Product Description SALSA® MLPA® Probemix P050-C1 CAH

To be used with the MLPA General Protocol.

Version C1. For complete product history see page 10.

Catalogue numbers:
- **P050-025R:** SALSA MLPA Probemix P050 CAH, 25 reactions.
- **P050-050R:** SALSA MLPA Probemix P050 CAH, 50 reactions.
- **P050-100R:** SALSA MLPA Probemix P050 CAH, 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.mlpa.com).

Certificate of Analysis: Information regarding storage conditions, quality tests, and a sample electropherogram from the current sales lot is available at www.mlpa.com.

Precautions and warnings: For professional use only. Always consult the most recent product description AND the MLPA General Protocol before use: www.mlpa.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 P050 CAH is a **research use only (RUO)** assay for the detection of large *CYP21A2* gene deletions, which are associated with Congenital Adrenal Hyperplasia (CAH). This probemix should be used in conjunction with sequence analysis. Some frequent point mutations in the *CYP21A2* gene can also be detected with this probemix, but reciprocal exchanges between *CYP21A2* and its pseudogene will be missed.

Congenital adrenal hyperplasia (CAH) is an autosomal recessive disorder which results from a deficiency in one of the enzymes involved in cortisol biosynthesis. CAH affects about 1 in 5,000 births, with a carrier frequency of 1 in 35. In about 95% of cases, CAH is caused by deficiency of the steroid 21-hydroxylating enzyme encoded by the *CYP21A2* gene. The inactive pseudogene *CYP21A1P* is located at a very close distance from *CYP21A2*. Both genes have 10 exons and are about 3.4 kb long. The great majority of the *CYP21A2* mutant alleles arise through recombination between *CYP21A2* and *CYP21A1P*. Approximately 20% of mutant alleles have DNA deletions of 30 kb that have been generated by unequal meiotic crossing-over, whereas 75% are due to gene conversion events, where an inactivating mutation is present in the *CYP21A2* gene that is usually only present in the *CYP21A1P* pseudogene.

Other genes in this unstable chromosomal region are the closely related complement genes *C4A* and *C4B* and the *TNXB* gene and its pseudogene *TNXA*. Orientation of these genes is as displayed in Figure 1.

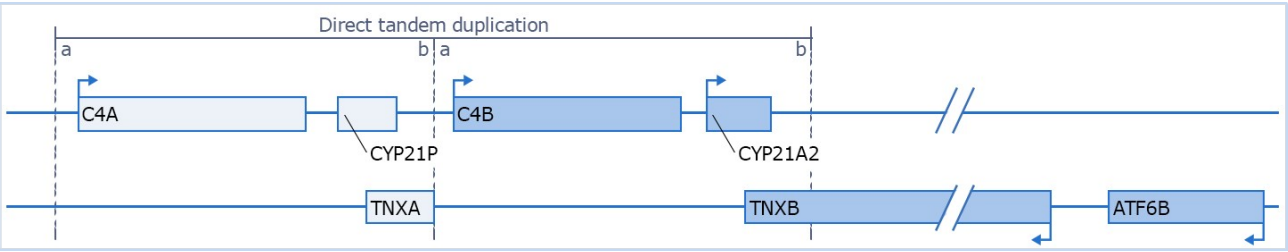

Figure 1. A schematic representation of the 151 kb chromosomal region with the duplicated gene cluster as it is present in the reference genome, containing the *TNXA* & *CYP21P* pseudogenes (light) and the *TNXB* & *CYP21A2* genes (dark). Arrows indicate direction of transcription. Note that the 3'-ends of *CYP21A2* and *TNXB* overlap. The figure represents genomic position chr6:31,946,000-32,097,000 of the GRCh37/hg19 assembly and is based on Gitelman et al. 1992.

