Product Description
SALSA® MLPA® Probemix P018-G2 SHOX

To be used with the MLPA General Protocol.

Version G2
For complete product history see page 14.

Catalogue numbers:
- **P018-025R:** SALSA MLPA Probemix P018 SHOX, 25 reactions.
- **P018-050R:** SALSA MLPA Probemix P018 SHOX, 50 reactions.
- **P018-100R:** SALSA MLPA Probemix P018 SHOX, 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. **Please note that copy number variation in healthy individuals has been reported for several genes outside of the *SHOX* region.**

Intended purpose
The SALSA MLPA Probemix P018 SHOX is an in vitro diagnostic (IVD)[1] or research use only (RUO) semi-quantitative assay[2] for the detection of deletions or duplications in the human short stature homeobox (*SHOX*) gene and its regulatory regions on Xp22.33/Yp11.32 in genomic DNA isolated from human peripheral whole blood specimens or buccal swabs. P018 SHOX is intended to confirm a potential cause for disorders associated with short stature, including Leri-Weill dyschondrosteosis (LWD), Langer mesomelic dysplasia (LMD) or Idiopathic short stature (ISS).

Copy number variations (CNVs) detected with P018 SHOX should be confirmed with a different technique. In particular, CNVs detected by only a single probe always require confirmation by another method. In the majority of patients, defects in the *SHOX* gene region are deletions. However, point mutations can occur which will not be detected by MLPA. It is therefore recommended to use this assay in combination with sequence analysis.

Assay results are intended to be used in conjunction with other clinical and diagnostic findings, consistent with professional standards of practice, including confirmation by alternative methods, clinical genetic evaluation, and counselling, as appropriate. The results of this test should be interpreted by a clinical molecular geneticist or equivalent.

This device is not intended to be used for standalone diagnostic purposes, pre-implantation or prenatal testing, population screening, or for the detection of, or screening for, acquired or somatic genetic aberrations.

[1] Please note that this probemix is for in vitro diagnostic (IVD) use in the countries specified at the end of this product description. In all other countries, the product is for research use only (RUO).

[2] To be used in combination with a SALSA MLPA Reagent Kit and Coffalyser.Net analysis software.

Clinical background
SHOX is located in the pseudoautosomal region 1 (PAR1) on the short arm of the X and Y chromosomes. Located upstream and downstream of *SHOX* are highly conserved non-coding elements (CNEs), some of which have been shown to be important *SHOX* enhancer sequences. Mutations in *SHOX* or its regulatory regions cause a range of disorders associated with short stature, including LWD, LMD, and ISS, as *SHOX* is a known transcription factor highly expressed in tissues responsible for bone development (Benito-Sanz et al. 2012b).

LWD is a dominant skeletal disorder characterised by short stature, mesomelic shortening of the limbs, and the characteristic Madelung deformity. LMD is a more severe form of LWD and is a result of mutations in both *SHOX* alleles (while LWD is associated with pathogenic variants in one *SHOX* allele) (Bertorelli et al. 2007, Campos-Barros et al. 2007, Shears et al. 2002, Zinn et al. 2002). ISS classifies individuals with a height below the third centile in whom no identifiable disorder is present. Heterozygous mutations of *SHOX* and/or its regulatory elements are detected in approximately 60% of LWD patients and approximately 5-15% of ISS cases. Homozygous or compound heterozygous mutations of *SHOX* and/or its enhancers are detected in 75% of LMD patients (Benito-Sanz et al. 2006, Benito-Sanz et al. 2012a, Chen et al. 2009, Huber et al. 2006).

In individuals with a *SHOX* related disorder, 70-80% of *SHOX* mutations are whole gene deletions, 2-6% are partial deletions, and 20-25% are point mutations, including small deletions or insertions (Binder 2011, Caliebe et al. 2012).

An extra copy of the *SHOX* gene and the entire *SHOX* regulatory region is present in individuals with tall stature and an additional X or Y chromosome, where all three copies of *SHOX* are fully expressed. When a duplication does not include all flanking regulatory elements, the effect on SHOX expression is difficult to predict. Duplications of *SHOX* alone or including various lengths of the *SHOX* regulatory elements have been reported in LWD and ISS patients, and in *SHOX*-specific cohorts, the frequency of these duplications has been estimated at 0.33% (Bunyan et al. 2023). Reported duplications include those extending upstream or downstream of the *SHOX* area (Bunyan et al. 2016, Bunyan et al. 2021, Bunyan et al. 2023), as well as those exclusively affecting downstream PAR1 regions (Eid et al. 2020, Hirschfeldova et al. 2012, Hirschfeldova and Solc 2017). In terms of the clinical significance of these types of duplications, since the occurrence is low, there has been limited evidence supporting the association between such CNVs and short stature. However, one study showed that there was a statistically significant increase in the frequency of these duplications in individuals with LWS or ISS compared to unaffected individuals (Hirschfeldova and Solc 2017).

