Product Description

SALSA® MLPA® Probemix P163-E1 GJB-WFS1-POU3F4

To be used with the MLPA General Protocol.

Version E1
For complete product history see page 13.

Catalogue numbers:

- **P163-025R:** SALSA MLPA Probemix P163 GJB-WFS1-POU3F4, 25 reactions.
- **P163-050R:** SALSA MLPA Probemix P163 GJB-WFS1-POU3F4, 50 reactions.
- **P163-100R:** SALSA MLPA Probemix P163 GJB-WFS1-POU3F4, 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.

Intended purpose
The SALSA MLPA Probemix P163 GJB-WFS1-POU3F4 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 *GJB2*, *GJB3*, *GJB6*, *WFS1*, and *POU3F4* genes, microdeletions upstream of *POU3F4*, and the presence of six specific mutations in the *GJB2* gene in genomic DNA isolated from human peripheral whole blood specimens. P163 GJB-WFS1-POU3F4 is intended to confirm a potential cause for and clinical diagnosis of hereditary hearing loss and Wolfram Syndrome type 1. This assay can also be used for molecular genetic testing of at-risk family members.

Copy number variations (CNVs) detected with P163 GJB-WFS1-POU3F4 should be confirmed with a different technique. In particular, CNVs detected by only a single probe always require confirmation by another method. Most defects in the *GJB2*, *GJB3*, *POU3F4* and *WFS1* genes are point mutations, the majority of 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

Hearing loss is a common congenital defect. The estimated incidence of permanent hearing loss at birth, defined as a sensorineural loss of ≥35 dB, is 1 in 500-1000 newborns, and approximately 50 to 60% of all hearing loss cases have been attributed to inherited or genetic factors. Inheritance of hearing loss can be autosomal recessive (75-85%), autosomal dominant (15-24%), X-linked (1-2%) or mitochondrial. More than 70% of hereditary hearing loss is nonsyndromic; the remaining 30% is accompanied by additional clinical findings and is considered syndromic.

In many populations, up to 50% of all cases of autosomal recessive nonsyndromic hearing loss are caused by mutations in the DFNB1 (Deafness, Nonsyndromic, autosomal recessive 1) locus on 13q12 (https://www.ncbi.nlm.nih.gov/books/NBK1272/). This locus contains the *GJB2* and *GJB6* genes, encoding connexin 26 and 30 protein, respectively. In Caucasians, the *GJB2* c.35delG is the most frequent mutation, comprising 70% of mutated DFNB1 alleles with a carrier rate of 2–4% in the northern European population. The c.235delC mutation is the most common variant in the Japanese population (carrier rate of 1-2%). With a carrier rate of ~7.5%, the c.167delT mutation is the most frequently occurring mutation in the Ashkenazi Jewish population. The c.101T>C mutation is associated with mild hearing loss and has a minor allele frequency of 1-2%. The IVS1+1G>A and c.313del14 mutations have been found with relatively high frequency among various populations, mainly in eastern Europe, Russia, the Middle East and Asia. Six large deletions in *GJB2* and/or *GJB6* have been shown to contribute to DFNB1 hearing loss and account for 1–10% of all the mutated DFNB1 alleles. The most common deletions of 309 kilobases (kb) [del(GJB6-D13S1830)] and 232 kb [del(GJB6-D13S1854)] truncate *GJB6* and the upstream *CRYL1* gene.

GJB3 is located on 1p34, which is the DFNA2B (Deafness, Nonsyndromic, autosomal dominant 2B) locus. Mutations in *GJB3* can cause nonsyndromic hearing loss, as well as the skin disorder erythrokeratodermia variabilis (https://www.ncbi.nlm.nih.gov/books/NBK1434/).

The *GJB2* and *GJB6* genes are also involved in DFNA3 (Deafness, Nonsyndromic, autosomal dominant 3). DFNA3 is caused by a heterozygous pathogenic mutation in one of the two genes. The relative prevalence of DFNA3 as a cause of autosomal dominant nonsyndromic hearing loss is not known, but it is extremely rare; only 14 pathogenic variants have been described worldwide. The majority of these pathogenic variants are described only in single families or simplex cases. Copy number changes in *GJB2* and *GJB6* related to DFNA3 have not been described (https://www.ncbi.nlm.nih.gov/books/NBK1536/).

