Product Description
SALSA® MLPA® Probemix P334-A5 Gonadal

To be used with the MLPA General Protocol.

Version A5
As compared to version A4, four reference probes have been replaced and one reference probe has been removed. For complete product history see page 7.

Catalogue numbers:
- **P334-025R:** SALSA MLPA Probemix P334 Gonadal, 25 reactions.
- **P334-050R:** SALSA MLPA Probemix P334 Gonadal, 50 reactions.
- **P334-100R:** SALSA MLPA Probemix P334 Gonadal, 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 P334 Gonadal is a **research use only (RUO)** assay for the detection of deletions or duplications in the *DMRT1*, *CYP17A1*, *SRD5A2* and *HSD17B3* genes, which are associated with gonadal development disorders.

The development and function of the mammalian gonad is influenced by several pathways. In the current SALSA MLPA P334 probemix, four genes (*DMRT1*, *CYP17A1*, *SRD5A2* and *HSD17B3*) have been included which are all known to have an effect on gonadogenesis. The effects of mutations or loss of function of these genes range from pseudohermaphroditism, synthesis of androgens and oestrogens to gonadal dysgenesis.

The *DMRT1* gene (5 exons), spans ~127 kb of genomic DNA and is located on chromosome 9p24.3, 0.8 Mb from the p-telomere. The *CYP17A1* gene (8 exons), spans ~7 kb of genomic DNA and is located on chromosome 10q24.32, 105 Mb from p-telomere. The *SRD5A2* gene (5 exons), spans ~56 kb of genomic DNA and is located on chromosome 2p23.1, 32 Mb from p-telomere. The *HSD17B3* gene (11 exons), spans ~67 kb of genomic DNA and is located on chromosome 9q22.32, 98 Mb from the p-telomere.

More information is available at https://www.ncbi.nlm.nih.gov/books/NBK1547/.

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 *DMRT1* exon numbering used in this P334-A5 Gonadal product description is the exon numbering from the NG_009221.1 sequence. The *CYP17A1* exon numbering used in this product description is the exon

numbering from the NG_007955.1 sequence. The *SRD5A2* exon numbering used in this product description is the exon numbering from the NG_008365.1 sequence. The *HSD17B3* exon numbering used in this product description is the exon numbering from the LRG_1296 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 NG or 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 P334-A5 Gonadal contains 35 MLPA probes with amplification products between 165 and 472 nucleotides (nt). This includes ten probes for the *DMRT1* gene (two probes for each exon), seven probes for the *CYP17A1* gene (one probe for each exon except exon 8), seven probes for the *SRD5A2* gene (one probe for each exon and two probes for exons 1 and 4) and three probes for the *HSD17B3* gene (for exons 1, 3 and 11). In addition, eight reference probes are included that detect autosomal chromosomal locations. Complete probe sequences and the identity of the genes detected by the reference probes are available online (www.mrcholland.com).

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

Length (nt)	Name
64-70-76-82	Q-fragments (only visible with <100 ng sample DNA)
88-96	D-fragments (low signal indicates incomplete denaturation)
92	Benchmark fragment
100	X-fragment (X chromosome specific)
105	Y-fragment (Y chromosome specific)

MLPA technique

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

MLPA technique validation

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

Required specimens

Extracted DNA, free from impurities known to affect MLPA reactions. For more information please refer to the section on DNA sample treatment found in the MLPA General Protocol.

Reference samples

A sufficient number (≥3) of reference samples should be included in each MLPA experiment for data normalisation. All samples tested, including reference DNA samples, should be derived from the same tissue type, handled using the same procedure, and prepared using the same DNA extraction method when possible. Reference samples should be derived from different unrelated individuals who are from families without a history of gonadal development disorders. 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 from the Coriell Institute in the table below have been tested with this P334-A5 probemix at MRC Holland and can be used as positive control samples. The quality of cell lines can change; therefore samples should be validated before use.

