Product Description
SALSA® MLPA® Probemix P154-C2 GPC3-GPC4

To be used with the MLPA General Protocol.

Version C2
For complete product history see page 7.

Catalogue numbers:
- **P154-025R:** SALSA MLPA Probemix P154 GPC3-GPC4, 25 reactions.
- **P154-050R:** SALSA MLPA Probemix P154 GPC3-GPC4, 50 reactions.
- **P154-100R:** SALSA MLPA Probemix P154 GPC3-GPC4, 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 P154 GPC3-GPC4 is a **research use only (RUO)** assay for the detection of deletions or duplications in the *GPC3* and *GPC4* genes, which are associated with Simpson-Golabi-Behmel syndrome type 1.

Simpson-Golabi-Behmel syndrome (SGBS) type 1 is an X-linked overgrowth syndrome associated with multiple congenital anomalies caused by a mutant X-linked recessive trait. Alternative names are: Bulldog syndrome; dysplasia gigantism syndrome X-linked (DGSX), Golabi-Rosen syndrome, Simpson dysmorphia syndrome (SDYS). SGBS shows a broad spectrum of clinical manifestations, varying from mild forms in carrier females to infantile lethal forms in affected males. The most consistent findings are pre- and postnatal macrosomia, characteristic coarse "bulldog-like" face and a complex assortment of congenital defects affecting the internal organs and skeleton. On some occasions, intellectual disability of variable degree is observed. SGBS is also associated with an increased risk of developing embryonal neoplasia, mostly Wilms and liver tumours. SGBS type 2 is a rarer, more lethal form with different genes involved.

SGBS type 1 is caused by mutations in the glypican 3 (*GPC3*) and/or glypican 4 (*GPC4*) genes, both located on chromosome Xq26. Around 43% of *GPC3* pathogenic variants is a deletion or duplication. Alternative names for the *GPC3* gene are *SGBS*, *SGBS1*, *SGB* and *DGSX*. The glypicans (GRIPS) are a family of cell surface heparan sulphate proteoglycans that are bound to the cell surface and they may play a role in the control of cell division and growth regulation. Glypicans are anchored to the peripheral membrane through glycosylphosphatidylinositol (GPI) linkage. Deletions in the *GPC3* gene have been found in a number of SGBS families supplying evidence that such mutations are responsible for SGBS. Duplication of the *GPC4* gene has been described in one SGBS family (Waterson et al. 2010). The tight clustering of *GPC3* and *GPC4* may be relevant for explaining the variability of the SGBS phenotype.

The *GPC3* gene (8 exons) spans ~450 kb of genomic DNA and is located on chromosome Xq26.2, 132 Mb from the p-telomere. The *GPC4* gene (9 exons) spans ~115 kb of genomic DNA and is located on chromosome Xq26.2, centromeric to *GPC3*.

More information is available at https://www.ncbi.nlm.nih.gov/books/NBK1219/.

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 *GPC3* exon numbering used in this P154-C2 GPC3-GPC4 product description is the exon numbering from the LRG_505 sequence. The *GPC4* exon numbering used in this P154-C2 GPC3-GPC4 product description is the exon numbering from the NG_012498.1 sequence. 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 or NG 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 P154-C2 GPC3-GPC4 contains 37 MLPA probes with amplification products between 136 and 454 nucleotides (nt). This includes 26 probes for the *GPC3* and *GPC4* genes: two probes for each exon of the *GPC3* gene (three probes for *GPC3* exon 1 and one for exon 5) and one probe for each exon of the *GPC4* gene (two probes for *GPC4* exon 1). In addition, 11 reference probes are included that detect locations on the X-chromosome. 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 of the same sex 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 Simpson-Golabi-Behmel syndrome. It is recommended to use samples of the same sex to facilitate interpretation. 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:

Copy Number status: Male samples	Final ratio
Normal	0.80 < FR < 1.20
Deletion	FR = 0
Duplication	1.65 < FR < 2.25
Ambiguous copy number	All other values

Copy Number status: Female samples	Final ratio
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 or in or near the *GPC3* 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.

Limitations of the procedure

- In most populations, the major cause of genetic defects in the *GPC3* and *GPC4* genes are small (point) mutations, most of which will not be detected by using SALSA MLPA Probemix P154 GPC3-GPC4.
- 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.

***GPC3* and *GPC4* mutation databases**

https://databases.lovd.nl/shared/genes/GPC3 and https://databases.lovd.nl/shared/genes/GPC4. We strongly encourage users to deposit positive results in the Leiden Open Variation Database. 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 *GPC4* exons 3 and 5 but not exon 4) to MRC Holland: info@mrcholland.com.