The P018 SHOX probemix can detect most deletions and duplications and therefore complements sequence analysis of *SHOX*.

More information is available on http://www.ncbi.nlm.nih.gov/books/NBK1215/.

Gene structure
The *SHOX* gene spans 35 kilobases (kb) of the pseudoautosomal region 1 (PAR1) located on Xp22.33 / Yp11.32 and contains 7 exons. The *SHOX* LRG_710 is available at www.lrg-sequence.org and is identical to GenBank NG_009385.2.

Transcript variants
For *SHOX*, two major transcript variants have been described: http://www.ncbi.nlm.nih.gov/gene/6473. *SHOX* transcript variant 1 (NM_000451.4, 7934 nt, coding sequence 108-986) represents the longer transcript and encodes the longer active isoform (SHOXa). The ATG translation start codon is located in exon 1 (LRG exon 2b) and the stop codon is located in exon 5 (LRG exon 6). *SHOX* transcript variant 2 (NM_006883.2, 1951 nt, coding sequence 692-1369) contains alternative 5' and 3' exons compared to transcript variant 1 and encodes a shorter isoform (SHOXb) with a different C-terminus than isoform SHOXa. Several regulatory sequences located outside of *SHOX* that affect *SHOX* transcription have been described (e.g. Benito-Sanz et al. 2012b, Durand et al. 2010, Fukami et al. 2006, Sandbacka et al. 2011).

Exon numbering
The *SHOX* exon numbering used in this P018-G2 SHOX product description is the exon numbering from the LRG_710 sequence. This exon numbering is different from the *SHOX* exon numbering in many articles where exon 7 is referred to as exon 6b. 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 P018-G2 SHOX contains 48 MLPA probes with amplification products between 124 and 504 nucleotides (nt). This includes 32 probes for the PAR1 region on chromosome Xp22 / Yp11, including at least one probe for each exon of *SHOX* transcript variant 1, and one probe for intron 6 only present in the *SHOXb* splice variant. Several probes are present for *SHOX* regulatory regions, located upstream and downstream of *SHOX*. Moreover, this probemix contains multiple flanking probes targeting the X chromosome outside the *SHOX* area: one probe detecting the area just before the *SHOX* upstream regulatory regions, five probes inside the PAR1 region but downstream of the *SHOX* area, and seven probes outside of PAR1. Flanking probes can be used to characterise larger deletions/duplications and to distinguish *SHOX* deletions from a Turner syndrome karyotype. In addition, nine 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 ten quality control fragments generating amplification products between 64 and 118 nt: four DNA Quantity fragments (Q-fragments), two DNA Denaturation fragments (D-fragments), one Benchmark fragment, and one chromosome X and two chromosome Y-specific fragments (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) (*AMOT* gene)
105	Y-fragment (Y chromosome specific) (*UTY* gene)
118	Y-fragments (Y chromosome specific) (*ZFY* gene)

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 reference probes over the experiment.

Required specimens
Extracted DNA from peripheral blood or buccal swab, 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 short stature. **Importantly, all reference samples need to be of the same sex for correct data analysis**. To facilitate interpretation, it is also recommended to use reference and patient samples of the same sex. 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 NA20212, NA20217, NA20218, and NA04626 from the Coriell Institute have been tested with this P018-G2 probemix at MRC Holland and can be used as a positive control samples. For details about genotype, affected probes and expected results, please see table below. The quality of cell lines can change; therefore samples should be validated before use.

Sample ID	Genotype	Affected probes	Expected final ratio
NA20212	Female sample: Heterozygous deletion (~0.9 Mb) of the *SHOX* gene and the upstream and downstream *SHOX* area.	09333-L10292 to 09335-L30792	0.5
NA20217	Male sample: Compound heterozygous deletion of *SHOX* and of *SHOX* downstream area.	01341-L20651 to 09338-L24247 13297-L24253 to 09335-L30792	0.5 0.5
NA20218	Female sample: Compound heterozygous deletions of upstream and downstream area of *SHOX* resulting in a homozygous deletion of the entire *SHOX* gene.	09333-L10292, 05642-L05096 to 14697-L24245 18889-L25087 to 09338-L24247	0.5 0
NA04626	Female sample: Heterozygous duplication of the *SHOX* gene and the upstream and downstream *SHOX* area.	09333-L10292 to 01156-L00659	1.5