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 *CYP21A2* exon numbering used in this P050-C1 CAH product description is the exon numbering from the RefSeq transcript NM_000500.9, which is identical to the exon numbering of NG_007941.3 sequence. The *TNXB* exon numbering used in this product description is the exon numbering from the RefSeq transcript NM_019105.8. The NM and NG sequences and exon numbering have been retrieved on 09/2019. As changes to the NCBI database can occur after release of this product description, exon numbering may not be up-to-date.

Probemix content: The SALSA MLPA Probemix P050-C1 CAH contains 27 MLPA probes with amplification products between 130 and 382 nucleotides (nt) This includes eight probes for the *CYP21A2* gene and four probes for the *CYP21A1P* pseudogene. The probemix furthermore contains six probes for the *TNXB* gene and one for the *ATF6B* gene in order to further define *CYP21A2* gene deletions. In addition, eight reference probes are included which detect eight different autosomal chromosomal locations. Complete probe sequences and the identity of the genes detected by the reference probes is available online (www.mlpa.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.mlpa.com.

Length (nt)	Name
64-70-76-82	Q-fragments (only visible with <100 ng sample DNA)
88-96	D-fragments (low signal of 88 nt and 96 nt fragment indicates incomplete denaturation)
92	Benchmark fragment
100	X-fragment (X chromosome specific)
105-118	Y-fragments (Y chromosome specific)

MLPA technique: The principles of the MLPA technique (Schouten et al. 2002) are described in the MLPA General Protocol (www.mlpa.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 unrelated individuals who are from families without a history of CAH. More information regarding the selection and use of reference samples can be found in the MLPA General Protocol.

Finding suitable reference samples for CAH-testing can be complicated. Perhaps due to the lack of selective pressure, many deletions, duplications and gene conversion events are present in the *CYP21A1P* pseudogene, thus complicating analysis. In our tests, only 21% of healthy DNA samples was heterozygous at the I2G locus and showed the usual number of two copies for all other autosomal sequences detected by the P050-C1 probes. The availability of reference samples carrying two copies of all probes greatly simplifies analysis of patient samples. A cell line derived reference selection DNA sample (SD039) is therefore included with the P050-C1 probemix.

Positive control DNA samples: MRC-Holland cannot provide positive DNA samples. Inclusion of a positive sample in each experiment is recommended. The Coriell Institute (https://catalog.coriell.org) and DSMZ (https://www.dsmz.de/home.html) have a diverse collection of biological resources which may be used as a positive control DNA sample in your MLPA experiments. Sample ID numbers NA14734 and NA12217 from the Coriell Institute have been tested with this P050-C1 probemix at MRC-Holland and can be used as a positive control sample to detect a homozygous deletion of the complete *CYP21A2* gene and smaller deletion (exons 1-4; heterozygous), respectively. Both samples have an increased *CYP21A1P* copy number. The quality of cell lines can change, therefore samples should be validated before use.

SALSA Reference Selection DNA SD039: The selection of suitable reference DNA samples that can be used with P050 CAH is complicated. To facilitate the selection of suitable reference DNA samples from your own sample collection, a reference selection DNA sample (catalogue number SD039) is provided with this probemix from MRC-Holland. Reference selection DNA SD039 should only be used for initial experiments on DNA samples from healthy individuals with the intention to identify suitable reference samples. **SD039 should not be used as a reference sample in subsequent experiments.** For further details, consult the Reference Selection DNA SD039 product description, available online: www.mlpa.com.

