Instructions for Use

SALSA® MLPA® Probemix P050 CAH

See also the MLPA General Protocol, and the product descriptions of the SALSA® MLPA® Reagent Kit, SALSA® Reference Selection DNA SD039 and the Coffalyser.Net Reference Manual.

Visit the SALSA® MLPA® Probemix P050 CAH product page on our website to find Certificates of Analysis and a list of related products.

Product Name	**SALSA® MLPA® Probemix P050 CAH**
Version	**D1**
Catalogue numbers	**P050-025R (25 reactions)** **P050-050R (50 reactions)** **P050-100R (100 reactions)**
Basic UDI-DI:	**872021148P0505K**
Ingredients	**Synthetic oligonucleotides, oligonucleotides purified from bacteria, Tris-HCl, EDTA**

Additional Test Components	**Catalogue numbers**
SALSA® MLPA® Reagent Kit	EK1-FAM EK1-CY5 EK5-FAM EK5-CY5 EK20-FAM
SALSA® Reference Selection DNA SD039	SD039

Storage and Shelf Life

Recommended conditions		

A shelf life of until the expiry date is guaranteed, also after opening when stored in the original packaging under recommended conditions. For the exact expiry date, see the label on the vial. This product should not be exposed to more than 25 freeze-thaw cycles. Do not use the product if the packaging is damaged or opened. Leave chemicals in original containers. Waste material must be disposed of in accordance with the national and local regulations.

Regulatory Status	
IVD	EUROPE CE 2797
RUO	ALL OTHER COUNTRIES

Label Symbols			
IVD	In Vitro Diagnostic	RUO	Research Use Only

More Information: **www.mrcholland.com**	
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E-mail	info@mrcholland.com (information & technical questions); order@mrcholland.com (orders)
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Any serious incident that has occurred in relation to this product should be reported to MRC Holland and the competent authority of the Member State in which the user and/or the patient is located.

Changes in this Product Version:
As compared to the C1 version, 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.

1. Intended Purpose

The SALSA MLPA Probemix P050 CAH is an in vitro diagnostic (IVD)[1] or research use only (RUO) semi-quantitative manual assay[2] for the detection of large deletions in the *CYP21A2* gene. This probemix can also detect copy number variations (CNVs) in the surrounding region to aid in interpretation. 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. In addition, this assay can be used for carrier testing. 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.

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. Additionally, most defects in the *CYP21A2* gene are point mutations, most of which will not be detected by MLPA. It is therefore recommended to always use this assay in combination with sequence analysis. Not all exons of the aforementioned genes are covered in this probemix.

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 on page 1 of this product description. In all other countries, the product is for research use only (RUO).

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

2. Sample requirements

Specimen	50-250 ng purified human genomic DNA, dissolved in 5 µl $TE_{0.1}$ buffer, pH 8.0-8.5
Collection method	Standard methods
Extraction method	Methods tested by MRC Holland: • QIAGEN Autopure LS (automated) and QIAamp DNA mini/midi/maxi kit (manual) • Promega Wizard Genomic DNA Purification Kit (manual) • Salting out (manual)

Sample types		
Test sample	• Provided by user	
Reference samples (required)	**For proper data analysis, reference samples that fulfil all criteria listed below must be identified before testing patient samples. See also the sample type SALSA Reference Selection DNA SD039 and the reference sample selection guide in the Appendix.** • Provided by user • Extraction method, tissue type, DNA concentration and treatment as similar as possible in all test and reference samples. Should have one copy of each wildtype allele at the I2G locus, *i.e.* one I2G-**C** allele and one I2G-**A** allele; and a normal copy number and ≤0.10 standard deviation for all other autosomal sequences detected by P050 CAH. • At least three* independent reference samples required in each experiment for proper data normalisation. Derived from unrelated individuals from families without a history of CAH.	
No-DNA control (preferably)	• Provided by user • $TE_{0.1}$ buffer instead of DNA • To check for DNA contamination	
Reference Selection DNA	• SALSA Reference Selection DNA SD039, available from MRC Holland. • Use SD039 in initial experiments to identify suitable reference samples. • Suitable reference samples provide results equivalent to Reference Selection DNA SD039 for each probe in this probemix. • **SD039 should never be used as a reference sample in the data analysis of patient samples.**	
Positive control samples (preferably)	• provided by user, or	
	Available from third parties	See the table of positive samples on the probemix product page on our website.

