Product Description
SALSA® MLPA® Probemix P050-D1 CAH

To be used with the MLPA General Protocol.

Version D1
As compared to version C1, I173N WT sequence probe detects the same sequence but now on the reverse strand. Five CYP21A2/CYP21A1P probes changed in length not in sequence detected. Two TNXB probes have been replaced. One reference probe has been replaced and three have been added. For complete product history see page 15.

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.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 and the complex genetic nature of the *CYP21A2-CYP21A1P* locus before drawing any conclusions from findings generated with this product. Please note that PCR amplification for sequence analysis of exons 1-3 of the *CYP21A2* gene can easily cause inaccurate sequencing results due to allele dropout, which may be inconsistent with MLPA results (For more details, see point 8 in section *Recommendations for the analysis of results of P050* on page 6).

This product requires the identification of suitable reference samples for proper data analysis. For more information, see section on reference samples (page 4).

Intended purpose
The SALSA MLPA Probemix P050 CAH is an in vitro diagnostic (IVD)[1] or research use only (RUO) semi-quantitative assay[2] for the detection of large deletions and large gene conversions in the *CYP21A2* gene and its surrounding region located on chromosome 6p21.3. P050 CAH is intended to confirm a potential cause for and clinical diagnosis of Congenital Adrenal Hyperplasia (CAH) and for molecular genetic testing of at-risk family members. This probemix is for use with genomic DNA isolated from human peripheral whole blood specimens or prenatal DNA isolated from (un)cultured amniotic fluid obtained in week 16 of the pregnancy or later and free from blood contamination, (un)cultured chorionic villi free from maternal contamination, or fetal blood.

Copy number variations (CNVs) detected with P050 CAH should always be confirmed with a different technique. In particular, CNVs detected by only a single probe always require confirmation by another method. Reciprocal exchanges between *CYP21A2* and its pseudogene *CYP21A1P* will not be detected. It is therefore recommended to always 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, parental evaluation, 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 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, Reference Selection DNA SD039 and Coffalyser.Net analysis software.

Clinical background
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 approximately 1:5,000 births, and among the general population the carrier frequency is estimated at 1:35. In ~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 closely upstream of *CYP21A2*. Both the gene and pseudogene have 10 exons and span ~3.2 kilobases (kb). The great majority of the *CYP21A2* mutant alleles arise through recombination between *CYP21A2* and *CYP21A1P*. In most populations ~65-75% of the pathogenic *CYP21A2* mutations are point mutations or small indels, the far majority (>90%) of which are microconversions where the *CYP21A2* gene has obtained a single small inactivating mutation from the *CYP21A1P* pseudogene. The frequency of large deletions and large conversions ranges from ~10 to 35%, two-thirds of which originate from unequal meiotic cross-overs resulting in intergenic deletions of 30 kb and the formation of a *CYP21A1P-CYP21A2* chimeric non-functional gene.

Other genes located in this chromosomal region, also referred to as RCCX module, are the closely related complement genes *C4A* and *C4B*, and the *TNXB* gene and its pseudogene *TNXA*. Some large rearrangements in this region affect both *CYP21A2* and *TNXB*. Orientation of these genes is as displayed in Figure 1. When both functional alleles of *CYP21A2* and both alleles of *TNXB* are lost this leads to the contiguous gene syndrome CAH-X, where the patient develops symptoms of classic-like Ehlers-Danlos syndrome (a disorder affecting the connective tissues) in combination with CAH. Note that for the detection of deletions or duplications in the *TNXB* gene the SALSA MLPA Probemix P155 EDS is recommended.

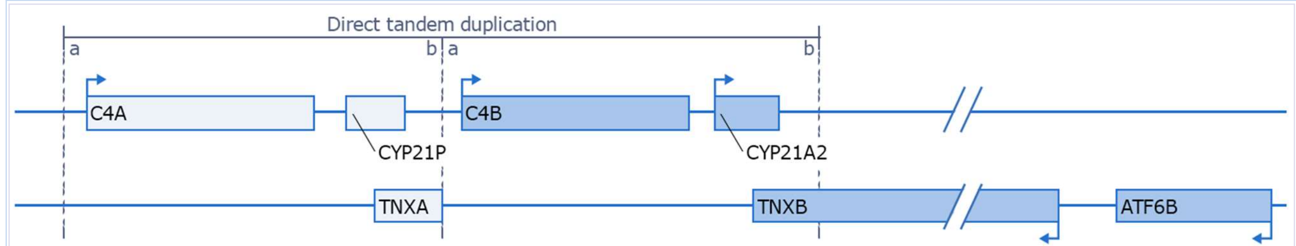

Figure 1. A schematic representation of the 151 kb chromosomal region with the duplicated gene cluster as it is present in the reference genome, containing the *TNXA* & *CYP21P* pseudogenes (light) and the *TNXB* & *CYP21A2* genes (dark). Arrows indicate direction of transcription. Note that the 3'-ends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

Gene structure
The *CYP21A2* gene spans 3.2 kb on chromosome 6p21.32 and contains 10 exons. The *CYP21A2* LRG_829 is available at www.lrg-sequence.org and is identical to GenBank NG_007941.3.