Sample name	Altered target genes in P334-A5	Expected copy number alteration
NA02819	*DMRT1*	Heterozygous duplication
NA03563	*DMRT1*	Heterozygous deletion
NA00959	*CYP17A1*	Heterozygous duplication
NA08386	*CYP17A1*	Heterozygous duplication
NA01353	*SRD5A2*	Heterozygous duplication
NA09834	*HSD17B3*	Heterozygous deletion

Data analysis

Coffalyser.Net software should be used for data analysis in combination with the appropriate lot-specific MLPA Coffalyser sheet. For both, the latest version should be used. Coffalyser.Net software is freely downloadable at www.mrcholland.com. Use of other non-proprietary software may lead to inconclusive or false results. For more details on MLPA quality control and data analysis, including normalisation, see the Coffalyser.Net Reference Manual.

Interpretation of results

The standard deviation of each individual probe over all the reference samples should be ≤0.10 and the final ratio (FR) of each individual reference probe in the patient samples should be between 0.80 and 1.20. When these criteria are fulfilled, the following cut-off values for the FR of the probes can be used to interpret MLPA results for autosomal chromosomes or pseudo-autosomal regions:

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

Note: The term “dosage quotient”, used in older product description versions, has been replaced by “final ratio” to become consistent with the terminology of the Coffalyser.Net software. (Calculations, cut-offs and interpretation remain unchanged.) Please note that the Coffalyser.Net software also shows arbitrary borders as part of the statistical analysis of results obtained in an experiment. As such, arbitrary borders are different from the final ratio cut-off values shown here above.2

- Arranging probes according to chromosomal location facilitates interpretation of the results and may reveal more subtle changes such as those observed in mosaic cases. Analysis of parental samples may be necessary for correct interpretation of complex results.
- False positive results: Please note that abnormalities detected by a single probe (or multiple consecutive probes) still have a considerable chance of being a false positive result. Sequence changes (e.g. SNVs, point mutations) in the target sequence detected by a probe can be one cause. Incomplete DNA denaturation (e.g. due to salt contamination) can also lead to a decreased probe signal, in particular for probes located in or near a GC-rich region. The use of an additional purification step or an alternative DNA extraction method may resolve such cases. Additionally, contamination of DNA samples with cDNA or PCR amplicons of individual exons can lead to an increased probe signal (Varga et al. 2012). Analysis of an independently collected secondary DNA sample can exclude these kinds of contamination artefacts.
- Normal copy number variation in healthy individuals is described in the database of genomic variants: http://dgv.tcag.ca/dgv/app/home. Users should always consult the latest update of the database and scientific literature when interpreting their findings.

- Not all abnormalities detected by MLPA are pathogenic. In some genes, intragenic deletions are known that result in very mild or no disease (as described for *DMD* by Schwartz et al. 2007). For many genes, more than one transcript variant exists. Copy number changes of exons that are not present in all transcript variants may not have clinical significance. Duplications that include the first or last exon of a gene (e.g. exons 1-3) might not result in inactivation of that gene copy.
- Copy number changes detected by reference probes or flanking probes are unlikely to have any relation to the condition tested for.
- False results can be obtained if one or more peaks are off-scale. For example, a duplication of one or more exons can be obscured when peaks are off-scale, resulting in a false negative result. The risk on off-scale peaks is higher when probemixes are used that contain a relatively low number of probes. Coffalyser.Net software warns for off-scale peaks while other software does not. If one or more peaks are off-scale, rerun the PCR products using either: a lower injection voltage or a shorter injection time, or a reduced amount of sample by diluting PCR products.

Limitations of the procedure

- In most populations, the major cause of genetic defects in the *DMRT1*, *CYP17A1*, *SRD5A2* and *HSD17B3* genes are small (point) mutations, most of which will not be detected by using SALSA MLPA Probemix P334 Gonadal.
- 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.