About half of all X-linked hearing loss cases are caused by pathogenic mutations in the *POU3F4* gene and upstream region of the gene. Several X-linked hearing loss patients and families possess deletions that do not affect the transcribed region of *POU3F4*, but instead remove different portions of DNA upstream of *POU3F4* where putative regulatory elements reside. *POU3F4* is located on the X chromosome, in a 3-Mb gene desert region enriched for highly conserved non-coding regions (HCNRs). Multiple enhancers, important for *POU3F4* expression, are defined within these HCNRs at 970, 920, 170 and 12 kb upstream of *POU3F4* (Naranjo et al. 2010, Song et al. 2010, Vore et al. 2005).

Wolfram Syndrome is a progressive neurodegenerative disorder characterised by the onset of diabetes mellitus and optic atrophy before the age of 15 and is typically associated with sensorineural hearing loss, progressive neurologic abnormalities, and other endocrine abnormalities. The estimated prevalence of Wolfram Syndrome type 1 is 1 in 500,000 people worldwide. Mutations in *WFS1* cause more than 90% of Wolfram Syndrome type 1 cases (https://www.ncbi.nlm.nih.gov/books/NBK4144/).

Gene structure

The *GJB2* gene (2 exons) spans ~5.5 kb of genomic DNA on chromosome 13q12. The *GJB2* LRG_1350 sequence is available at www.lrg-sequence.org and is identical to GenBank NG_008358.1. The *GJB3* gene (2 exons) spans ~5.1 kb of genomic DNA on chromosome 1p34. The *GJB6* gene (5 exons) spans ~10 kb of genomic DNA on chromosome 13q12. The *GJB6* LRG_1395 sequence is available at www.lrg-sequence.org and is identical to GenBank NG_008323.1. The *POU3F4* gene (1 exon) spans ~3.9 kb of genomic DNA on chromosome Xq21. The *WFS1* gene (8 exons) spans ~33.4 kb of genomic DNA on chromosome 4p16. The *WFS1* LRG_1417 sequence is available at www.lrg-sequence.org and is identical to GenBank NG_011700.1.

Transcript variants

For *GJB2,* one transcript variant has been described encoding the full length protein (NM_004004.6; 2290 nt; coding sequence 179-859; https://www.ncbi.nlm.nih.gov/gene/2706). This sequence is a reference standard in the NCBI RefSeq project. The ATG translation start site and stop codon are located in exon 2.

For *GJB3,* two transcript variants have been described, of which transcript variant 1 represents the longer transcript (NM_024009.3; 2192 nt; coding sequence 591-1403; https://www.ncbi.nlm.nih.gov/gene/2707). This sequence is a reference standard in the NCBI RefSeq project. The ATG translation start site and stop codon are located in exon 2.

For *GJB6,* four transcript variants have been described, of which transcript variant 1 represents the longest transcript (NM_001110219.3; 2064 nt; coding sequence 546-1331; https://www.ncbi.nlm.nih.gov/gene/10804). This sequence is a reference standard in the NCBI RefSeq project. The ATG translation start site and stop codon are located in exon 5.

For *POU3F4,* one transcript variant has been described encoding the full length protein (NM_000307.5; 3838 nt; coding sequence 36-1121; https://www.ncbi.nlm.nih.gov/gene/5456). This sequence is a reference standard in the NCBI RefSeq project. The ATG translation start site and stop codon are located in exon 1.

For *WFS1,* two transcript variants have been described, of which transcript variant 1 represents the longer transcript (NM_006005.3; 3640 nt; coding sequence 171-2843; https://www.ncbi.nlm.nih.gov/gene/7466). This sequence is a reference standard in the NCBI RefSeq project. The ATG translation start site is located in exon 2 and the stop codon is located in exon 8.