The *TNXB* gene spans 68 kb on chromosome 6p21.32 and contains 44 exons. No LRG is available for *TNXB*; the GenBank chromosomal sequence is NG_008337.2.

Transcript variants
For *CYP21A2,* multiple variants have been described. Transcript variant 1 encodes the longer isoform *a* (NM_000500.9; 2006 nucleotides (nt); coding sequence (CDS) 9-1496; https://www.ncbi.nlm.nih.gov/gene/1589). This sequence is a reference standard in the NCBI RefSeq project. The ATG translation start site is located in exon 1 and the stop codon is located in exon 10.

For *TNXB,* multiple variants have been described. Transcript variant XB encodes isoform 1 (NM_019105.8; 13091 nt; CDS 167-12895; https://www.ncbi.nlm.nih.gov/gene/7148). 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 44.

Exon numbering

The *CYP21A2* exon numbering used in this P050-D1 CAH product description is the exon numbering from the LRG_829 sequence. The exon numbering is the same as RefSeq transcript NM_000500.9 and the LRG genomic sequence is identical to NG_007941.3.

The *TNXB* exon numbering used in this P050-D1 CAH product description is the exon numbering from the NG_008337.2 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 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 P050-D1 CAH contains 30 MLPA probes with amplification products between 130 and 382 nt. This includes eight probes for the *CYP21A2* gene and four probes for the *CYP21A1P* pseudogene. Furthermore the probemix contains six probes for the *TNXB* gene and one for the *ATF6B* gene in order to further delineate *CYP21A2* gene deletions. In addition, eleven reference probes are included that detect autosomal chromosomal locations. Complete probe sequences and the identity of the genes detected by the reference probes are available online (www.mrcholland.com).

This probemix contains ten quality control fragments generating amplification products between 64 and 118 nt: four DNA Quantity fragments (Q-fragments), two DNA Denaturation fragments (D-fragments), one Benchmark fragment, and one chromosome X and two chromosome Y-specific fragments (see table below). More information on how to interpret observations on these control fragments can be found in the MLPA General Protocol and online at www.mrcholland.com.

Length (nt)	Name
64-70-76-82	Q-fragments (only visible with <100 ng sample DNA)
88-96	D-fragments (low signal indicates incomplete denaturation)
92	Benchmark fragment
100	X-fragment (X chromosome specific)
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.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 from human peripheral whole blood specimens or prenatal DNA isolated from (un)cultured amniotic fluid obtained in week 16 of the pregnancy or later and free from blood contamination, (un)cultured chorionic villi free from maternal contamination, or fetal 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 preferably 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 CAH. More information regarding the selection and use of reference samples can be found in the MLPA General Protocol (www.mrcholland.com).

Finding suitable reference samples for CAH-testing can be difficult. Deletions, duplications and gene conversion events are often observed in the *CYP21A1P* pseudogene, thereby complicating analysis. Reference samples should have one of each wild type (WT) allele at the I2G locus, *i.e.* one I2G-C allele (detected by probe 21552-L32321) and one I2G-A allele (detected by probe 21552-L20299). Two copies should be present for all other autosomal sequences detected by the other P050-D1 probes. See page 8 for further information on the I2G locus and other targeted loci. In our experience, approximately 1 in 5 (~20%) DNA samples from the general European population can be a suitable reference sample. SALSA Reference Selection DNA SD039 (provided with this probemix; see section *SALSA Reference Selection DNA SD039* below for more information) should be used in initial experiments to select suitable reference samples from your own sample collection. Suitable reference samples provide results equivalent to Reference Selection DNA SD039 for each probe in this probemix, including the two probes for both I2G WT alleles.

SALSA Reference Selection DNA SD039
As described above the selection of suitable reference DNA samples for P050 CAH is complicated. To facilitate this selection 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 and it **should never be used as a reference sample in subsequent experiments.** For further details, consult the Reference Selection DNA SD039 product description, available online: 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 NA14734 and NA12217 from the Coriell Institute have been tested with this P050-D1 probemix at MRC Holland. NA14734 has a homozygous deletion of the complete *CYP21A2* gene, of which the obtained result is shown in the table below. NA12217 has a typical, more complex, genotype: a large deletion/conversion spanning exon 1-3 on one allele, and a micro-conversion of the I173N locus in exon 4 on the second allele. How these different alleles translate to the Final Ratio (FR) of the probes is shown below. **Note that this and other complex genotypes *cannot be determined with MLPA alone* and require allele/locus-specific long range PCR amplification, sequencing data and often parental evaluation for full delineation of the genotype.** The quality of cell lines can change; therefore samples should be validated before use.