Performance characteristics

In individuals with a *SHOX* related disorder, 70-80% of all mutations are whole gene deletions and 2-6% are partial deletions, both of which can be detected by this MLPA probemix (Binder 2011, Caliebe et al. 2012). Duplications of *SHOX* have also been reported in LWD and ISS patients (Benito-Sanz et al. 2011b) and can be detected by this probemix. The analytical sensitivity and specificity for the detection of deletions/duplications in *SHOX* and its surrounding enhancer regions is very high and can be considered >99% (based on a 2006-2022 literature review).

Analytical performance can be compromised by: SNVs or other polymorphisms 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 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.

Note: Slope correction in samples with large deletions: The slope correction algorithm in Coffalyser.Net has been optimised to give the best possible results in as many situations as possible. However, the slope correction algorithm may confuse a large deletion for sloping, which can lead to over- or under-correction. Since large deletions are often detected when using the P018 probemix, this issue may occur relatively

frequently. Incorrectly applied slope correction can cause an FSLP warning in Coffalyser.Net or ambiguous results for multiple probes. If you suspect that slope correction was incorrectly applied we recommend contacting info@mrcholland.com for assistance.

Interpretation of results
The expected results for *SHOX*-specific MLPA probes are allele copy numbers of 2 (normal), 0 (homozygous deletion), 1 (heterozygous deletion), 3 (heterozygous duplication), and 4 (heterozygous triplication/homozygous duplication). Please see Table 3 for examples of potential results.

The standard deviation of each individual reference 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 when **reference samples of the same sex** have been used:

Copy number status		Final ratio (FR)
Pseudoautosomal sequences in males and females and X chromosome sequences in females	**X chromosome sequences in males**	
Normal	Normal	0.80 < FR < 1.20
Homozygous deletion	Deletion	FR = 0
Heterozygous deletion		0.40 < FR < 0.65
Heterozygous duplication		1.30 < FR < 1.65
Heterozygous triplication/homozygous duplication	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 the above mentioned final ratios can be affected in mosaic cases.

- 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.

P018 specific notes:

- Short stature and skeletal deformities due to *SHOX* defects are pseudoautosomal dominant disorders. Therefore, a heterozygous mutation of *SHOX* is expected to result in these phenotypes.
- A recurrent 47.5 kb deletion downstream of *SHOX* has been described by Benito-Sanz et al. (2012b). This 47.5 kb deletion is covered by three probes in this P018-G2 probemix (Table 2).
- Complete or partial duplications found within *SHOX* or its surrounding regulatory regions have been found in LWD and ISS patients (Benito-Sanz et al. 2011b).
- Breakpoints of partial *SHOX* deletions have been reported to frequently occur in intron 3 (Benito-Sanz et al. 2017).
- A partial *SHOX* deletion encompassing the last 20 nt of *SHOX* exon 3 and part of intron 3 has been described (Funari et al. 2019). This deletion will not be detected by P018-G2 SHOX as there is no probe present that targets this region.
- Deletion of the *SHOX* exon 6 and intron 6 probes, which are located downstream of the stop codon of transcript variant 1 (*SHOXa*), may not affect *SHOX* gene function.
- Please note that single exon deletions have a considerable chance of being a false positive result, either due to non-pathogenic copy number variants (Benito-Sanz et al. 2011a), mutations within the probe binding site (Barca-Tierno et al. 2011) or due to impurities in the DNA.
- Not all copy number changes detected by SHOX AREA probes will affect *SHOX* gene function. Analysis of family members may be required for correct interpretation of results.
- Large deletions and duplications interrupted by one or more probes with a normal copy number have been reported and might be the result of an inversion followed by a deletion/duplication or may indicate compound heterozygosity (Dupont et al. 2007). In these cases, parental evaluation can assist data interpretation.
- Flanking probes have been included in this probemix to help determine the extent of a deletion/duplication. Copy number changes detected by flanking probes only have been reported in healthy individuals and are unlikely to be related to short stature. However, several genes detected by the flanking probes have been associated with other disorders (Balasubramanian and Crowley Jr 2017, Mehta and Ebens 2021, Mullighan et al. 2009, Nguyen et al. 2022, Russell et al. 2009).

Limitations of the procedure

- The SALSA MLPA Probemix P018 SHOX will not detect point mutations in the *SHOX* gene, which are the second most common cause of genetic defects in *SHOX*.
- 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.