Exon numbering

The exon numbering used in this P163-E1 GJB-WFS1-POU3F4 product description is from:

Gene	RefSeq transcript	Remarks
GJB2	NM_004004.6	Identical to LRG_1350
GJB3	NM_024009.3	-
GJB6	NM_001110219.3	Identical to LRG_1395
POU3F4	NM_000307.5	-
WFS1	NM_006005.3	Identical to LRG_1417

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 P163-E1 GJB-WFS1-POU3F4 contains 51 MLPA probes with amplification products between 121 and 500 nucleotides (nt). This includes two probes for *GJB2* and five probes for *GJB6*, and several other probes are present for the 13q12 region. Four *GJB3* probes are present, covering the two exons. Two probes are included covering exon 1 of the *POU3F4* gene, as well as seven probes targeting the conserved regions up to 1 Mb upstream of *POU3F4*. This probemix contains nine probes for the *WFS1* gene; one probe for each exon and two probes for exon 8. Furthermore, this probemix contains six probes detecting the wild type sequence of the c.313del14, c.235delC, c.167delT, c.101T>C, c.35delG and IVS1+1G>A mutations in the *GJB2* gene and a reduced signal can point towards the presence of this mutation or a (partial) deletion of *GJB2*. In addition, ten 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 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 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
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 hereditary hearing loss or Wolfram 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. Sample ID numbers NA23835, HG00478, NA12606 and NA22601 from the Coriell Institute have been tested with this P163-E1 probemix at MRC Holland and can be used as a positive control sample (see table below). The quality of cell lines can change; therefore samples should be validated before use.

Sample name	Source	Altered target genes in P163-E1	Expected copy number alteration
NA23835	Coriell Institute	*GJB2*	c.35delG and c.101T>C mutations
HG00478	Coriell Institute	*GJB2*	c.235delC mutation
NA12606	Coriell Institute	*GJB2*, *GJB6* and flanking region	Heterozygous duplication of 42 Mb, affecting all probes as listed in Table 2d
NA22601	Coriell Institute	*WFS1*	Heterozygous *WFS1* deletion

Performance characteristics

The frequency of large deletions or duplications in *GJB2* and/or *GJB6* is 1–10% of all the mutated DFNB1 alleles. The most frequent mutation is the c.35delG in *GJB2* comprising 70% of mutated DFNB1 alleles in some populations with a carrier rate of 1–3% in the general population. No deletions or duplications in the *GJB3* gene have been described so far. The frequency of deletions or duplications in *POU3F4* or upstream of this gene in X-linked hearing loss cases is ~41%. The frequency of *WFS1* deletions or duplications in Wolfram Syndrome 1 cases is ~10%. The analytical sensitivity and specificity for the detection of deletions or duplications in the *GJB2*, *GJB3*, *GJB6*, *POU3F4* and *WFS1* genes is very high and can be considered >99% (based on a 2007-2022 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.

Interpretation of results

The expected results for the probes detecting autosomal sequences are allele copy numbers of 2 (normal), 1 (heterozygous deletion), or 3 (heterozygous duplication). The same results can be expected for the X-chromosome-specific probes in female samples. For the X-chromosome-specific probes in male samples, expected copy numbers are 1 (normal), 0 (deletion) or 2 (duplication).

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 when **reference samples of the same sex** have been used:

Copy number status		Final ratio (FR)
Autosomal sequences 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

The P163 probemix contains six probes detecting the wildtype sequence at the location of relatively common point mutations in the *GJB2* gene. If one of these probes show a FR lower than 0.65, this can be due to a *GJB2* deletion or due to the presence of the corresponding mutation. Other variants near the ligation site can also cause a lowered signal. A positive result must be confirmed by another method.

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 *WFS1* 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.

P163 specific notes:

- Deletion of a probe's recognition sequence on the X-chromosome will lead to the complete absence of the corresponding probe amplification product in males, whereas female heterozygotes are recognisable by a 35-60% reduction in relative peak height.
- Six probes are present detecting the wild type sequence of the c.313del14, c.235delC, c.167delT, c.101T>C, c.35delG and IVS1+1G>A mutations in the *GJB2* gene and a reduced signal can point towards the presence of this mutation or a deletion of *GJB2*.
- A map that depicts the location of the *GJB2*, *GJB6* and *CRYL1* genes in relation to common deletions can be found in Hoefsloot et al. 2013.
- Multiple enhancers (HCNRs 81676, 81728, 81866 and 82478), important for *POU3F4* expression, are targeted by seven probes (Table 1 and Table2c).