It is strongly advised to use test DNA samples and reference DNA samples extracted with the same method and derived from the same source of tissue. For certain applications, the selection of suitable reference DNA samples is complicated. Inclusion of one reaction with 5 µl SD039 Reference Selection DNA facilitates the identification of suitable reference DNA samples. For further instructions, consult the SD039 Reference Selection DNA product description provided. SD039 contains human genomic DNA purified from a selected cell line. This cell line contains one copy/cell for the two *CYP21A2* alleles at the I2G mutation location and one copy/cell for the sequence detected by the chromosome Y specific MLPA probe in the P050-C1 probemix. Furthermore, it contains two copies/cell of each sequence detected by the other MLPA probes in this probemix, including the six other *CYP21A2* probes and the four *CYP21A1P* pseudogene-specific MLPA probes. **This product is for research use only (RUO).**

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.mlpa.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 dosage quotient (DQ) of each individual reference probe in the patient samples should be between 0.80 and 1.20. When suitable reference samples are used that are similar in copy number to SD039, the following DQ values are expected:

Copy Number status	Dosage quotient normal probes	Dosage quotient I2G probes
0 copies	DQ = 0	DQ = 0
1 copy	0.40 < DQ < 0.65	0.80 <DQ <1.20
2 copies	0.80 < DQ < 1.20	1.75 < DQ < 2.15
3 copies	1.30 < DQ < 1.65	
4 copies	1.75 < DQ < 2.15	
Ambiguous copy number	All other values	All other values

- 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. Incomplete DNA denaturation (e.g. due to salt contamination) can 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.
- When running MLPA products, the capillary electrophoresis protocol may need optimization. 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: lower injection voltage / injection time settings, or a reduced amount of sample by diluting PCR products.

Recommendations for the analysis of results of P050:

1. Inclusion of suitable reference samples in your experiment is essential.
2. Data analysis should be performed as described above, e.g. with the use of the Coffalyser.Net software which is available free of charge on www.mlpa.com.
3. The analysed data should be arranged according to chromosomal location, similar to Table 2.
4. Signals of the probes detecting the *CYP21A2* wildtype sequence should be the main focus. **These are the important probes. All other probes are only included for accurate result interpretation.**
5. In the great majority of cases, an increased copy number detected by one or more of the *CYP21A2* probes will not be pathogenic and can be ignored. Many samples from healthy individuals show a duplication of one or more of the *CYP21A2* probe signals. Usually, this is accompanied by a decrease in copy number of the corresponding *CYP21A1P* sequence. In such cases, the pseudogene most likely has acquired the sequence of the wildtype *CYP21A2* gene, which has no clinical consequences.
6. DNA samples from healthy individuals should have a copy number of two or more for all *CYP21A2*-specific probes. Samples with a complete, or almost complete, *CYP21A2* gene deletion or a large gene conversion event will have a decreased probe signal for most *CYP21A2* probes. Please note that in samples with a large *CYP21A2* deletion, some probes may show a normal copy number. The most likely explanation for this is that one of the pseudogene copies has acquired the *CYP21A2* wildtype sequence at one or more probe locations.
7. Smaller gene conversion events will usually result in a decreased probe signal of one, or a small number of the *CYP21A2* probes. In most cases, the corresponding pseudogene-specific sequence will show an increased copy number. As explained in the section below, not all *CYP21A2* defects are expected to be detected as some pathogenic gene conversion events can be masked. Sequence analysis in combination with MLPA is required to detect all *CYP21A2* point mutations.
8. Please note that all *CYP21A1P*, *TNXB* and *ATF6B* probes are so-called flanking probes. They are included to facilitate the determination of the extent of a deletion/duplication and to help interpret copy number changes detected by the *CYP21A2* probes. Copy number alterations of flanking and reference probes are unlikely to be related to the condition tested.
9. In some cases, parental sample analysis might be required for proper interpretation of results.

Please note that this P050-C1 MLPA probemix will not detect all *CYP21A2* gene conversion events.
In order to detect large *CYP21A2* rearrangements, this MLPA probemix determines the copy number of the wildtype *CYP21A2* sequences at the location of several frequent mutations, such as I172N and the 8 nt deletion in exon 3. However, MLPA does not determine the chromosomal location of these sequences. As a result, many of the I172N and other point mutations in the *CYP21A2* gene will be detected by MLPA but others are missed as they are obscured by reciprocal sequence changes in the pseudogene. In fact, the *CYP21A1P* pseudogene very frequently contains the wildtype *CYP21A2* sequence at certain locations. A combination of MLPA and sequence analysis of the *CYP21A2* gene is therefore required for the detection of all mutations. Furthermore, when two different point mutations in *CYP21A2* are detected, MLPA results cannot indicate whether these inactivate the same or different *CYP21A2* alleles.