***SHOX* mutation database**

https://databases.lovd.nl/shared/genes/SHOX. 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 *SHOX* exons 3 and 5 but not exon 4) to MRC Holland: info@mrcholland.com.

Table 1. SALSA MLPA Probemix P018-G2 SHOX

Length (nt)	SALSA MLPA probe	Chromosomal position (hg18)[a]		
		Reference	*SHOX* region/PAR1	Outside PAR1
64-118	Control fragments – see table in probemix content section for more information			
124	Reference probe 15370-L13762	7q		
130	**SHOX AREA probe** 18885-L24430		CNE-3	
136	**SHOX AREA probe** 05642-L05096		6.2 kb after CNE2	
142 « ¬	**IL3RA probe** 13597-L15055		Xp22.33 / Yp11.32	
148	**SHOX AREA probe** 05648-L06218		Downstream	
154	**SHOX AREA probe** 13821-L14642		CNE3	
160	Reference probe 04966-L04696	1p		
166	**SHOX probe** 01145-L00702		**Exon 1**	
172	**SHOX AREA probe** 18886-L24431		CNE4	
178	**SHOX AREA probe** 05649-L20176		Downstream	
185	**SHOX AREA probe** 06293-L20177		CNE9	
191	Reference probe 06057-L05512	4p		
199	**SHOX AREA probe** 13296-L20175		CNE5	
204	**SHOX probe** 01146-L06220		**Exon 2**	
211 ¬	**PPP2R3B probe** 09333-L10292		Xp22.33 / Yp11.32	
219	Reference probe 03247-L02684	13q		
226 ◊	**SHOX probe** 09336-L20178		**Exon 6**	
231	**SHOX probe** 09337-L00911		**Exon 6**	
238 ¬ x	**ANOS1 probe** 06402-L09795			Xp22.31
245	**SHOX probe** 01147-L00802		**Exon 3**	
254 ¬ x	**ARSF probe** 16846-L20647			Xp22.33
261	Reference probe 00587-L20649	18q		
266	**SHOX probe** 01341-L20651		Upstream	
274 ¬ x	**FANCB probe** 03906-L03066			Xp22.2
283 ¬ x	**NLGN4X probe** 05587-L04577			Xp22.31
290	**SHOX AREA probe** 06291-L06222		CNE9	
300 ∫	**SHOX probe** 01148-L15501		**Exon 4**	
310 ¬	**ASMT probe** 01153-L00712		Xp22.33 / Yp11.31	
318 ^	**SHOX AREA** probe 05645-L05099		2 kb before CNE8	
328 ¬ x	**PRKX probe** 16898-L19768			Xp22.33
337 ¥	**SHOX probe** 21538-L30066		**Exon 5**	
346	Reference probe 06560-L06118	1q		
355 ¬ +	**VAMP7 probe** 01156-L00659			Xq28 / Yq12 (PAR2)
364	**SHOX AREA probe** 18889-L25087		CNE-5	
379	**SHOX AREA probe** 14697-L24245		Downstream	
389 ¬	**CSF2RA probe** 10251-L24246		Xp22.33 / Yp11.32	
395	**SHOX probe** 09338-L24247		Intron 6	
403 ¬	**CRLF2 probe** 13911-L19678		Xp22.33 / Yp11.32	
412	Reference probe 09793-L12593	15q		
420 ¬ x	**AIFM1 probe** 00820-L25090			Xq25
427	**SHOX AREA probe** 18891-L25088		CNE-2	
439 ^	**SHOX AREA probe** 05646-L24249		5.4 kb after CNE8	
445 ¥	**SHOX AREA probe** 09335-L30792		Downstream	
456 ¬	**ZBED1 probe** 16858-L25227		Xp22.33 / Yp11.31	
466	**SHOX AREA probe** 13297-L24253		6 kb before CNE9	
476	Reference probe 09888-L10301	16p		
481 ^	**SHOX AREA probe** 18893-L25091		CNE7	
504	Reference probe 09870-L19465	2p		

[a] See section Exon numbering on page 3 for more information.

CNE = Conserved Non-coding DNA Element. Locations of the upstream regulatory regions (CNE-2, CNE-3 and CNE-5) are based on Durand et al. (2010) and Benito-Sanz et al. (2012b). Locations of the downstream regulatory regions (CNE2 through CNE9) are based on Benito-Sanz et al. (2012b) and Fukami et al. (2006).

¥ Changed in version G2. Minor alteration, no change in sequence detected.

« 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.