Table 1. SALSA MLPA Probemix P154-C2 GPC3-GPC4

Length (nt)	SALSA MLPA probe	Chromosomal position (hg18)[a]		
		Reference	*GPC3*	*GPC4*
64-105	Control fragments – see table in probemix content section for more information			
136	Reference probe 13085-L14304	Xq		
143 «	**GPC3 probe** 19921-L27354		**Exon 1**	
154	**GPC3 probe** 04999-L18914		**Exon 5**	
160	Reference probe 06110-L20285	Xq		
166	**GPC3 probe** 04993-L06900		**Exon 2**	
171	**GPC3 probe** 05001-L04387		**Exon 6**	
184	**GPC4 probe** 05592-L04518			**Exon 9**
191	**GPC3 probe** 05003-L06901		**Exon 7**	
196	**GPC4 probe** 05593-L04512			**Exon 3**
202	**GPC3 probe** 04997-L04383		**Exon 4**	
211	**GPC3 probe** 05005-L21998		**Exon 8**	
222	**GPC4 probe** 05120-L04975			**Exon 1**
229 «	**GPC3 probe** 04992-L04378		**Exon 1**	
238	**GPC4 probe** 19920-L04509			**Exon 1**
247	Reference probe 07866-L07677	**Xp**		
256	**GPC3 probe** 04994-L04380		**Exon 2**	
265	**GPC3 probe** 05002-L18076		**Exon 6**	
274	Reference probe 07667-L07373	**Xp**		
283	**GPC3 probe** 04996-L04382		**Exon 3**	
291	**GPC3 probe** 05004-L04390		**Exon 7**	
301 «	**GPC3 probe** 12848-L14719		**Exon 1**	
310	**GPC3 probe** 04998-L04384		**Exon 4**	
319	**GPC3 probe** 05006-L04392		**Exon 8**	
328	Reference probe 02921-L02315	**Xp**		
337	**GPC4 probe** 05121-L04511			**Exon 2**
346	**GPC4 probe** 05125-L04515			**Exon 6**
355	**GPC3 probe** 05590-L06902		**Exon 3**	
364 *	Reference probe 15361-L17195	Xq		
373	**GPC4 probe** 05126-L04516			**Exon 7**
391	Reference probe 03520-L02313	**Xp**		
400	**GPC4 probe** 15946-L18087			**Exon 8**
409	Reference probe 04127-L08388	Xq		
418	**GPC4 probe** 05124-L04514			**Exon 5**
427	**GPC4 probe** 05945-L05371			**Exon 4**
436	Reference probe 02914-L02308	Xq		
445	Reference probe 13208-L14529	**Xp**		
454 *	Reference probe 15583-L17438	Xq		

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

* New in version C2.
« 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.

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. P154-C2 probes arranged according to chromosomal location

Table 2a. *GPC3*

Length (nt)	SALSA MLPA probe	*GPC3* exon[a]	Ligation site NM_004484.4	Partial sequence[b] (24 nt adjacent to ligation site)	Distance to next probe
		start codon	*146-148 (Exon 1)*		
301 «	12848-L14719	Exon 1	8-7 *reverse*	AGTTTCCTCGCA-GCTACCTGGGCG	0.2 kb
143 «	19921-L27354	Exon 1	192-193	GGCGATGCTGCT-CAGCTTGGACTT	0.1 kb
229 «	04992-L04378	Exon 1	270-271	CCGCTCCTTCTT-CCAGAGACTGCA	32.1 kb
166	04993-L06900	Exon 2	347-348	TATGTCTCCCTA-AGGGCCCAACAT	0.1 kb
256	04994-L04380	Exon 2	422-421 *reverse*	TGCAGACTGAAG-CAGCTGTTCCAT	199.0 kb
355	05590-L06902	Exon 3	522-523	CAAGAACTACAC-CAATGCCATGTT	0.5 kb
283	04996-L04382	Exon 3	1001-1002	TGCAAGGCTGTA-TGGCAGGTGTGG	53.7 kb
202	04997-L04383	Exon 4	1201-1202	TGTGCCCATTCT-CAACAACGCCAA	0.1 kb
310	04998-L04384	Exon 4	1296-1295 *reverse*	TTCGGCTGGATA-AGGTTTCTTCAT	7.5 kb
154	04999-L18914	Exon 5	1342-1343	AAGTCTTTCATC-AGCTTCTATAGT	30.6 kb
171	05001-L04387	Exon 6	1457-1458	AAAAGGCAGCAA-GGAATGGAATGA	0.1 kb
265	05002-L18076	Exon 6	1538-1537 *reverse*	GTGCTTCAGTTT-GTCAATAATTTG	65.2 kb
191	05003-L06901	Exon 7	1581-1582	CATGTCTATGCC-CAAAGGTAGAGT	0.1 kb
291	05004-L04390	Exon 7	1713-1712 *reverse*	AGTTACCTGCAA-GGAAGCGGAGCT	60.2 kb
211	05005-L21998	Exon 8	1740-1741	TGATCTGGATGT-GGATGATGCGCC	0.1 kb
319	05006-L04392	Exon 8	1820-1821	TCGGGAACGTTC-ATTCCCCGCTGA	121.0 kb to *GPC4* exon 1
		stop codon	*1886-1888 (Exon 8)*		