Probe[a]		Suitable reference sample[b]			NA14734			NA12217[e]		
Length (nt)	Gene, exon, & target	Allele 1	Allele 2	FR[c]	Allele 1	Allele 2	FR	Allele 1	Allele 2	FR
292	*CYP21A1P* exon 1 (-113 SNP)	1[d]	1	**1.0**	1	1	**1.0**	**2**	1	**1.5**
184	*CYP21A1P* exon 3 (del8bp)	1	1	**1.0**	1	1	**1.0**	**2**	1	**1.5**
175	*CYP21A1P* exon 4 (I173N)	1	1	**1.0**	1	1	**1.0**	1	**2**	**1.5**
220	*CYP21A1P* exon 7 (F308+T)	1	1	**1.0**	1	1	**1.0**	1	1	**1.0**
307	*CYP21A2* exon 1 (-113 SNP)	1	1	**1.0**	**0**	**0**	**0**	**0**	1	**0.5**
253	*CYP21A2* exon 3 I2G-C	**1**	**0**	**1.0**	**0**	**0**	**0**	**0**	1	**1.0**
258	*CYP21A2* exon 3 I2G-A	**0**	**1**	**1.0**	**0**	**0**	**0**	**0**	0	**0**
190	*CYP21A2* exon 3 (del8bp)	1	1	**1.0**	**0**	**0**	**0**	**0**	1	**0.5**
157	*CYP21A2* exon 4 (I173N)	1	1	**1.0**	**0**	**0**	**0**	1	**0**	**0.5**
232	*CYP21A2* exon 6 (V238E-WT)	1	1	**1.0**	**0**	**0**	**0**	1	1	**1.0**
238	*CYP21A2* exon 6 (M240K-WT)	1	1	**1.0**	**0**	**0**	**0**	1	1	**1.0**
214	*CYP21A2* exon 7 (F308+T)	1	1	**1.0**	**0**	**0**	**0**	1	1	**1.0**
148	*TNXB* exon 35 (probe 1)	1	1	**1.0**	1	1	**1.0**	1	1	**1.0**
318	*TNXB* exon 35 (probe 2)	1	1	**1.0**	1	1	**1.0**	1	1	**1.0**
355	*TNXB* exon 31	1	1	**1.0**	1	1	**1.0**	1	1	**1.0**
326	*TNXB* exon 29	1	1	**1.0**	1	1	**1.0**	1	1	**1.0**
202	*TNXB* exon 20	1	1	**1.0**	1	1	**1.0**	1	1	**1.0**
364	*TNXB* exon 19	1	1	**1.0**	1	1	**1.0**	1	1	**1.0**

[a] For more information see Table 1 & 2.
[b] Selected using SALSA Reference Selection DNA SD039.
[c] Final ratio (FR), see section *Interpretation of Results* below for more details on this term.
[d] Indicates the number of targets for that probe present on the allele.
[e] This complex genotype cannot be determined with MLPA alone and requires complementary methods such as allele/locus-specific long range PCR amplification and sequencing data for full delineation of the genotype.

Performance characteristics

It is estimated that 10-35% of pathogenic *CYP21A2* alleles are large deletions or large gene conversions (https://www.ncbi.nlm.nih.gov/books/NBK1171/). Analytical performance for the detection of large deletions and large gene conversions in *CYP21A2* is very high and can be considered >99% (based on a literature review covering 2015 to 2022 and an Analytical Performance Study performed in 2021).

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 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:

<table>
<tr><th>Copy number status</th><th>Final ratio (FR) target probes (excluding I2G probes)</th><th>FR I2G probes*</th></tr>
<tr><td>0 copies</td><td>FR = 0</td><td>0.00 ≤ FR < 0.15</td></tr>
<tr><td>1 copy</td><td>0.40 < FR < 0.65</td><td>0.70 < FR < 1.30</td></tr>
<tr><td>2 copies</td><td>0.80 < FR < 1.20</td><td>1.60 < FR < 2.40</td></tr>
<tr><td>3 copies</td><td>1.30 < FR < 1.65</td><td rowspan="2">FR > 2.6 indicates presence of 3 or more copies of this allele</td></tr>
<tr><td>4 copies</td><td>1.75 < FR < 2.15</td></tr>
<tr><td>Ambiguous copy number</td><td>All other values</td><td>All other values</td></tr>
</table>

* The copy number detected by the I2G probes should be added up to obtain the total number of WT alleles present in the test sample. For example, an FR between 0.70 and 1.20 for each I2G probe means that there is 1 copy of each allele present in the test sample. Another example: an FR between 1.60 and 2.40 for the I2G-C probe and FR=0 for the I2G-A probe means that the sample contains two I2G-C alleles and no I2G-A allele.

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. 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.

Recommendations for the analysis of results of P050:

1. Inclusion of sufficient (≥3) suitable reference samples in your experiment is essential. SD039 should only be used in initial experiment for the selection of suitable reference samples. SD039 should never be used as a reference sample in normal experiments.
2. Data analysis should be performed as described above with the use of the Coffalyser.Net software which is available free of charge on www.mrcholland.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 probes detect the clinically relevant genomic targets. All other probes are only included for accurate result interpretation. Please note that all *CYP21A1P*, *TNXB* and *ATF6B* probes are so-called flanking probes, which are included to facilitate the determination of the extent of a deletion/conversion and to help interpret copy number changes detected by the *CYP21A2* probes. Copy number alterations of only flanking and/or reference probes are unlikely to be related to the condition tested.
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 most likely explanation is that the pseudogene 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 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* mutations.
8. Please note that PCR amplification for sequence analysis of exons 1-3 of the *CYP21A2* gene can easily result in inaccurate results due to allele dropout, which may lead to inconsistencies between MLPA and sequencing results. The C allele of the I2G polymorphism results in a potential G-quadruplex structure which can lower efficiency of PCR amplification, thereby increasing the chance of allele dropout (White and Speiser 2000).
9. In some cases, parental sample analysis might be required for proper interpretation of results.