Limitations of the procedure

- In most populations, the major cause of genetic defects in the *GJB2*, *GJB3*, *POU3F4* and *WFS1* genes are small (point) mutations, most of which will not be detected by using SALSA MLPA Probemix P163 GJB-WFS1-POU3F4.
- 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.

Leiden Open Variation Database (LOVD)

https://databases.lovd.nl/shared/genes/ We strongly encourage users to deposit positive results in the LOVD for the corresponding gene. 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 *WFS1* exons 3 and 5 but not exon 4) to MRC Holland: info@mrcholland.com.

Table 1. SALSA MLPA Probemix P163-E1 GJB-WFS1-POU3F4

Length (nt)	SALSA MLPA probe	Chromosomal position (hg18)[a]					
		reference	*GJB2*	*GJB6*	*GJB3*	*WFS1*	*POU3F4*
64-105	Control fragments – see table in probemix content section for more information						
121 *	Reference probe 19041-L31238	5q					
127 ¥ +	**POU3F4 probe** 21761-L13889						Exon 1
132 *	**WFS1 probe** 21747-L30412					Exon 8	
138	Reference probe 00662-L21335	6p					
146 ¥ + Ø	**POU3F4 probe** 21762-L14398						Upstream
151 ¥ Δ	**WFS1 probe** 05375-L30787					Exon 1	
157	**GJB3 probe** 14206-L21338				Exon 1		
162 ∫	**GJB6 probe** 05370-L04761			Exon 2			
166	**WFS1 probe** 05377-L11414					Exon 3	
172 ∞	**GJB2 probe** 09862-L09256		WT at c.35delG, ex2				
178 ¥	**GJB2 probe** 21979-L19379		Exon 1				
184	**WFS1 probe** 05379-L04770					Exon 5	
190	**GJB3 probe** 05367-L04758				Exon 1		
196 ∫	**GJB6 probe** 05371-L04762			Exon 3			
202 * + Ø	**POU3F4 probe** 21748-L30413						Upstream
208 Δ	**WFS1 probe** 06699-L04772					Exon 7	
216	Reference probe 05707-L06963	3q					
223 « ¬	**PSPC1 probe** 02399-L06768		13q12				
229 Δ	**WFS1 probe** 05376-L04767					Exon 2	
235 *	Reference probe 15108-L25275	8p					
241 ¥	**GJB3 probe** 05368-L31484				Exon 2		
247 ¥	**GJB2 probe** 22362-L31524		Exon 1				
252	**WFS1 probe** 05378-L04769					Exon 4	
259 *	**GJB6 probe** 22189-L31241			Exon 5			
267 ¥ ∞	**GJB2 probe** 22413-L31695		WT at IVS1+1G>A				
274 ∞	**GJB2 probe** 13150-L14407		WT at c.101T>C, ex2				
282 ∫	**GJB6 probe** 06701-L04763			Exon 4			
292 ¥	**GJB3 probe** 05369-L23138				Exon 2		
303 *	Reference probe 05697-L05139	12q					
312 ¥ ∞	**GJB2 probe** 09118-L30816		WT at c.313del14, ex2				
319	**WFS1 probe** 06702-L09985					Exon 6	
328	**GJB2** probe 05365-L04756		Exon 2				
337	**GJB6 probe** 05374-L04765			Exon 5			
346	Reference probe 02473-L01917	15q					
355 ¬	**LATS2 probe** 06704-L03639		13q12				
364 ¥ + Ø	**POU3F4 probe** 21763-L14400						Upstream
373 *	Reference probe 08878-L08934	2p					
390 ¥ + Ø	**POU3F4 probe** 21764-L14397						Upstream
396 ¥ ∞	**GJB2 probe** 13126-L30618		WT at c.167delT, ex2				
402 ∞	**GJB2 probe** 13127-L15168		WT at c.235delC, ex2				
409	Reference probe 02669-L02136	11q					
418 + Ø	**POU3F4 probe** 13139-L14396						Upstream
425 *	**WFS1 probe** 21749-L30414					Exon 8	
436 +	**POU3F4 probe** 12771-L13887						Exon 1
445 * ∫	**CRYL1 probe** 21750-L30617		13q12, *CRYL1* exon 3				
453 *	Reference probe 16287-L18579	20q					
463 + Ø	**POU3F4 probe** 13142-L14399						Upstream
471 ¥ ¬	**ZMYM2 probe** 21765-L30428		13q12				
481 + Ø	**POU3F4 probe** 13144-L14401						Upstream
493 * ∫	**CRYL1 probe** 21751-L30416		13q12, *CRYL1* exon 8				
500	Reference probe 10218-L14675	7q					