¬ Flanking probe. Included to help determine the extent of a deletion/duplication. Copy number alterations of only the flanking or reference probes are unlikely to be related to the condition tested.

x X-chromosome, outside PAR region. Gives 50% reduced signal in males as compared to females.

∫ A founder *SHOX* point mutation in the Spanish Gypsy population, c.508G>C (p.A170P), results in a strongly reduced probe signal of the 300 nt exon 4 probe (Barca-Tierno et al. 2011).

^ The 481, 318 and 439 nt probes are within the recurrent ~47.5 kb deletion region described by Benito-Sanz et al. (2012b).

+ The VAMP7 probe at 355 nt is located very close to the X and Y q-telomere in PAR2.

◊ From product description version 05 onwards the 226 nt probe is considered an exon 6 probe to align with the LRG exon numbering.

SNVs located in the target sequence of a probe can influence probe hybridization and/or probe ligation. Please note: not all known SNVs are mentioned in the tables above. Single probe aberration(s) must be confirmed by another method.

Table 2. Xp / Yp probes arranged according to chromosomal location

Length (nt)	SALSA MLPA probe	Gene/Exon[a]	Ligation site	Partial sequence[b] (24 nt adjacent to ligation site)	Distance to next probe
p-telomere ---------- **Start of PAR1** ----------					227.0 kb
211 ¬	09333-L10292	*PPP2R3B* gene	Xp22.33 / Yp11.32	CGTCCGAGTTCC-ACTCGCGCTACA	91.6 kb
***SHOX* region**					
364	18889-L25087	*SHOX* AREA	CNE-5	GAAATGTTAACA-GCTCCCCGAGCT	61.7 kb
130	18885-L24430	*SHOX* AREA	CNE-3	ATGGCAGAGCAT-TTGTACCCCTGG	56.3 kb
427	18891-L25088	*SHOX* AREA	CNE-2	TACACCGTTATG-CGGATGCTCGTT	63.5 kb
266	01341-L20651	*SHOX* LOC159015	Upstream of SHOX	GCCTGGAACAGA-ACTTCCGCGGGG	4.7 kb
		SHOX	**NM_000451.4**		
		start codon	*108-110 (Exon 2b)*		
166	01145-L00702	Exon 1	6.3 kb before exon 2b (NM_006883.2: 99-100)	TTTCTACTGCAA-ACAGAAATGGGA	6.7 kb
204	01146-L06220	Exon 2	336-337	ACCACGTAGACA-ATGACAAGGAGA	3.6 kb
245	01147-L00802	Exon 3	448-449	CGGGCAGACCAA-GCTGAAACAGAG	6.2 kb
300 ∫	01148-L15501	Exon 4	614-615	CAGAACCGGAGA-GCCAAGTGCCGC	0.2 kb
337	21538-L30066	Exon 5	677-678	ACAGCCAACCAC-CTAGACGCCTGC	3.6 kb
231	09337-L00911	Exon 6	922-923	AAGCAACAGCAA-GAATTCCAGCAT	6.4 kb
226 ◊	09336-L20178	Exon 6	7370-7371	TGGCTTCACGAG-TTCAGCCCATTG	6.4 kb
		stop codon	*984-986 (Exon 6)*		
395	09338-L24247	*SHOX* Intron 6	1.4 kb before exon 7	TCCCACATTCTT-GGAATCACAATG	56.9 kb
136	05642-L05096	*SHOX* AREA	6.2 kb after CNE2	GCAGCAGTGAAA-GTGAGCATTCCC	19.8 kb
154	13821-L14642	*SHOX* AREA	CNE3	GATGGCTGATAA-TTACTCCGTATG	19.4 kb
172	18886-L24431	*SHOX* AREA	CNE4	GCCTCCGATACA-GTTTACGGCTTC	37.4 kb
199	13296-L20175	*SHOX* AREA	CNE5	GGAAAACCACGT-TCCTATCGATCC	29.6 kb
481 ^	18893-L25091	*SHOX* AREA	CNE7	CAGACCAGGTCT-CCTGTTTCATGT	28.1 kb
318 ^	05645-L05099	*SHOX* AREA	2 kb before CNE8	TGTTCCCACCGT-AAAACTCACTCC	8.4 kb
439 ^	05646-L24249	*SHOX* AREA	5.4 kb after CNE8	TGCATGTCTGCT-TTTTGAATGGCC	10.7 kb
466	13297-L24253	*SHOX* AREA	6 kb before CNE9	TACAGCAAATGA-TACGTATAAATT	6.3 kb
290	06291-L06222	*SHOX* AREA	CNE9	CTTGAAAGGGCA-GGAACTCTAATT	0.4 kb
185	06293-L20177	*SHOX* AREA	CNE9	TAATTGATGAGA-TGCAGAAGCCAG	15.4 kb
148	05648-L06218	Xp22-PAR1	Downstream	TGGTGCTGAAAT-GAGGAAGCCCTG	48.7 kb
178	05649-L20176	Xp22-PAR1	Downstream	TGAGGAGGTACC-TCAAAGCTAAAC	64.4 kb
445	09335-L30792	Xp22-PAR1	Downstream	GAAATTCAGTTT-TAATAACACAGA	66.0 kb
379	14697-L24245	Xp22-PAR1	Downstream	CTCTGGTGAGAT-GCCATCTAGAGA	338.0 kb
403 ¬	13911-L19678	*CRLF2* gene	Xp22.33 / Yp11.32	GAATGCCAGCAA-ATACTCCAGGAC	73.9 kb
389 ¬	10251-L24246	*CSF2RA* gene	Xp22.33 / Yp11.32	GACAAGCCTTCT-GCTCTGTGAGTT	69.8 kb
142 « ¬	13597-L15055	*IL3RA* gene	Xp22.33 / Yp11.32	TGCACAGATAAG-TTTGTCGTCTTT	280.7 kb
310 ¬	01153-L00712	*ASMT* gene	Xp22.33 / Yp11.31	GACATCCCAGAA-GTGGTGTGGACG	706.4 kb
456 ¬	16858-L25227	*ZBED1* gene	Xp22.33 / Yp11.31	TCGTCAAGAGCA-ACACGGAGCAGA	593.8 kb
---------- **End of PAR1** ----------					
254 ¬ x	16846-L20647	*ARSF* gene	Xp22.33	CATCCATATAAT-TATGGGTTTGAC	536.9 kb
328 ¬ x	16898-L19768	*PRKX* gene	Xp22.33	CGATTAGGAAAC-ATGAAGGTCAGT	2.6 **M**b
283 ¬ x #	05587-L04577	*NLGN4X* gene	Xp22.31	GACGGCTTGGGT-GATGCACGAAAT	2.3 **M**b
238 ¬ x #	06402-L09795	*ANOS1* gene	Xp22.31	GTTTCCTGAAGC-GTGTGCCCACAA	6.3 **M**b
274 ¬ x	03906-L03066	*FANCB* gene	Xp22.2	TCTCATCAGAAT-TCTCCCTATAAA	114.3 **M**b
420 ¬ x	00820-L25090	*AIFM1* gene	Xq25	TATTGGTCTTGT-GGACAGTAGTTT	25.7 **M**b