Detection of large *CYP21A2* rearrangements

The P050-D1 probemix is intended to detect large rearrangements, which can be subdivided in *CYP21A2* gene deletions, most of which lead to *CYP21A1P-CYP21A2* chimeras, and large *CYP21A2-CYP21A1P* gene conversions. Large deletions of ~30 kb are the most abundant type and have breakpoints somewhere between exons 3 and 8 of the *CYP21A1P* pseudogene and the corresponding point in *CYP21A2*, thus yielding a single remaining but inactive chimeric 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 will suffer from the salt wasting form of CAH. 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 I173N and the 8 nt deletion in exon 3, that are the result of microconversion events where the *CYP21A1P* (mutation) sequence replaces the *CYP21A2* (wildtype) sequence. These specific loci represent the limited sequence differences between *CYP21A2* and *CYP21A1P* and are therefore the few available probe targets to specifically determine the *CYP21A2* copy number. MLPA does not determine the chromosomal location of these sequences.

Large gene conversions span multiple of the common mutation sites. Clinically relevant large gene conversions result in a decreased copy number of the *CYP21A2* probes, often in combination with increased copy number of the equivalent *CYP21A1P* probes. An example of a gene conversion spanning exon 4-7: 1 copy will be detected by the *CYP21A2* exon 4 (I173N), *CYP21A2* exon 6 V238E and M240K) and *CYP21A2* exon 7 (F308+T) probes, in combination with 3 copies detected by the *CYP21A1P* exon 4 (I173N) and *CYP21A1P* exon 7 (F308+T) probes.

Large deletions of the *CYP21A2* gene different from the *CYP21A1-CYP21A2* chimeras are very rare but may occur and will result in a decreased copy number of the *CYP21A2* specific probe signals of the affected loci without increased signals of the equivalent *CYP21A1P* probes.

It should be noted that gene conversions can be obscured due to reciprocal sequence changes in the pseudogene. In fact, the *CYP21A1P* pseudogene very frequently contains the wildtype *CYP21A2* sequence at certain locations, which may mask the loss of *CYP21A2* sequences. A combination of at least MLPA and sequence analysis of the *CYP21A2* gene is required for the detection of all mutations, and in addition allele-specific PCR is required for the delineation of all possible large rearrangements in the RCCX module and in case of complex cases. Furthermore, when two different mutations in *CYP21A2* are detected, MLPA results cannot indicate whether these mutations are located *in cis* or *in trans*.

Typical results obtained on DNA samples from healthy individuals
When internally testing DNA samples from 145 healthy individuals, we found that ~20% of these DNA samples were suitable reference samples. In the same sample set we observed, among other deviations, the following copy number variants:

- Only one pseudogene copy in 32 samples (22%). In most cases this was combined with a normal *CYP21A2* copy number, resulting in a total combined number (1x*CYP21A1P* + 2x*CYP21A2*) of three copies. In three samples tested (2%), no pseudogene copies were present at all. In 20 samples (13%), we detected three pseudogene copies and one sample (<1%) contained four copies.
- The presence of three copies of the complete *CYP21A2* gene was observed in six samples (4%).
- Five samples (3%) appeared to be carrier for a CAH-associated mutation: one sample contained only a single *CYP21A2* allele, two samples (1%) had only one wildtype sequence at the I173N location, and two samples contained only one copy of the -113 bp (in respect to start codon, see next section for more details on the '-113 SNP') wildtype sequence.

Our internal tests indicate that 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-D1.

Probe target loci used to detect large *CYP21A1P-CYP21A2* rearrangements
Seven specific loci are targeted to detect large *CYP21A2* rearrangements:

1. **SNP at 113 bp before the start codon** (-113 SNP; c.-113G>A; rs1246774295). The 307 nt probe detects the wildtype *CYP21A2* sequence. The 292 nt probe detects the *CYP21A1P* pseudogene (mutant) sequence. Transcription of the *CYP21A2* gene appears to be reduced several fold when the *CYP21A2* upstream region contains the -113 SNP pseudogene-specific sequence.
2. **I2G locus** at 13 bp before exon 3 (intron 2 splice mutation; IVS-12A/C>G; c.293-13A/C>G; rs6467). The *CYP21A2* gene has two wildtype alleles at this location: C and A, for both of which a probe is present. Both a C-to-G and an A-to-G mutation at nucleotide -13 will cause the severe 21-OH deficiency (note that there is no probe present for the mutated allele I2G-G). 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 253 nt and 258 nt probes. The Reference Selection DNA sample SD039 is heterozygous for the two wildtype-specific sequences at this I2G location.
3. **8 bp deletion** in exon 3 (del8bp in short; c.332_339del; p.Gly111Valfs*21; rs387906510). 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-specific sequence containing the 8 nt deletion.
4. **I173N mutation** in exon 4 (c.518T>A; p.Ile173Asn; rs6475). The 157 nt probe detects the wildtype I173N sequence. The 175 nt probe detects the *CYP21A1P* pseudogene (mutant) sequence. Although the I173N 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.
5. **V238E mutation** in exon 6 (c.713T>A; p.Val238Glu; rs12530380). This is one of the three so called exon 6 cluster mutations. The 232 nt probe detects the V238E wildtype sequence. No probe is present for the corresponding *CYP21A1P* pseudogene (mutant) sequence. The signal of this probe is