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

* New in version E1.
¥ Changed in version E1. Minor alteration, no change in sequence detected.
+ Probe targets the X chromosome.
∞ Wild-type sequence detected. A lowered probe signal can be due to a *GJB2* deletion or due to the presence of the mutation. Other variants near the ligation site can also cause a lowered signal. A positive result must be confirmed by another method.
« Probe located in or near a GC-rich region. A low signal can be caused by salt contamination in the DNA sample leading to incomplete DNA denaturation, especially of GC-rich regions.
¬ Flanking probe. Included to help determine the extent of a deletion/duplication. Copy number alterations of only the flanking probes are unlikely to be related to the condition tested.
Δ SNP rs542977017 influences the 151 nt probe signal. SNP rs147734431 influences the 208 nt probe signal. SNP rs550975729 influences the 229 nt probe signal. In case of apparent deletions, it is recommended to sequence the region targeted by these probes.
Ø Probe targeting the HCNRs: 81676 is covered by 146 nt and 364 nt, 81728 is covered by 390 nt and 418 nt, 81866 is covered by 463 nt and 481 nt and 82478 is covered by 202 nt.
∫ Probes 282, 196, 162, 493 and 445 nt are expected to be affected by del(GJB6-D13S1830). Probes 282, 196, 162 and 493 nt are expected to be affected by del(GJB6-D13S1854).

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. P163-E1 probes arranged according to chromosomal location

Table 2a. *GJB3*, 1p34

Length (nt)	SALSA MLPA probe	*GJB3* exon[a]	Ligation site NM_024009.3	Partial sequence[b] (24 nt adjacent to ligation site)	Distance to next probe
157	14206-L21338	Exon 1	310-311	AGCCCAGCCCAT-GGCCACCGATGC	0.8 kb
190	05367-L04758	Exon 1	570 nt after exon 1, 39-40 in NM_001005752.1	CCATGGGAGTGT-GTCAGGTGGAAG	2.8 kb
		start codon	*591-593 (exon 2)*		
241	05368-L31484	Exon 2	1003-1004	CCTCATCTTCAA-GCTCATCATTGA	1.0 kb
		stop codon	*1401-1403 (exon 2)*		
292	05369-L23138	Exon 2	2019-2020	GGAAGCCCTCTT-TCCCCAAATCCT	

Table 2b. *WFS1*, 4p16

Length (nt)	SALSA MLPA probe	*WFS1* exon[a]	Ligation site NM_006005.3	Partial sequence[b] (24 nt adjacent to ligation site)	Distance to next probe
151 Δ	05375-L30787	Exon 1	21-22	GCCGCGCTAGCC-GGCTCTTCAGCA	7.7 kb
		start codon	*171-173 (exon 2)*		
229 Δ	05376-L04767	Exon 2	260-261	CTCAATGCCACA-GCCTCGTTGGAG	9.6 kb
166	05377-L11414	Exon 3	425-426	GGAGACATGGAA-ATCCCCTTTGAA	1.9 kb
252	05378-L04769	Exon 4	537-538	AAGAACTCAACA-GCTGCACCGCTG	2.3 kb
184	05379-L04770	Exon 5	720-721	CCCTGGTCATGT-ACTGGAAGCTCA	0.6 kb
319	06702-L09985	Exon 6	811-812	AGATGGAGGGGC-GCAGCCAGGCCC	3.2 kb
208 Δ	06699-L04772	Exon 7	977-978	CAGGACGACGAA-GATGATGACGAG	5.6 kb
425	21749-L30414	Exon 8	1073-1074	GAGATCAAGGAG-TACCTGATTGAC	1.0 kb
132	21747-L30412	Exon 8	2037-2038	CTGTGGTGGGGA-TGGTGAAGTCCC	
		stop codon	*2841-2843 (exon 8)*		