Typical results obtained on DNA samples from healthy individuals:
When testing DNA samples from 145 healthy individuals, we found that only 21% of these DNA samples have two copies per cell for all sequences detected by the P050-C1 probes. In the same sample set we observed, among other "abnormalities", the following copy number variants:

- Only one pseudogene copy in 32 samples. In most cases this was combined with a normal *CYP21A2* copy number, resulting in a total combined number (1x*CYP21A1P* + 2x*CYP21A2*) of only three copies. In three samples tested, no pseudogene copies were present. In 20 samples, we detected three pseudogene copies and one sample even contained four copies.
- The presence of three copies of the complete *CYP21A2* gene in six samples.
- Five samples tested appeared to be carrier for a (mild) CAH-related mutation: one sample contained only a single *CYP21A2* allele, two samples had only one wildtype sequence at the I172N location, and two samples contained only one copy of the -113 bp (in respect to start codon) wildtype sequence.

Our tests indicate that apparently a large percentage of the pseudogene *CYP21A1P* copies have acquired the typical wildtype *CYP21A2* sequence at the P30L, V281L, Q318X and/or R356W locations. This is not expected to have any consequences. For these sequences there are no probes included in P050-C1.

***CYP21A2* mutations that can be detected with the P050-C1 MLPA probemix:**

1. **Large rearrangements,** which can be subdivided in *CYP21A2* gene deletions and large 21A2/21A1P gene conversions (delA2 or LGC). Deletions typically extend approximately 30 kb and have their breakpoint somewhere between exons 3 and 8 of the *CYP21A1P* pseudogene and ending somewhere at the corresponding point in *CYP21A2*, thus yielding a single remaining gene in which the 5' end corresponds to the *CYP21A1P* pseudogene and the 3' end corresponds to the *CYP21A2* gene. As a consequence, no residual enzyme activity remains and patients with a homozygous deletion suffer from the salt wasting form of CAH.
2. **SNP at 113 bp before the start codon.** The 309 nt probe detects the wildtype *CYP21A2* sequence. The 292 nt probe detects the *CYP21A1P* pseudogene sequence. Transcription of the *CYP21A2* gene appears to be reduced several fold when the *CYP21A2* upstream region containing the -113 SNP has been replaced by the pseudogene-specific upstream sequence.
3. **I2G sequence** at 13 bp before exon 3 (intron 2 splice mutation; IVS-12A/C-G; A/C655G(i2); I2Splice; IVS2-655 C/AG). The *CYP21A2* gene has two wildtype alleles at this location: C and A. Both a C-to-G and an A-to-G mutation at nucleotide -13 will cause the severe 21-OH deficiency. This is the most frequent disease-causing mutation. Gene conversion resulting in the I2G mutation will result in a lower combined copy number of the 254 nt and 259 nt probes. The reference DNA sample SD039 is heterozygous for the two wildtype-specific sequences at this I2G location. No probe is present for the pseudogene-specific sequence.
4. **8 bp deletion** in exon 3 (del8bp; 706_713del8). This deletion results in a frame shift and the resulting protein product has no enzymatic activity. The 190 nt probe detects the wildtype sequence. The 184 nt probe detects the *CYP21A1P* pseudogene sequence containing the 8 nt deletion. This mutation abolishes enzymatic activity.
5. **I172N mutation** in exon 4. The 265 nt probe detects the wildtype I172N sequence. The 272 nt probe detects the *CYP21A1P* pseudogene sequence. Although the I172N mutation results in a very low (~1%) residual enzymatic activity, it seems that this low activity allows sufficient aldosterone synthesis to prevent significant salt wasting in most cases.
6. **V237E mutation** in exon 6. This is one of the three so called exon 6 cluster mutations. The 232 nt probe detects the V237E wildtype sequence. No probe is present for the corresponding *CYP21A1P*

pseudogene sequence. The signal of this probe might also be influenced by the I236N mutation; we did not have samples available to verify this. The cluster of three exon 6 missense mutations (I236N, V237E, M239K) abolishes enzymatic activity.