moderately affected by the M240K mutation (confirmed by testing samples with the M240K mutation and not the V238E mutation). This probe might also be influenced by the I237N mutation, but we do not have samples available to verify this. The cluster of three exon 6 missense mutations (I237N, V238E, M240K) abolishes enzymatic activity.

6. **M240K mutation** in exon 6 (c.719T>A; p.Met240Lys; rs6476). This is also one of the three so called exon 6 cluster mutations. The 238 nt probe detects the M240K wildtype sequence. No probe is present for the corresponding *CYP21A1P* pseudogene (mutant) sequence. The signal of this probe might also be influenced by the V238E and/or the I237N mutations; we do not have samples available to verify this.
7. **F308+T mutation** in exon 7 (c.923dup; p.Leu308Phefs*6; rs267606756). The 214 nt probe detects the wildtype F308+T sequence. The 220 nt probe detects the *CYP21A1P* pseudogene (mutant) 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 P31L, V281L, Q319*, P454S or R357W 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: Baumgartner-Parzer (2020), 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. A subset of CAH patients also have genetic deletions of the *TNXB* gene. This contiguous gene syndrome is referred to as CAH-X. Biallelic mutations in *TNXB* that do not affect *CYP21A2* gene function are associated with the autosomal recessive disorder classic-like Ehlers-Danlos syndrome.

Limitations of the procedure

In most populations, the major cause of genetic defects in the *CYP21A2* are small (point) mutations, for the detection of which, SALSA MLPA Probemix P050 CAH is not suited.

- 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.
- Reciprocal exchanges between *CYP21A2* and its pseudogene will not be detected.

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.

Copy number changes detected by more than one consecutive probe should always 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

The *CYP21A2* page on the LOVD is 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 SNVs and unusual results to MRC Holland: info@mrcholland.com.

Table 1. SALSA MLPA Probemix P050-D1 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
157 ¥	**CYP21A2 probe** 22959-L32396		**Exon 4, I173N location**		
166 ¥	Reference probe 05721-L31493	7q			
175 ¥ Δ	**CYP21A1P probe** 22961-L32398			Exon 4, I173N location	
184 Δ	**CYP21A1P probe** 15221-L20262			Exon 3, del8bp location	
190	**CYP21A2 probe** 15221-L20261		**Exon 3, del8bp location**		
202 *	**TNXB probe** 22962-L32399				Exon 20
208 *	Reference probe 13384-L25019	6q			
214	**CYP21A2 probe** 17261-L21169		**Exon 7, F308+T location**		
220 Δ	**CYP21A1P probe** 17261-L21170			Exon 7, F308+T location	
226	Reference probe 14471-L16191	4q			
232 »	**CYP21A2 probe** 17270-L16990		**Exon 6, V238E location**		
238 ∫	**CYP21A2 probe** 17271-L16989		**Exon 6, M240K location**		
244	Reference probe 16307-L19696	13q			
253 ¥ ∞	**CYP21A2 probe** 21552-L32321		**Exon 3, I2G location, C-allele**		
258 ¥ ∞ +	**CYP21A2 probe** 21552-L20299		**Exon 3, I2G location, A-allele**		
267 *	Reference probe 14758-L32312	9q			
279	Reference probe 04988-L20303	8q			
292 ¥ Δ	**CYP21A1P probe** 22963-L32401			Exon 1, -113 bp SNP	
307 ¥ ±	**CYP21A2 probe** 22964-L32402		**Exon 1, -113 bp SNP**		
318	**TNXB probe** 15230-L14636				Exon 35
326 *	**TNXB probe** 22965-L32568				Exon 29
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 *	Reference probe 05953-L30687	2p			
382	Reference probe 13329-L14755	18q			

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

* New in version D1.
¥ Changed in version D1. Minor alteration, no change in sequence detected.
∞ The *CYP21A2* gene has a polymorphism at the I2G locus. The 253 and 258 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 Selection DNA SD039 contains one copy for each of these two alleles, a probe ratio of 1 corresponds to 1 copy for these probes.**

+ Note that in the absence of the I2G A-allele sometimes a small background signal (up to ~10%) at 258 nt can be detected.
Δ 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.
± SNP rs544417036 and rs573835051 could influence the 307 nt probe signal. In case of apparent deletions, it is recommended to sequence the region targeted by this probe.
» The 232 nt probe signal (V238E, exon 6 cluster) is moderately affected by the presence of the M240K mutation (rs6476) and might also be affected by the I237N mutation (rs111647200). The V238E mutation is often present together with the M240K and I237N mutations.
∫ The 238 nt probe signal (M240K, exon 6 cluster) might be affected by the V238E and/or the I237N mutation (rs12530380 and rs111647200 respectively). The M240K mutation is often present together with the V238E and I237N mutations.

