570-L10024	Exon 5	941-942	CTGCTGTGCAAT-GCTCGGTGCAAC	5.0 kb
328	09571-L10025	Exon 6	1027-1028	GGAGATGATCAT-CAGCATGGACAG	2.0 kb
176	12644-L13936	Exon 7	75 nt before exon 7	CAGAGCAGAAGT-GGCAGTGCCCAC	3.6 kb
232	09573-L19084	Exon 8	1378-1379	TCCTACATTCCT-AGCCTCCAAAAT	1.2 kb
283	09574-L18997	Exon 9	1523-1524	TTCTCCCTGGAA-AGAGCTCAGCCC	1.9 kb
142	09575-L19386	Exon 10	4 nt after exon 10	AAGCGGACGTAA-GTGGATCGAGAT	3.2 kb
391	09576-L10030	Exon 11	1678-1679	AGGAGGAGTGAA-AGGCCTCATCAT	2.3 kb
249	09577-L10031	Exon 12	1807-1808	GTTCCTAGGTAA-GTCCATGGCCTA	4.8 kb
409	09579-L10034	Exon 13	2049-2050	GGGAGCCTCGTT-TCAACCAGAACG	0.6 kb
196	09580-L19083	Exon 14	2226-2227	TCCATGAGTACA-ATCAGGACCTGA	2.0 kb
310	20619-L18798	Exon 15	2333-2334	ACCTGTGTGGAT-GTCTTCCGTCCC	1.1 kb
214	09582-L14062	Exon 16	2484-2485	GCATCCAGTACT-TCAGGTGAGGGC	1.0 kb
166	12571-L13621	Exon 17	3251-3252	CTTCCCAATGGA-AGTGGCTTAAGA	
		stop codon	*2631-2633 (Exon 17)*		

[a] See section Exon numbering on page 1 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.

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

References

- 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 P120 PANK2/PLA2G6

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- Yamashita C et al. (2017). Mutation screening of PLA2G6 in Japanese patients with early onset dystonia-parkinsonism. *J Neural Transm*, 124(4), 431-435.
- Yang D et al. (2022). Genetic mutation spectrum of pantothenate kinase-associated neurodegeneration expanded by breakpoint sequencing in pantothenate kinase 2 gene. *Orphanet J Rare Dis*, 17(1), 1-6.

P120 product history	
Version	*Modification*
B2	The length of a number of probes has been adjusted.
B1	Two new probes for exon 1 of PANK2 have been included, one probe for exon 8 of PANK2 has been removed and eight reference probes have been replaced.
A1	First release.

Implemented changes in the product description
Version B2-02 – 22 November 2022 (04P) - Product description rewritten and adapted to a new template. - Ligation sites of the probes targeting the PANK2 and PLA2G6 genes updated according to new version of the NM_ reference sequence. - Incomplete DNA denaturation warnings in both Table 1 and 2 removed. Version B2-01 — 4 July 2019 (02P) - Product description rewritten and adapted to a new template.

- Ligation sites of the probes targeting the PANK2 gene updated according to new version of the NM_ reference sequence. Version 08 – 24 August 2015 (54) - 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). - Added references on page 2. - Exon numbering of PANK2 and PLA2G6 updated according to the NM_reference sequence.

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