Table 2c. *POU3F4* region, Xq21

Length (nt)	SALSA MLPA probe	*POU3F4* exon[a]	Ligation site NM_000307.5	Partial sequence[b] (24 nt adjacent to ligation site)	Distance to next probe
146 Ø	21762-L14398	Upstream	974 kb before exon 1	TGCCCTTCCTTT-ATTGAAGGGTTA	0.1 kb
364 Ø	21763-L14400	Upstream	974 kb before exon 1	TGACCCCTAGCA-CTGCTGCTGTAG	52.0 kb
390 Ø	21764-L14397	Upstream	922 kb before exon 1	GCCAATTTCTAA-AGTTACGCTTTT	0.3 kb
418 Ø	13139-L14396	Upstream	921 kb before exon 1	TTCCGGCCAAAC-CTCTTTGGATTG	137.3 kb
463 Ø	13142-L14399	Upstream	784 kb before exon 1	CTTCATGGCCAA-TGATCCATTCCA	0.2 kb
481 Ø	13144-L14401	Upstream	784 kb before exon 1	AAAAAGACCATA-ATTTGTTGAAGC	613.5 kb
202 Ø	21748-L30413	Upstream	170 kb before exon 1	TGTCATGGATGC-AGCACAGCCCAA	170.2 kb
		start codon	*36-38 (exon 1)*		
436	12771-L13887	Exon 1	63-64	CGAATCCCTACA-GCATTCTCAGTT	1.0 kb
127	21761-L13889	Exon 1	1112-1113	ACATCTTGCCAT-GATCTCTGACTG	
		stop codon	*1119-1121 (exon 1)*		

Table 2d. 13q12 region

Length (nt)	SALSA MLPA probe	Gene / exon[a]	Ligation site	Partial sequence[b] (24 nt adjacent to ligation site)	Distance to next probe
		Centromere			
223 « ¬	02399-L06768	*PSPC1*		TTCGCTACACAT-GGAGCAGCCTTG	221.2 kb
471 ¬	21765-L30428	*ZMYM2*		AGACTCTGAAAT-TCAGATTGCTAA	194.3 kb
		GJB2	**NM_004004.6**		
328	05365-L04756	Exon 2	1838-1839	TTTATTGACACA-GTACCATTTAAT	1.3 kb
		stop codon	*857-859 (exon 2)*		
312 ∞	09118-L30816	WT sequence at c.313del14 mutation, exon 2	495-496	GAAGAGGAAGTT-CATCAAGGGGGA	0.1 kb
402 ∞	13127-L15168	WT sequence at c.235delC mutation, exon 2	413-412 reverse	GATCAGCTGCAG-GGCCCATAGCCG	0.1 kb
396 ∞	13126-L30618	WT sequence at c.167delT mutation, exon 2	345-346	CTGCAACACCCT-GCAGCCAGGCTG	0.1 kb
274 ∞	13150-L14407	WT sequence at c.101T>C mutation, exon 2	279-278 reverse	CAACGAGGATCA-TAATGCGAAAAA	0.1 kb
172 ∞	09862-L09256	WT sequence at c.35delG mutation, exon 2	208-209	CAGACGATCCTG-GGGGGTGTGAAC	3.2 kb
		start codon	*179-181 (exon 2)*		
267 ∞	22413-L31695	WT sequence at IVS1+1G>A mutation, intron 1	1 nt after exon 1	TCCCGACGCAGG-TGAGCCCGCCGG	0.1 kb
247	22362-L31524	Exon 1	81-82	ACCCGCCTAGGA-GCGCAGGAGCCC	0.1 kb
178	21979-L19379	Upstream	55 nt before exon 1	TCCCAGTCTCCG-AGGGAAGAGGCG	29.0 kb
		GJB6	**NM_001110219.3**		
337	05374-L04765	Exon 5	2021-2022	TGAAATGTGGAT-GTGATTGCCTCA	0.8 kb
		stop codon	*1329-1331 (exon 5)*		
259	22189-L31241	Exon 5	1189-1190	CTACCTGCTGCT-GAAAGTGTGTTT	6.9 kb
		start codon	*546-548 (exon 5)*		
282 ∫	06701-L04763	Exon 4	416-417	ACTGTGCACCAT-TGGCTTCTAGGC	1.2 kb
196 ∫	05371-L04762	Exon 3	113 nt before exon 3	AAAGCTGGGAAG-ACGCTGGTCAGT	0.3 kb