Table 2b. *GPC4*

Length (nt)	SALSA MLPA probe	*GPC4* exon[a]	Ligation site NM_001448.3	Partial sequence[b] (24 nt adjacent to ligation site)	Distance to next probe
		start codon	*525-527 (Exon 1)*		
238	19920-L04509	Exon 1	349-350	ACGCGAGGCCCA-GCTCTACTTTTC	0.3 kb
222	05120-L04975	Exon 1	622-623	TTGCTCGGAAGT-GCGACGTCTTTA	75.6 kb
337	05121-L04511	Exon 2	713-714	TGTCCCCAGGGT-TCTACCTGCTGC	15.0 kb
196	05593-L04512	Exon 3	1060-1061	GGTGAACTCCCA-GTACCACTTTAC	13.0 kb
427	05945-L05371	Exon 4	1322-1323	GTGAAGCCATGT-TACAACTACTGC	5.2 kb
418	05124-L04514	Exon 5	1429-1430	GGCAGAGAGGCT-AGAGGGTCCTTT	0.3 kb
346	05125-L04515	Exon 6	1639-1640	TCACCCCGAGGA-ACGCCCAACCAC	1.0 kb
373	05126-L04516	Exon 7	1769-1770	AGGATGGCTGCA-GGAAACGGCAAT	1.4 kb
400	15946-L18087	Exon 8	1818-1817 *reverse*	TGCAAACAGGTA-CCTGGAATGTAA	0.7 kb
184	05592-L04518	Exon 9	2390-2391	TGTGCATTGAGT-TGGTTCCTGCTC	
		stop codon	*2193-2195 (Exon 9)*		

[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.

« 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.

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.
- Waterson J et al. (2010). Novel duplication in glypican-4 as an apparent cause of Simpson-Golabi-Behmel syndrome. *Am J Med Genet A.* 152:3179-3181.

Selected publications using SALSA MLPA Probemix P154 GPC3-GPC4

- Cottereau E et al. (2014). Duplication of exon 2 of the GPC3 gene in a case of Simpson-Golabi-Behmel syndrome. *Am J Med Genet A* 164:282-284.
- Mateos M et al. (2013). Simpson-Golabi-Behmel Syndrome Type 1 and Hepatoblastoma in a Patient With a Novel Exon 2-4 Duplication of the GPC3 Gene. *Am J Med Genet A.* 161:1091-1095.
- Nakane T et al. (2018). Hyperechoic renal medullary pyramids in a boy with Simpson-Golabi-Behmel syndrome. *Clin Dysmorphol*. 27:25-26.
- Vaisfeld A et al. (2017). Simpson-Golabi-Behmel syndrome in a female: A case report and an unsolved issue. *Am J Med Genet A*. 173:285-288.

P154 product history	
Version	*Modification*
C2	Two additional reference probes have been added.
C1	One target and one reference probe were removed. In addition, four reference probes have been replaced. Furthermore the control fragments have been adjusted (QDX2).
B1	One of the *GPC3* exon 1 probes, the *GPC4* exon 8 probe and four reference probes have been replaced. One extra *GPC3* exon 1 probe and four extra control fragments at 88-96-100-105 nt have been included.
LOT0806	One new probe added for *GPC4* exon 4 and several small changes in length or amplification characteristics of several probes.
LOT1005	First release

Implemented changes in the product description

Version C2-02 – 19 November 2024 (04P)
- The term 'mental retardation' is considered outdated and was updated to 'intellectual disability' where appropriate.

Version C2-01– 16 February 2021 (04P)
- Product description rewritten and adapted to a new template.
- Ligation sites of the probes targeting the *GPC3* and *GPC4* genes updated according to new version of the NM_ reference sequence.
- Small changes of probe lengths in Table 1 and 2 in order to better reflect the true lengths of the amplification products.

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