7. **M239K mutation** in exon 6. This is one of the three so called exon 6 cluster mutations. The 238 nt probe detects the M239K wildtype sequence. No probe is present for the corresponding *CYP21A1P* pseudogene sequence. The signal of this probe might also be influenced by the V237E and/or the I236N mutations; we did not have samples available to verify this.
8. **F306+T mutation** in exon 7. The 214 nt probe detects the wildtype F306+T sequence. The 220 nt probe detects the *CYP21A1P* pseudogene sequence. This mutation results in a frame shift which abolishes enzymatic activity.

Most CAH patients are compound heterozygotes for two of the abovementioned mutations or the P30L, V281L, Q318X, P453S or R356W mutations. Each unique combination of alleles results in a different residual enzyme activity. This in turn determines the clinical form of the congenital adrenal hyperplasia: salt-wasting (SW), simple virilising (SV), nonclassic late onset (NC; attenuated; acquired) and cryptic.

More information: White and Speiser (2000) and http://www.ncbi.nlm.nih.gov/books/NBK1171/.

TNXB gene

The *TNXB* gene spans ~68 kb of genomic DNA and has 44 exons. Many *CYP21A2-CYP21A1P* gene conversions extend into the *TNXB* gene and inactivate that copy of the gene. Haploinsufficiency of the *TNXB* gene can cause the hypermobility type of Ehlers-Danlos syndrome (Zweers et al. 2003). Disruption of both copies of *TNXB* is the cause of a recessive form of the Ehlers-Danlos syndrome (MIM 606408). For the detection of TNXB copy number variations the P155 EDS probemix is recommended.

Limitations of the procedure:

- 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. SNPs, point mutations, small indels) in the target sequence detected by a probe can cause false positive results. Mutations/SNPs (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.
- Reciprocal exchanges between *CYP21A2* and its pseudogene will be missed.

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.

***CYP21A2* mutation database:** https://databases.lovd.nl/shared/genes/CYP21A2. We strongly encourage users to deposit positive results in the *CYP21A2* 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 SNPs and unusual results to MRC-Holland: info@mlpa.com.

Table 1. SALSA MLPA Probemix P050-C1 CAH

Length (nt)	SALSA MLPA probe	Chromosomal position (hg18)[a]			
		reference	*CYP21A2*	*CYP21A1P*	other
64-118	Control fragments – see table in probemix content section for more information				
130	Reference probe 00797-L13645	5q			
135	Reference probe 16316-L21434	3q			
148	**TNXB probe** 19037-L14637				Exon 35
166	Reference probe 10729-L11311	6p			
184 +	**CYP21A1P probe** 15221-L20262			Exon 3, del8bp location	
190	**CYP21A2 probe** 15221-L20261		**Exon 3, del8bp location**		
214	**CYP21A2 probe** 17261-L21169		**Exon 7, F306+T location**		
220 +	**CYP21A1P probe** 17261-L21170			Exon 7, F306+T location	
226	Reference probe 14471-L16191	4q			
232 »	**CYP21A2 probe** 17270-L16990		**Exon 6, V237E location**		
238 †	**CYP21A2 probe** 17271-L16989		**Exon 6, M239K location**		
244	Reference probe 16307-L19696	13q			
254 ∞	**CYP21A2 probe** 16645-L20231		**Exon 3, I2G location, C-allele**		
259 ∞	**CYP21A2 probe** 16645-L20299		**Exon 3, I2G location, A-allele**		
265	**CYP21A2 probe** 15220-L20667		**Exon 4, I172N location**		
272 +	**CYP21A1P probe** 15220-L20668			Exon 4, I172N location	
279	Reference probe 04988-L20303	8q			
292 +	**CYP21A1P probe** 15945-L18079			Exon 1, -113 bp SNP	
309	**CYP21A2 probe** 15944-L18351		**Exon 1, -113 bp SNP**		
318	**TNXB probe** 15230-L14636				Exon 35
326 #	**TNXB probe** 19038-L17756				Exon 23
336	Reference probe 09027-L09281	1q			
346	**ATF6B probe** 01979-L20800				Exon 1B
355	**TNXB probe** 15232-L01515				Exon 31
364	**TNXB probe** 15235-L04400				Exon 19
373 #	**TNXB probe** 15233-L15002				Exon 26
382	Reference probe 13329-L14755	18q			