Length (nt)	SALSA MLPA probe	Gene/Exon[a]	Ligation site	Partial sequence[b] (24 nt adjacent to ligation site)	Distance to next probe
---------- Start of PAR2 ----------					
355 ¬ +	01156-L00659	VAMP7 gene	Xq28 / Yq12	TGTGGGAAAAGT-GTTTCCATTCTG	98 kb
q-telomere					

[a] See section Exon numbering on page 3 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.

Notes:

- The 105 nt chromosome Y-specific control fragment targets the *UTY* gene, located ~11 Mb from the PAR region.
- The 118 nt chromosome Y-specific control fragment targets the *ZFY* gene, located just outside the PAR region at ~470 kb distance from the 456 nt ZBED1 probe. A small signal for the 118 nt fragment may be observed in some female samples.

CNE = Conserved Non-coding DNA Element. Locations of the upstream regulatory regions (CNE-2, CNE-3 and CNE-5) are based on Durand et al. (2010) and Benito-Sanz et al. (2012b). Locations of the downstream regulatory regions (CNE2 through CNE9) are based on Benito-Sanz et al. (2012b) and Fukami et al. (2006).

« 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.

¬ Flanking probe. Included to help determine the extent of a deletion/duplication. Copy number alterations of only the flanking or reference probes are unlikely to be related to the condition tested.

x X-chromosome, outside PAR region. Gives 50% reduced signal in males as compared to females.

∫ A founder *SHOX* point mutation in the Spanish Gypsy population, c.508G>C (p.A170P), results in a strongly reduced probe signal of the 300 nt exon 4 probe (Barca-Tierno et al. 2011).

^ The 481, 318 and 439 nt probes are within the recurrent ~47.5 kb deletion region described by Benito-Sanz et al. (2012b).

+ The VAMP7 probe at 355 nt is located very close to the X and Y q-telomere in PAR2.

◊ From product description version 05 onwards the 226 nt probe is considered an exon 6 probe to align with the LRG exon numbering.