***DMRT1*, *CYP17A1*, *SRD5A2* and *HSD17B3* mutation databases**

https://databases.lovd.nl/shared/genes/DMRT1
https://databases.lovd.nl/shared/genes/CYP17A1
https://databases.lovd.nl/shared/genes/SRD5A2
https://databases.lovd.nl/shared/genes/HSD17B3

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 *CYP17A1* exons 4 and 6 but not exon 5) to MRC Holland: info@mrcholland.com.

Table 1. SALSA MLPA Probemix P334-A5 Gonadal

Length (nt)	SALSA MLPA probe		Chromosomal position (hg18)[a]			
		Reference	*DMRT1*	*CYP17A1*	*SRD5A2*	*HSD17B3*
64-105	Control fragments – see table in probemix content section for more information					
165 *	Reference probe 09632-L09917	17q				
173	**DMRT1** probe 20614-L28621		**Exon 4**			
184	**DMRT1** probe 13067-L14286		**Exon 5**			
190	**CYP17A1** probe 13068-L14287			**Exon 3**		
196	**SRD5A2** probe 12920-L28622				**Exon 1**	
203 *	Reference probe 10873-L11543	15q				
208	**DMRT1** probe 13069-L14288		**Exon 3**			
214	**CYP17A1** probe 20615-L28623			**Exon 7**		
220	**HSD17B3** probe 12921-L14072					**Exon 3**
232	**SRD5A2** probe 12922-L14073				**Exon 4**	
238	**HSD17B3** probe 12923-L14074					**Exon 11**
246	**DMRT1** probe 13071-L14290		**Exon 1**			
256	**DMRT1** probe 13072-L14291		**Exon 3**			
262 *	Reference probe 12434-L13435	14q				
274	**CYP17A1** probe 13073-L14292			**Exon 2**		
281	**SRD5A2** probe 12924-L14075				**Exon 1**	
292	**DMRT1** probe 13074-L14293		**Exon 2**			
301	**CYP17A1** probe 13075-L14294			**Exon 5**		
310	**SRD5A2** probe 12925-L14076				**Exon 5**	
319	**CYP17A1** probe 13431-L14298			**Exon 1**		
328 *	Reference probe 19756-L26539	9q				
337	**CYP17A1** probe 13076-L14295			**Exon 4**		
346	**HSD17B3** probe 12926-L14077					**Exon 1**
355	**DMRT1** probe 13077-L14296		**Exon 5**			
362	**SRD5A2** probe 12927-L14078				**Exon 4**	
382	**DMRT1** probe 13078-L14297		**Exon 2**			
391	**SRD5A2** probe 12928-L14079				**Exon 2**	
409	Reference probe 09615-L09910	20p				
418	**SRD5A2** probe 12930-L14081				**Exon 3**	
427	Reference probe 12652-L13726	18q				
436	**DMRT1** probe 13080-L14299		**Exon 1**			
445	Reference probe 19796-L26602	1q				
454	**DMRT1** probe 13082-L14301		**Exon 4**			
461	**CYP17A1** probe 21731-L14302			**Exon 6**		
472	Reference probe 09945-L10404	8q				

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

* New in version A5.

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. P334-A5 probes arranged according to chromosomal location