a) See above section on exon numbering for more information.

∞ The *CYP21A2* gene has a SNP at the I2G mutation site. The 254 and 259 nt probes both detect wildtype sequences. **The copy number detected by these two probes should be combined. Many normal samples will have no signal for one of these two probes. As reference DNA SD039 contains one copy for each of these two alleles, a probe ratio of 1 corresponds to 1 copy for these probes.**

+ The *CYP21A1P* gene has a highly variable copy number in normal individuals. In our tests: 0 copies in 5% of samples; 1 copy in 22%; 2 copies in 56%; 3 copies in 15% and 4 copies in rare cases.

† The 238 nt probe at the M239K location (exon 6 cluster) might also be affected by the V237E and/or the I236N mutation. The M239K mutation is often present together with the V237E and I236N mutations.

» The 232 nt probe at the V237E location (exon 6 cluster) might also be affected by the I236N mutation. The V237E mutation is often present together with the M239K and I236N mutations.

This probe's specificity relies on a single nucleotide difference compared to a homologous sequence. As a result, an apparent duplication of only this probe can be the result of a non-significant single nucleotide sequence change in the highly homologous sequence.

Note 1: Amino acid numbering used in this P050-C1 CAH product description is according to the commonly used numbering in literature and OMIM. Please note that the amino acid numbering in the NM_000500.9 sequence is different. Please notify us of any mistakes. The identity of the genes detected by the reference probes is available on request: info@mlpa.com.