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.

Note: Amino acid numbering used in this P050-D1 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@mrcholland.com.

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

Length (nt)	SALSA MLPA probe	Gene exon[a]	Ligation site	Partial sequence[b] (24 nt adjacent to ligation site)	Distance to next probe
***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 Δ¬	22963-L32401	Exon 1	105 nt before ex 1 -113 bp SNP	GGTCGGTGGGAA-GGCACCTGAGGG	0.9 kb
184 Δ¬	15221-L20262	Exon 3	350-351 del8bp location	TGTCGTTGGTCT-CTGCTCTGGAAA	0.3 kb
175 Δ¬	22961-L32398	Exon 4	526-527 I173N location	CTGCAGCATCAA-CTGTTACCTCAC	0.8 kb
220 Δ¬	17261-L21170	Exon 7	927-928 F308+T location	GGCCGTGGTTTT-TTTTGCTTCACC	30.8 kb
***CYP21A2* probes** Ligation sites of the *CYP21A2* probes are shown in the NM_000500.9 reference sequence.					
		Start codon	*9-11*		
307 ±	22964-L32402	Exon 1	105 nt before ex 1, reverse, **-113 bp SNP**	CTTCAGGTACCC-TCCCACCGACCC	0.8 kb
253 ∞	21552-L32321	Exon 3	13 nt before ex 3, reverse, **C-allele at I2G location**	TGCAGGAGGAGG-TGGGGGCTGGAG	0.1 kb
258 ∞ +	21552-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
157	22959-L32396	Exon 4	526-525, reverse **I173N wildtype**	TGAGGTAACAGA-TGATGCTGCAGG	0.4 kb
232 »	17270-L16990	Exon 6	721-722 **V238E wildtype**	GGATCACATCGT-GGAGATGCAGCT	0.0 kb
238 ∫	17271-L16989	Exon 6	727-726, reverse, **M240K wildtype**	GCCTCAGCTGCA-TCTCCACGATGT	0.4 kb
214	17261-L21169	Exon 7	927-928 **F308+T wildtype**	GGGCCGTGGTTT-TTTTGCTTCACC	3.6 kb
		stop codon	*1494-1496*		

Length (nt)	SALSA MLPA probe	Gene exon[a]	Ligation site	Partial sequence[b] (24 nt adjacent to ligation site)	Distance to next probe
***TNXB* Probes** Ligation sites are shown in the NM_019105.8 reference sequence.					
		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	2.3 kb
326 ¬	22965-L32568	Exon 29	16 nt before exon 29, reverse	AAAGAGCAGAGC-AGGCCCATGGGT	13.8 kb
202 ¬	22962-L32399	Exon 20	7017-7018	AGCCCCAGGAAA-GGATGAAGAAAT	2.6 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 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.

* New in version D1.
¥ Changed in version D1. Minor alteration, no change in sequence detected.
∞ The *CYP21A2* gene has a polymorphism at the I2G locus. The 253 and 258 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 Selection DNA SD039 contains one copy for each of these two alleles, a probe ratio of 1 corresponds to 1 copy for these probes.**
\+ Note that in the absence of the I2G A-allele sometimes a small background signal (up to ~10%) at 258 nt can be detected.
∆ 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.
± SNP rs544417036 and rs573835051 could influence the 307 nt probe signal. In case of apparent deletions, it is recommended to sequence the region targeted by this probe.
» The 232 nt probe signal (V238E, exon 6 cluster) is moderately affected by the presence of the M240K mutation (rs6476) and might also be affected by the I237N mutation (rs111647200). The V238E mutation is often present together with the M240K and I237N mutations.
∫ The 238 nt probe signal (M240K, exon 6 cluster) might be affected by the V238E and/or the I237N mutation (rs12530380 and rs111647200 respectively). The M240K mutation is often present together with the V238E and I237N mutations.
¬ 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.
¤ The 148 and 318 nt *TNXB* exon 35 probes are located within the 121 nt sequence that is absent in the *TNXA* pseudogene.

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

Complete probe sequences are available at www.mrcholland.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.
P495 CYP11A1-CYP11B1-CYP11B2	Contains probes the *CYP11A1, CYP11B1*, and *CYP11B2* genes, which are associated with related disorders.

References

- Baumgartner-Parzer S et al. (2020). EMQN best practice guidelines for molecular genetic testing and reporting of 21-hydroxylase deficiency. *Eur J Hum Genet.* 28:1341-67.
- Gitelman SE et al. (1992). Mechanism and consequences of the duplication of the human C4/P450c21/gene X locus. *Mol Cell Biol*. 12:2124-2134.
- 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.
- White PC and Speiser PW (2000). Congenital adrenal hyperplasia due to 21-hydroxylase deficiency. *Endocr Rev.* 21:245-291.