Table 2a. *DMRT1* gene

Length (nt)	SALSA MLPA probe	*DMRT1* exon[a]	Ligation site NM_021951.3	Partial sequence[b] (24 nt adjacent to ligation site)	Distance to next probe
		start codon	*143-145 (Exon 1)*		
436	13080-L14299	Exon 1	167-168	AGGCATTCAGCA-AGCCCTCTACAC	0.3 kb
246	13071-L14290	Exon 1	473-474	TGATCGCCGAGA-GGCAGCGCGTGA	4.8 kb
292	13074-L14293	Exon 2	40 nt before exon 2	GCAAAGCTGATT-CTGGAGTGCTGG	0.1 kb
382	13078-L14297	Exon 2	596-597	AAAGAGAGAACA-ATGGCAGTAACC	46.9 kb
256	13072-L14291	Exon 3	705-706	CATCCAGGATAT-TCCTGCTGTCAC	0.2 kb
208	13069-L14288	Exon 3	857-858	TGGCTGCTGATT-CTGCTTCTGGGG	22.8 kb
454	13082-L14301	Exon 4	1071-1072	GTTCTTCACTTT-TGAGGATGCTCC	0.1 kb
173	20614-L28621	Exon 4	56 nt after exon 4	TGGCTATCCAGT-ATTGGGTCATTA	51.0 kb
355	13077-L14296	Exon 5	1135-1136	AGCAGTCAAGAT-TCTGGCTTGGTT	0.2 kb
184	13067-L14286	Exon 5	1310-1311	TAACAGGCTTAT-TCCACTTTCCAT	
		stop codon	*1262-1264 (Exon 5)*		

Table 2b. *CYP17A1* gene

Length (nt)	SALSA MLPA probe	*CYP17A1* exon[a]	Ligation site NM_000102.4	Partial sequence[b] (24 nt adjacent to ligation site)	Distance to next probe
		start codon	*53-55 (Exon 1)*		
319	13431-L14298	Exon 1	312-313	GGAGGTGCTTAT-TAAGAAGGGCAA	1.8 kb
274	13073-L14292	Exon 2	427-428	CAGCTGCATCGA-AGGCTGGCGATG	0.4 kb
190	13068-L14287	Exon 3	588-589	AACCAATGTCAT-CTCCTTGATCTG	0.8 kb
337	13076-L14295	Exon 4	46 nt before exon 4	GCAGCTGGAGAA-GCAAAATGGAAG	1.0 kb
301	13075-L14294	Exon 5	864-865	GCAAGCCAAGAT-GAACTCAGATAA	0.5 kb
461	21731-L14302	Exon 6	1026-1027	TCTTCAGGTGAA-GAAGAAGCTCTA	1.1 kb
214	20615-L28623	Exon 7	1237-1238	GTTATCATCAAT-CTGTGGGCGCTG	
	No probe	Exon 8			
		stop codon	*1577-1579 (Exon 8)*		

Table 2c. *SRD5A2* gene

Length (nt)	SALSA MLPA probe	*SRD5A2* exon[a]	Ligation site NM_000348.4	Partial sequence[b] (24 nt adjacent to ligation site)	Distance to next probe
		start codon	*39-41 (Exon 1)*		
281	12924-L14075	Exon 1	86-87	GGCAGCGCCACT-TTGGTCGCCCTT	0.4 kb
196	12920-L28622	Exon 1	191 nt after exon 1	AGAGAGTTGACA-AGCGGCTGCGGC	46.8 kb
391	12928-L14079	Exon 2	425-426	CAAGGCTACTAT-CTGATTTACTGT	2.3 kb
418 #	12930-L14081	Exon 3	566-567	CCTGGAGAAATC-AGCTACAGGATT	1.8 kb
362	12927-L14078	Exon 4	84 nt before exon 4	TCTTGCTGCCTT-TGTGTATTTTGG	0.4 kb
232	12922-L14073	Exon 4	161 nt after exon 4	TGCTGGGCTTGT-ATTGAAGTTGGA	3.4 kb
310	12925-L14076	Exon 5	1275-1276	AGACCCAGAGCA-AACCCACTCCCA	
		stop codon	*801-803 (Exon 5)*		

Table 2d. *HSD17B3* gene

Length (nt)	SALSA MLPA probe	*HSD17B3* exon[a]	Ligation site NM_000197.2	Partial sequence[b] (24 nt adjacent to ligation site)	Distance to next probe
		start codon	*73-75 (Exon 1)*		
346	12926-L14077	Exon 1	198-199	TTGCCAAAGTCT-TTCTTGCGGTCA	47.1 kb
220	12921-L14072	Exon 3	303-304	AATGTTGTCCTT-ATTAGCCGGACG	19.5 kb
238	12923-L14074	Exon 11	1090-1091	ACCAGCACTTCA-ACCTAGTCCGCT	
		stop codon	*1003-1005 (Exon 11)*		

[a] See section Exon numbering on page 1 for more information.
[b] Only partial probe sequences are shown. Complete probe sequences are available at www.mrcholland.com. Please notify us of any mistakes: info@mrcholland.com.