Table 2. *CYP21A2* region probes arranged according to chromosomal location

Length (nt)	SALSA MLPA probe	Gene/ Exon[a]	Ligation site	Partial sequence (24 nt adjacent to ligation site)[b]	Distance to next probe
***CYP21A1P* probes** Ligation sites of both the *CYP21A1P* and *CYP21A2* probes are shown according to the NM_000500.9 *CYP21A2* reference sequence (start codon: 9-11). The *CYP21A1P* probes will have one or more mismatches as compared to this sequence.					
292 ¬	15945-L18079	Exon 1	105 nt before ex 1 -113 bp SNP	GGTCGGTGGGAA-GGCACCTGAGGG	0.9 kb
184 ¬	15221-L20262	Exon 3	350-351 del8bp location	TGTCGTTGGTCT-CTGCTCTGGAAA	0.3 kb
272 ¬	15220-L20668	Exon 4	526-525, reverse, I172N location	TGAGGTAACAGT-TGATGCTGCAGG	0.8 kb
220 ¬	17261-L21170	Exon 7	927-928 F306+T location	GGCCGTGGTTTT-TTTTGCTTCACC	30.8 kb
***CYP21A2* probes** Ligation sites of the *CYP21A2* probes are shown in the NM_000500.9 reference standard in the NCBI RefSeqGene project.					
		start codon	*9-11*		
309	15944-L18351	Exon 1	105 nt before ex 1, reverse, **-113 bp SNP**	CTTCAGGTACCC-TCCCACCGACCC	0.8 kb
254 ∞ ∫	16645-L20231	Exon 3	13 nt before ex 3, reverse, **C-allele at I2G location**	TGCAGGAGGAGG-TGGGGGCTGGA	0.1 kb
259 ∞	16645-L20299	Exon 3	13 nt before ex 3, reverse, **A-allele at I2G location**	TGCAGGAGGAGT-TGGGGGCTGGAG	
190	15221-L20261	Exon 3	350-351 **del8bp wildtype**	GGAGACTACTCC-CTGCTCTGGAAA	0.3 kb
265	15220-L20667	Exon 4	526-527 **I172N wildtype**	CTGCAGCATCAT-CTGTTACCTCAC	0.4 kb
232 »	17270-L16990	Exon 6	721-722 **V237E wildtype**	GGATCACATCGT-GGAGATGCAGCT	0.0 kb
238 †	17271-L16989	Exon 6	727-726, reverse, **M239K wildtype**	GCCTCAGCTGCA-TCTCCACGATGT	0.4 kb
214	17261-L21169	Exon 7	927-928 **F306+T wildtype**	GGGCCGTGGTTT-TTTTGCTTCACC	3.6 kb
		stop codon	*1494-1496*		
***TNXB* Probes** Ligation sites are shown in the NM_019105.8 reference standard in the NCBI RefSeqGene project.					
		stop codon	*12893-12895 (exon 44)*		
148 ¤ ¬	19037-L14637	Exon 35	11670-11669 reverse	GGAAGCCTGTGA-GAGGCTCACTCT	0.1 kb
318 ¤ ¬	15230-L14636	Exon 35	11608-11609	CAGGGGCTGATC-CCAGGCGCTCGC	2.6 kb
355 ¬	15232-L01515	Exon 31	10554-10555	CTCCAGCTCTCT-GCGCCTGTCCTG	6.3 kb
373 # ¬	15233-L15002	Exon 26	9262-9261 reverse	GTCACACCCACA-GCGGACACTGGG	4.1 kb
326 # ¬	19038-L17756	Exon 23	8072-8073	GGGAGCTGACCA-TGACAGATGCCA	8.3 kb
364 ¬	15235-L04400	Exon 19	6745-6746	GCTCCTCTTGCA-AAGCTGCGCCTA	63.1 kb
		start codon	*167-169 (exon 2)*		
346 ¬	01979-L20800	*ATF6B* gene		GACAACCTGCTT- AGCCCGGAGGAC	-

a) See above section on exon numbering for more information.

b) Only partial probe sequences are shown. Complete probe sequences are available at www.mlpa.com. Please notify us of any mistakes: info@mlpa.com.

† The 238 nt probe at the M239K location (exon 6 cluster) might also be affected by the V237E and/or the I236N mutation. The M239K mutation is often present together with the V237E and I236N mutations.

» The 232 nt probe at the V237E location (exon 6 cluster) might also be affected by the I236N mutation. The V237E mutation is often present together with the M239K and I236N mutations.

¤ The 148 and 318 nt *TNXB* exon 35 probes are located within the 121 nt sequence that is absent in the *TNXA* pseudogene.

∞ The *CYP21A2* gene has a SNP at the I2G mutation site. The 254 and 259 nt probes both detect wildtype sequences. **The copy number detected by these two probes should be combined. Many normal samples will have no signal for one of these two probes. As reference DNA SD039 contains one copy for each of these two alleles, a probe ratio of 1 corresponds to 1 copy for these probes.**

¬ Flanking probe. All *CYP21A1P*, *TNXB* and *ATF6B* probes should be regarded as "flanking probes". They are included to interpret copy number changes detected by the *CYP21A2* probes and to determine the extent of deletions/duplications. Copy number alterations of flanking and reference probes are unlikely to be related to the condition tested.