Selected publications using SALSA MLPA Probemix P050 CAH

- Castro PS et al. (2019). High frequency of non-classical congenital adrenal hyperplasia form among children with persistently elevated levels of 17-hydroxyprogesterone after newborn screening. *J Pediatr Endocrinol Metab.* 32:499-504.
- Chang Z et al. (2021). Genetic aetiology of primary adrenal insufficiency in Chinese children. *BMC Med Genomics.* 14:172.
- Chi DV et al. (2019). Novel variants of CYP21A2 in Vietnamese patients with congenital adrenal hyperplasia. *Mol Genet Genomic Med*. 7:e623.
- de Carvalho DF et al. (2016). Molecular CYP21A2 diagnosis in 480 Brazilian patients with congenital adrenal hyperplasia before newborn screening introduction. *Eur J Endocrinol*. 175:107-116.
- Concolino P et al. (2009). Multiplex ligation-dependent probe amplification (MLPA) assay for the detection of CYP21A2 gene deletions/duplications in congenital adrenal hyperplasia: first technical report. *Clin Chim Acta*. 402:164-170.
- Concolino P (2019). Issues with the Detection of Large Genomic Rearrangements in Molecular Diagnosis of 21-Hydroxylase Deficiency. *Mol Diagn Ther*. 23:563-567.
- Costa-Barbosa FA et al. (2021). Reassessment of predictive values of ACTH-stimulated serum 21-deoxycortisol and 17-hydroxyprogesterone to identify CYP21A2 heterozygote carriers and nonclassic subjects. *Clin Endocrinol (Oxf).* 95:677-685.
- Dubey S et al. (2017). Prenatal diagnosis of steroid 21-hydroxylase-deficient congenital adrenal hyperplasia: Experience from a tertiary care centre in India. *Indian J Med Res*. 145:194-202.
- Fernandez CS et al. (2020). Genetic characterization of a large cohort of Argentine 21-hydroxylase Deficiency. *Clin Endocrinol (Oxf)*. 93:19-27.
- Gangodkar P et al. (2021). Clinical application of a novel next generation sequencing assay for CYP21A2 gene in 310 cases of 21- hydroxylase congenital adrenal hyperplasia from India. *Endocrine.* 71:189-198.
- Gao Y et al. (2021). Bone mineral density and trabecular bone score in patients with 21-hydroxylase deficiency after glucocorticoid treatment. *Clin Endocrinol (Oxf).* 94:765-773.
- Gao Y et al. (2020). The prevalence of heterozygous CYP21A2 deficiency in patients with idiopathic acne, hirsutism, or both. *Endocrine*. 67:665-672.
- Gao Y et al. (2020). The Prevalence of the Chimeric TNXA/TNXB Gene and Clinical Symptoms of Ehlers-Danlos Syndrome with 21-Hydroxylase Deficiency. *J Clin Endocrinol Metab*. 105:2288-2299.