The specificity of this probe relies on a single nucleotide difference between 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.

SNVs located in the target sequence of a probe can influence probe hybridization and/or probe ligation. Please note: not all known SNVs are mentioned in the tables above. Single probe aberration(s) must be confirmed by another method.

Table 3. Examples of final ratios from potential results

Type of test sample	Probes	RATIO: when compared to		Explanation
		Male ref	Female ref	
Normal FEMALE DNA	All PAR1/PAR2 probes, incl. SHOX AREA, Xp22	1	1	PAR1 & PAR2 regions are located on X **and** Y: normal females: 2 copies; normal males: 2 copies.
	X-chromosome probes OUTSIDE PAR1/2 region	**2**	1	Sequences present only on X: normal females: 2 copies; normal males: 1 copy.
	105 & 118 nt Y-chromosome probes outside PAR1 + PAR2	**0**	-	Sequences present only on Y: normal females: 0 copies; normal males: 1 copy.
Normal MALE DNA	All PAR1/PAR2 probes, incl. SHOX AREA, Xp22	1	1	PAR1 & PAR2 regions are located on X **and** Y: normal females: 2 copies; normal males: 2 copies.
	X-chromosome probes OUTSIDE PAR1/2 region	1	**0.5**	Sequences present only on X: normal females: 2 copies; normal males: 1 copy.
	105 & 118 nt Y-chromosome probes outside PAR1 + PAR2	1	**∞**	Sequences present only on Y: normal females: 0 copies; normal males: 1 copy.
FEMALE DNA with a deletion that includes one or more exons of the SHOX gene	One or more SHOX probes + possibly one or more SHOX AREA and/or Xp22 probes	**0.5**	**0.5**	Heterozygous deletion in essential *SHOX* area in PAR1. PAR1 region is located on X **and** Y. **This typically causes LWD or short stature.**
	Deletions extending outside PAR1: one or more of the 6 chromosome X probes	**1**	**0.5**	Heterozygous deletion outside PAR1. Sequences present only on X.

Type of test sample	Probes	RATIO: when compared to		Explanation
		Male ref	Female ref	
	(marked x in Table 2) also show a lower copy number			
MALE DNA with a deletion that includes one or more exons of the SHOX gene	One or more SHOX probes + possibly one or more SHOX AREA and/or Xp22 probes	**0.5**	**0.5**	Heterozygous deletion in essential *SHOX* area in PAR1. PAR1 region is located on X **and** Y. **This typically causes LWD or short stature.**
	Deletions extending outside PAR1: one or more of the 6 chromosome X probes (marked x in Table 2) also show a lower copy number	**0**	**0**	Heterozygous deletion outside PAR1. Sequences present only on X.
FEMALE or MALE DNA with a deletion in the area upstream or downstream of SHOX, but not including SHOX gene probes	One or more probes upstream or downstream of SHOX, indicated with *SHOX* AREA in table 2	**0.5**	**0.5**	Heterozygous deletion. PAR1 region is located on X **and** Y. **Deletions in this region have been associated with LWD & ISS** (Benito-Sanz et al. 2005, Benito-Sanz et al. 2012a, Benito-Sanz et al. 2012b, Chen et al. 2009). **Not all deletions detected by these probes will result in LWD or ISS!**
FEMALE sample with Turner syndrome (45,X)	All PAR1/PAR2 probes, incl. SHOX AREA, Xp22	**0.5**	**0.5**	PAR1 & PAR2 regions are located on X **and** Y. Only one X is present in this case.
	X-chromosome probes OUTSIDE PAR1/2 region	1	**0.5**	Probe sequences located only on X. Only one X is present in this case.
	105 & 118 nt Y-chromosome probes outside PAR1 + PAR2	**0**	-	Probe sequences located only on Y. No Y present in this case.
MALE sample with Klinefelter syndrome (47,XXY)	All PAR1/PAR2 probes, incl. SHOX AREA, Xp22.	**1.5**	**1.5**	PAR1 & PAR2 regions are located on X **and** Y. Three instead of the normal two copies present.
	X-chromosome probes OUTSIDE PAR1/2 region	**2**	1	Probe sequences present only on X. Two Xs are present in this case.
	105 & 118 nt Y-chromosome probes outside PAR1 + PAR2	1	**∞**	Probe sequences present only on Y. One Y present in this case.
FEMALE sample with Triple X-syndrome (47,XXX).	All PAR1/PAR2 probes, incl. SHOX AREA, Xp22.	**1.5**	**1.5**	PAR1 & PAR2 regions are located on X **and** Y. Three instead of the normal two copies present.
	X-chromosome probes OUTSIDE PAR1/2 region	**3**	**1.5**	Probe sequences present only on X. Three Xs are present in this case.
	105 & 118 nt Y-chromosome probes outside PAR1 + PAR2	**0**	-	Probe sequences located only on Y. No Y present in this case.