∫ Probe signal is very sensitive to pipetting mistakes of the hybridisation mastermix.

This probe's specificity relies on a single nucleotide difference compared to a homologous sequence. As a result, an apparent duplication of only this probe can be the result of a non-significant single nucleotide sequence change in the highly homologous sequence.

Note 1: Amino acid numbering used in this P050-C1 CAH product description is according to the commonly used numbering in literature and OMIM. Please note that the amino acid numbering in the NM_000500.9 sequence is different. Please notify us of any mistakes. The identity of the genes detected by the reference probes is available on request: info@mlpa.com.

Related SALSA MLPA probemixes

- P155 EDS: Contains probes for the *COL3A1* and *TNXB* genes.
- P185 Intersex: Contains probes for the *NR0B1*, *NR5A1*, *SOX9* and *WNT4* genes.
- P312 POR: Contains probes for the *POR* gene.
- P334 Gonadal: Contains probes for the *DMRT1*, *CYP17A1*, *SRD5A2* and *HSD17B3* genes.

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Selected publications using SALSA MLPA Probemix P050 CAH

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- Wang R et al. (2016). 21-hydroxylase deficiency-induced congenital adrenal hyperplasia in 230 Chinese patients: Genotype-phenotype correlation and identification of nine novel mutations. *J Steroids.* 2016.01.007*.*
- Xu Z et al. (2013). Comprehensive mutation analysis of the CYP21A2 gene: an efficient multistep approach to the molecular diagnosis of congenital adrenal hyperplasia. *J Mol Diagn*. 15:745-53.

P050 Product history	
Version	*Modification*
C1	Probemix was completely redesigned.
B3	The 88 nt and 96 nt control fragments have been replaced (QDX2).
B2	Extra control fragments have been added at 88, 96, 100 and 105 nt.
B1	One target probe was replaced, several reference probes were replaced.
A2	Multiple target probes and reference probes replaced.
A1	First release.

Implemented changes in the product description

Version C1-04 – 01 July 2020 (02P)
- Minor corrections were implemented in section Positive control DNA samples.

Version C1-03 — 09 October 2019 (02P)
- Product description rewritten and adapted to a new template.
- Ligation sites of the probes targeting the *TNXB* gene updated according to new version of the NM_019105.8 reference sequence.
- Figure 1 and its legend have been updated.
- Exon numbering section has been included.
- Warning added to Table 1 and 2 for probe specificity relying on a single nucleotide difference between target gene and related gene or pseudogene.

Version C1-02 – 23 January 2019 (01P)
- Several minor textual changes.
- Dosage quotient I2G probes added to Table X.
- Size and number of exons for the *TNXB* gene updated.
- The NM sequence used for the *CYP21A2* and *CYP21A1P* probes updated to NM_000500.9.
- The NM sequence used for the *ATF6B* probe updated to NM_004381.5. Subsequently, the position to exon 1B and the ligation site in Table 2 updated.
- A few distances between probes in Table 2updated.
- Warning added to probe 16645-L20231 (254 nt) in Table 2.
- Probemix P312 POR added to the related SALSA MLPA probemixes section.
- Two references added to the selected publications section.

Version C1-01 – 17 May 2018 (01P)
- Product description restructured and adapted to a new template.
- Ligation sites of the probes targeting the *CYP21A2* gene updated according to the new version of the NM_ reference sequence.

Version 35 – 16 August 2017 (55)
- Electropherogram picture adjusted from male DNA to SD039.
- Minor textual and layout changes.

Version 34 – 7 July 2017 (55)
- Product description adapted to a new product lot (lot number added, new picture included).

Version 33 – 20 February 2017 (55)
- Note on deviating amino acid numbers changed.
- Several minor textual changes.

Version 32 – 07 February 2017 (55)
- Note on deviating amino acid numbers added.
- Small changes to Table 2.

Version 31 (53)
- Product description adapted to a new product lot (lot number added, new picture included).

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