Length (nt)	SALSA MLPA probe	Gene / exon[a]	Ligation site	Partial sequence[b] (24 nt adjacent to ligation site)	Distance to next probe
162 ∫	05370-L04761	Exon 2	121 nt after exon 2	TGTTTAGTGTCT-GATTGAACGTAA	172.9 kb
493 ∫	21751-L30416	*CRYL1* exon 8	NM_015974.3; 987-988	TGCCTCATGAGA-CTCGCCAAGTTG	85.3 kb
445 ∫	21750-L30617	*CRYL1* exon 3	NM_015974.3; 280-281	AAGAGCAGCTGT-CACTCATCAGTG	485.6 kb
355 ¬	06704-L03639	*LATS2*		CTGAGCACGCAT-TTTACGAATTCA	

[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.

∞ Wild-type sequence detected. A lowered probe signal can be due to a *GJB2* deletion or due to the presence of the mutation. Other variants near the ligation site can also cause a lowered signal. A positive result must be confirmed by another method.
« Probe located in or near a GC-rich region. A low signal can be caused by salt contamination in the DNA sample leading to incomplete DNA denaturation, especially of GC-rich regions.
¬ Flanking probe. Included to help determine the extent of a deletion/duplication. Copy number alterations of only the flanking probes are unlikely to be related to the condition tested.
Δ SNP rs542977017 influences the 151 nt probe signal. SNP rs147734431 influences the 208 nt probe signal. SNP rs550975729 influences the 229 nt probe signal. In case of apparent deletions, it is recommended to sequence the region targeted by these probes.
Ø Probe targeting the HCNRs: 81676 is covered by 146 nt and 364 nt, 81728 is covered by 390 nt and 418 nt, 81866 is covered by 463 nt and 481 nt and 82478 is covered by 202 nt.
∫ Probes 282, 196, 162, 493 and 445 nt are expected to be affected by del(GJB6-D13S1830). Probes 282, 196, 162 and 493 nt are expected to be affected by del(GJB6-D13S1854).

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.

Related SALSA MLPA probemixes

P153 EYA1	Contains probes for *EYA1*, involved in Branchiootorenal syndrome.
P186 PAX3-MITF-SOX10	Contains probes for *PAX3*, *MITF* and *SOX10,* involved in Waardenburg Syndrome.
P191/P192 X-linked Alport syndrome mix 1/2	Contains probes for *COL4A5*, involved in Alport syndrome.
P280 SLC26A4	Contains probes for *SLC26A4*, involved in Pendred syndrome & DFNB4
P292 PCDH15	Contains probes for *PCDH15*, involved in Usher syndrome.
P361/P362 USH2A	Contains probes for *USH2A*, involved in Usher syndrome.
P381/P382 COL11A1	Contains probes for *COL11A1*, involved in Marshall and type II Stickler syndrome.
P461 STRC-CATSPER2-OTOA	Contains probes for *CATSPER2*, *STRC* and *OTOA*, involved in Deafness Infertility Syndrome (DIS).