Related SALSA MLPA probemixes

P026 Sotos syndrome	Contains probes for *NSD1* and *NFIX*, involved in Sotos syndrome.
P216 Growth Hormone Deficiency mix -1	Contains probes for *GH1*, *PROP1*, *POU1F1*, *GHRHR*, *HESX1*, *LHX3* and *LHX4*, involved in Growth Hormone Deficiency (GHD).
P217 IGF1R	Contains probes for *IGF1R, IGFBP3* and *IGFALS* involved in growth and development.
P262 GHI	Contains probes for *IGF1*, *GHR*, *JAK2* and *STAT5B*, related to growth hormone insensitivity and short stature.
P329 CRLF2-CSF2RA-IL3RA	Contains probes for the PAR1 genes *CRLF2*, *CSF2RA* and *IL3RA*, linked to B-cell acute lymphoblastic leukaemia (ALL).
P360 Y-Chromosome Microdeletions	Contains probes for the Y chromosomal regions AZFa, AZFb, AZFc, associated with spermatogenetic failure in infertile men.

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P018 product history	
Version	*Modification*
G2	One SHOX probe and one SHOX area probe have been adjusted. Minor alteration, no change in sequence detected.
G1	One SHOX area probe has been replaced (CNE4) and four new probes have been included for the upstream *SHOX* enhancer sequences CNE-2, CNE-3 and CNE-5 and the downstream CNE7 enhancer sequence.
F1	Three new probes near the PAR1 boundary have been included. One probe (GPR143) has been removed. The 88 and 96 nt control fragments have been replaced (QDX2).
E1	Six probes located on chromosome X and six reference probes have been replaced.

D1	One target and six reference probes have been replaced.
C1	Several reference probes replaced. Variable probes in the SHOX downstream region removed. Extra control fragments added.
B1	Many probes outside the *SHOX* gene have been added.
A1	First release.

Implemented changes in the product description

Version G2-08 – 23 May 2025 (04P)
- Extended information on *SHOX* duplications added to Clinical background section, and related publications added to References section.
- Warning for salt sensitivity removed for the 211 nt probe in Table 1 and Table 2.
- Selected publications list refined to 11 articles.

Version G2-07 – 20 March 2024 (04P)
-In section Positive control DNA samples, NA04626 was added. The genotype of this sample has been specified in the associated table in that same section.
-Morocco has been removed from the list of countries in which the product is IVD-registered.

Version G2-06 – 21 March 2023 (04P)
- In section Reference samples, clarification added that all reference samples need to be the same sex for correct data analysis.
- In section Interpretation of results, expected final ratios for X chromosome probes in males added to the table.
- Minor textual and layout changes.

Version G2-05 – 22 February 2022 (04P)
- Product description adapted to a new template.
- Multiple minor textual changes.
- Transcript variants section updated according to *SHOX* LRG_710.
- P018 specific notes added about breakpoints in *SHOX* intron 3, exon 3/intron 3 deletions that are not detected by P018-G2, and note about flanking probes rephrased.
- Exon numbering of the *SHOX* gene has been changed to align with the LRG exon numbering: the 226 nt probe is now considered an exon 6 probe (was intron 6 probe).
- Chromosomal band of the 420 nt probe (00820-L25090) updated.
- Ligation sites of the probes targeting the *SHOX* gene updated according to new version of the NM_ reference sequence.
- Multiple small changes in Table 2.
- Selected publications updated.
- UK has been added to the list of countries in Europe that accept the CE mark.

Version G2-04 — 13 November 2020 (02P)
- Product description rewritten and adapted to a new template.
- Various minor textual changes.
- Note about a small signal for the ZFY probe in some females added.
- Information about slope correction in samples with large deletions added.
- Clarification added about the purpose of flanking probes and their relation to other disorders.
- Link to mutation database updated.
- Note added about copy number variation observed in healthy individuals for a number of probes outside the *SHOX* region.
- *SHOX* region information in Table 1 updated.
- Ligation sites of flanking probes in Table 2 updated.
- 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	
	MRC Holland bv; Willem Schoutenstraat 1 1057 DL, Amsterdam, The Netherlands
E-mail	info@mrcholland.com (information & technical questions) order@mrcholland.com (orders)
Phone	+31 888 657 200

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