- Hong G et al. (2015). CYP21A2 mutation analysis in Korean patients with congenital adrenal hyperplasia using complementary methods: sequencing after long-range PCR and restriction fragment length polymorphism analysis with multiple ligation-dependent probe amplification assay. *Ann Lab Med*. 35:535-539.
- Hou L et al. (2019). Analysis of phenotypes and genotypes in 84 patients with 21-Hydroxylase deficiency in southern China. *Steroids*. 151:108474.
- Kurzynska A et al. (2022). Molecular analysis and genotype-phenotype correlations in patients with classical congenital adrenal hyperplasia due to 21-hydroxylase deficiency from southern Poland - experience of a clinical center. *Hormones (Athens)*.1-9.
- Lidaka L et al. (2021). Non-Classical Congenital Adrenal Hyperplasia-Causing Alleles in Adolescent Girls with PCOS and in Risk Group for PCOS Development. *Diagnostics (Basel).* 11:980.
- Liu Y et al. (2020). The spectrum of CYP21A2 gene mutations in patients with classic salt wasting form of 2l-hydroxylase deficiency in a Chinese cohort. *Mol Genet Genomic Med.* 8:e1501.
- Liu J et al. (2018). Identification of a novel compound heterozygous mutation of the CYP21A2 gene causing 21hydroxylase deficiency in a Chinese pedigree. *Mol Med Rep*. 17:4265-4272.
- Meinel J et al. (2021). Establishment of Clinical and Lab Algorithms for the Identification of Carriers of Mutations in CYP21A2 - A Study of 365 Children and Adolescents. *Exp Clin Endocrinol Diabetes*. *129*:492-499.
- Moura-Massari VO et al. (2016). The Presence of Clitoromegaly in the Nonclassical Form of 21-Hydroxylase Deficiency Could Be Partially Modulated by the CAG Polymorphic Tract of the Androgen Receptor Gene. *PLoS One*. 11:e0148548.
- Nan MN et al. (2021). Comprehensive Genetic Testing of CYP21A2: A Retrospective Analysis in Patients with Suspected Congenital Adrenal Hyperplasia. *J Clin Med.* 10:1183.
- Navardauskaite R et al. (2021). Impact of Newborn Screening on Clinical Presentation of Congenital Adrenal Hyperplasia. *Medicina (Kaunas).* 57:1035.
- Neocleous V et al. (2019). The Spectrum of Genetic Defects in Congenital Adrenal Hyperplasia in the Population of Cyprus: A Retrospective Analysis. *Horm Metab Res*. 51:586-594.
- Oriolo C et al. (2020). Steroid biomarkers for identifying non-classic adrenal hyperplasia due to 21-hydroxylase deficiency in a population of PCOS with suspicious levels of 17OH-progesterone. *J Endocrinol Invest*. 43:1499-1509.
- Pignatelli D et al. (2019). The Complexities in Genotyping of Congenital Adrenal Hyperplasia: 21-Hydroxylase Deficiency. *Front Endocrinol (Lausanne)*. 10:432.
- Prado MJ et al. (2017). Development of CYP21A2 Genotyping Assay for the Diagnosis of Congenital Adrenal Hyperplasia. *Mol Diagn Ther*. 21:663-675.
- Ravichandran L et al. (2021). Allele-specific PCR and Next-generation sequencing based genetic screening for Congenital Adrenal Hyperplasia in India. *Eur J Med Genet*. 64:104369.
- Saraf S et al. (2022). Characterization of the CYP21A2 Gene Mutations in Children with Classic Congenital Adrenal Hyperplasia. *Indian J Pediatr*.1-6.
- Shima R et al. (2020). Timing of hyponatremia development in patients with salt-wasting-type 21-hydroxylase deficiency. *Clin Pediatr Endocrinol*. 29:105-110.
- Soveizi M et al. (2020). p.Gln318X and p.Val281Leu as the Major Variants of CYP21A2 Gene in Children with Idiopathic Premature Pubarche. Int J Endocrinol. 2020:4329791.
- Su L et al. (2018). Clinical presentation and mutational spectrum in a series of 166 patients with classical 21-hydroxylase deficiency from South China. *Clin Chim Acta.* 486:142-150.
- Turan I et al. (2020). 21-Hydroxylase deficiency: Mutational spectrum and Genotype-Phenotype relations analyses by next-generation sequencing and multiplex ligation-dependent probe amplification. *Eur J Med Genet*. 63:103782.
- Umana-Calderon A et al. (2021). CYP21A2 mutations in pediatric patients with congenital adrenal hyperplasia in Costa Rica. Mol Genet Metab Rep. 27:100728.
- Wang X et al. (2020). Neonatal Screening and Genotype-Phenotype Correlation of 21-Hydroxylase Deficiency in the Chinese Population. *Front Genet.* 11:623125.

- 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 J et al. (2019). Identification of novel and rare CYP21A2 variants in Chinese patients with congenital adrenal hyperplasia due to 21-hydroxylase deficiency. *Clin Biochem*. 68:44-49.
- Yoon JY et al. (2021). Genotype and clinical outcomes in children with congenital adrenal hyperplasia. *Pediatr Int*. 63:658-663.

P050 product history	
Version	*Modification*
D1	I173N WT sequence probe detects the same sequence but now on the reverse strand. Five CYP21A2/CYP21A1P probes changed in length not in sequence detected. Two TNXB probes have been replaced. One reference probe has been replaced and three have been added
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 D1-02 – 24 November 2022 (04P)
- Two instances of the F306+T mutation in Table 2 were corrected to F308+T, in line with the current clinical standards of the *CYP21A2* mutation nomenclature.
- SALSA MLPA Probemix P495 CYP11A1-CYP11B1-CYP11B2 has been added to the *Related SALSA MLPA probemixes.*

Version D1-01 – 06 May 2022 (04P)
- P050-D1 is now CE marked.
- Product description adapted to a new product version (version number changed, changes in Table 1 and Table 2).
- Table was added to section *Positive control DNA samples.*
- Cut-off values for the FR of the I2G-probes are adjusted.
- *CYP21A2* mutation nomenclature updated to current clinical standards (Baumgartner-Parzer *et al.* 2020).
- The references and the list with selected publications were curated.
- The product description has been rewritten, including the new Intended Purpose and Clinical background, and minor textual and layout changes throughout the document.

Version C1-05 – 01 September 2021(04P)
- Product description rewritten and adapted to a new template.
- *Reference samples* section was edited to better explain the selection of reference samples.
- *SALSA Reference Selection DNA SD039* section is updated.
- Section *TNXB gene* updated according to current scientific literature and guidelines.
- Various minor textual or layout changes.

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.
- 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 2 updated.
- 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.

More information: www.mrcholland.com; www.mrcholland.eu	
	MRC Holland bv; Willem Schoutenstraat 1 1057 DL, Amsterdam, The Netherlands
E-mail	info@mrcholland.com (information & technical questions) order@mrcholland.com (orders)
Phone	+31 888 657 200

IVD	EUROPE* CE
RUO	ALL OTHER COUNTRIES

*comprising EU (candidate) member states and members of the European Free Trade Association (EFTA), and the UK. The product is for RUO in all other European countries.