This probe's specificity relies on a single nucleotide difference compared to 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. Single probe aberration(s) must be confirmed by another method.

Related SALSA MLPA probemixes

- P185 Intersex: Contains probes for the *NR0B1*, *NR5A1*, *SOX9* and *WNT4* genes.

References

- Schouten JP et al. (2002). Relative quantification of 40 nucleic acid sequences by multiplex ligation-dependent probe amplification. *Nucleic Acids Res.* 30:e57.
- Schwartz M et al. (2007). Deletion of exon 16 of the dystrophin gene is not associated with disease. *Hum Mutat.* 28:205.
- Varga RE et al. (2012). MLPA-based evidence for sequence gain: pitfalls in confirmation and necessity for exclusion of false positives. *Anal Biochem.* 421:799-801.

Selected publications using SALSA MLPA Probemix P334 Gonadal

- Andonova S et al. (2017). New territory for an old disease: 5-alpha-reductase type 2 deficiency in Bulgaria. *Sex Dev*. 11:21-28.
- Lima AC et al. (2015). Rare double sex and mab-3-related transcription factor 1 regulatory variants in severe spermatogenic failure. *Andrology* 3:825-833.
- Machado AZ et al. (2012). Absence of inactivating mutations and deletions in the DMRT1 and FGF9 genes in a large cohort of 46, XY patients with gonadal dysgenesis. *Eur J Med Genet*. 55:690-694.
- Neocleous V et al. (2012). Duplication of exons 3-10 of the HSD17B3 gene: A novel type of genetic defect underlying 17β-HSD-3 deficiency. *Gene*. 499:250-255.
- Papi G et al. (2018). 46,XY Disorder of Sex Development Caused by 17α-Hydroxylase/17, 20-Lyase Deficiency due to Homozygous Mutation of CYP17A1 Gene: Consequences of Late Diagnosis. *Case Rep Endocrinol*. 2018.
- Petri C et al. (2014). 17α-hydroxylase deficiency diagnosed in early infancy caused by a novel mutation of the CYP17A1 gene. *Horm Res Paediatr*. 81:350-355.
- Turkkahraman D et al. (2015). Identification of a novel large CYP17A1 deletion by MLPA analysis in a family with classic 17α-hydroxylase deficiency. *Sex Dev*. 9:91-97.

P334 product history	
Version	*Modification*
A5	Four reference probes have been replaced and one reference probe has been removed.
A4	One reference probe has been replaced and one probe length has been adjusted.
A3	One reference probe has been replaced and two reference probes have been removed.
A2	The 88 and 96 nt DNA Denaturation fragments have been replaced (QDX2).
A1	First release.

Implemented changes in the product description
Version A5-01 – 01 September 2021 (04P) - Product description rewritten and adapted to a new template. - Product description adapted to a new product version (version number changed, changes in Table 1 and Table 2). - Ligation sites of the probes targeting the *DMRT1*, *CYP17A1*, *SRD5A2* and *HSD17B3* genes updated according to new versions of the NM_ reference sequences. - Small changes of probe lengths in Table 1 and 2 in order to better reflect the true lengths of the amplification products. - Warning added to Table 2 for probe specificity relying on a single nucleotide difference between target gene and related gene or pseudogene. *Version 08 – 12 October 2017 (55)* - Product description adapted to a new product version (version number changed, lot number added, small changes in Table 1 and Table 2, new picture included). - References included on page 2. - Various minor textual changes.

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