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P163 product history	
Version	*Modification*
E1	Two new probes for *WFS1*, one new probe upstream of *POU3F4*, and two new probes for *CRYL1* have been added. One probe for *GJB6* is replaced by a new probe. Several reference probes have been replaced and some probes have a change in length, but no change in sequence detected.
D1	One probe upstream of *GJB2* has been removed. One reference probe and two control fragments at 88 and 96 nt have been replaced.
C1	The three mutation-specific probes have been replaced by probes detecting the wild-type sequence at these mutations. In addition, probes for the wild-type sequence of the *GJB2* 167delT and 235delC mutations and eight probes for the *POU3F4* gene have been included. Two reference probes have been added, and three reference probes have been replaced. Finally, two control fragments at 100 and 105 nt have been added (X/Y specific).
B1	Three *GJB2* probes have been added that are specific for the c.35delG, IVS1+1G>A and 313del14 mutations. In addition, two DNA Denaturation control fragments (D-fragments) at 88 and 96 nt are now present.
A1	First release.

Implemented changes in the product description

Version E1-05 — 19 May 2023 (04P)
- Positive control DNA samples table updated, for NA12606 sample altered target genes further delineated including *GJB2, GJB6 and flanking region*.
- Warning for probe located in or near a GC-rich region, has been removed from Table 1 and 2 for 493 nt 21751-L30416 probe.
- Added P163 specific note and a footnote under Table 1 and Table 2c for multiple enhancers targeted.
- A footnote added under Table 1 and Table 2d for probes affected by del(GJB6-D13S1830) and del(GJB6-D13S1854).
- Selected publication using P163 updated.
- Updated the name for the related SALSA MLPA probemixes P191/P192.
- Various minor textual and lay-out changes.

Version E1-04 — 28 October 2021 (04P)
- Product description rewritten and adapted to a new template.
- UK has been added to the list of countries in Europe that accept the CE mark.
- Added exon numbers and ligation sites of the probes targeting the *CRYL1* gene in Table 2.
- Small changes of probe lengths in Table 1 and 2 in order to better reflect the true lengths of the amplification products.
- Added P163 specific note with reference to a map that depicts the location of common deletions.
- P461 probemix added to 'Related probemixes'.
- New reference added.
- Various minor textual changes.

Version E1-03 — 23 October 2020 (02P)
- Wording of the intended use has been updated to the new template. The content of the intended use did not change.
- Ligation sites of the probes targeting the *POU3F4* gene updated according to new version of the NM_ reference sequence.
- Exon numbering of the *GJB6* gene has been changed.
- Warning added to Table 1 and 2 for the 149 nt 05375-L30787, 208 nt 06699-L04772 and 229 nt 05376-L04767 probes, of which the signal is influenced by presence of SNPs in the target DNA.

- Added information about DFNA3 and the frequency of point mutations in the *GJB2* gene to the clinical background section.
- Added the *WFS1* LRG_1417 to the gene structure section.
- Added one new positive sample to the table on page 5.
- Added information on how to interpret results of the probes detecting the wildtype sequences at the location of frequent mutations in the *GJB2* gene.
- Removed link to the connexin deafness mutation database and included link to the LOVD.
- Removed the flanking probe symbols from the CRYL1 probes, since this gene contains cis-regulatory elements. Copy number changes are therefore related to the condition tested.
- New references added.
- Various minor textual changes.

Version E1-02 — 11 May 2020 (02P)

- Changes in Table 2d: the 24 nt sequence of the 259 nt 22189-L31241 probe has been corrected. The distances to the next probe for the 337 nt 05374-L04765 and the 259 nt 22189-L31241 probes have been corrected.

Version E1-01 — 04 July 2019 (02P)

- Product description adapted to a new product version (version number changed, changes in Table 1 and Table 2).
- P163-E1 is now CE marked.
- Probemix name changed to GJB-WFS1-POU3F4.
- Product description rewritten and adapted to a new template.
- For uniformity, the chromosomal locations and bands in this document are now all based on hg18 (NCBI36).
- Ligation sites of the probes targeting the *GJB2*, *GJB3* and *GJB6* genes updated according to new version of the NM_reference sequence.
- Exon numbering of the *GJB6* gene has been changed.
- Various minor textual changes.

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