*When testing >21 samples, include one extra reference for each 7 test samples.

3. Test Procedure

See the MLPA General Protocol.

4. Quality Control, Data Analysis, and Troubleshooting

Quality Control Fragments in the Probemix	
Length (nt)	**Function**
64-70-76-82	DNA quantity control fragments
88-96	DNA denaturation control fragments
92	Benchmark fragment
100	Chromosome X presence control fragment
105-118	Chromosome Y presence control fragments

Coffalyser.Net should be used for data analysis in combination with the appropriate product and lot-specific Coffalyser sheet. See the Coffalyser.Net Reference Manual for details on data analysis and quality control.

For troubleshooting help, see the additional resources offered on our support portal.

5. Interpretation of Results

Determining Typical Values in Normal and Affected Populations
The typical final ratio (FR) values stated in the copy number tables were determined in a validation study with samples containing abnormal copy numbers. The standard deviation of each individual probe over all the reference samples was ≤0.10.

Expected Results of Reference Probes

Final Ratio (FR)	Copy Number	Description
0.80-1.20	2	Normal

Typical Results of Probes Targeting Two Copies (6p21.3 region, excluding I2G probes)

FR	Copy Number	Description
0	0	Homozygous deletion
0.40 – 0.65	1	Heterozygous deletion
0.80 – 1.20	**2**	**Normal in reference samples**
1.30 – 1.65	3	Heterozygous duplication
1.75 – 2.15	4	Homozygous duplication or Heterozygous triplication
All other values	-	Ambiguous

Typical Results of individual I2G probes

FR I2G probes*	Copy Number
0.00 - 0.15	0
0.70 - 1.30	1
1.60 - 2.40	2
FR > 2.6 indicates presence of 3 or more copies of this allele	3
	4
All other values	-

The total number of WT alleles present in the test sample is the sum of the copy number detected by **I2G-C and I2G-A** probes.

Interpretation of I2G probes

Length (nt)	I2G Probes	Copy number		Sum of I2G-C and I2G-A	Description
253	*CYP21A2* I2G-**C**	0		0	No WT alleles
258	*CYP21A2* I2G-**A**	0			
253	*CYP21A2* I2G-**C**	1	0	1	One WT allele
258	*CYP21A2* I2G-**A**	0	1		
253	*CYP21A2* I2G-**C**	1		**2**	Two WT alleles **Normal in reference samples**
258	*CYP21A2* I2G-**A**	1			
253	*CYP21A2* I2G-**C**	2	0	**2**	Two WT alleles
258	*CYP21A2* I2G-**A**	0	2		
253	*CYP21A2* I2G-**C**	2	1	3	Three WT alleles
258	*CYP21A2* I2G-**A**	1	2		

The tables illustrate the relationship between final probe ratio and corresponding copy number. Test results are expected to center around these values. Ambiguous values can indicate a technical problem, but may also reflect a biological cause such as mosaicism or a SNV influencing a single probe. It is important to use Coffalyser.Net to determine the significance of values found.

For a detailed interpretation guide, see the **Appendix**.

Examples of MLPA results obtained with this probemix can be found on the P050 CAH product page on www.mrcholland.com.

6. Performance Characteristics

<table>
<tr><th>Study</th><th>Description</th></tr>
<tr><td>Expected values for copy number in normal and affected populations</td><td>To determine the expected values in normal and affected populations a study was conducted on over 1500 MLPA reactions using samples with and without abnormal copy numbers. When the standard deviation of each individual probe over all the reference samples is ≤0.10, the ranges stated in the copy number table in the product description can be used. Cut-off values for copy number determination were verified with P050 CAH in 56 samples from healthy individuals with normal copy number and six samples with known CNVs. The expected FRs for the corresponding copy number were found in all samples tested, with the exception of 8/744 ambiguous or incorrect measurements. Ambigous measurements were only slightly outside of the expected cut-off range and therefore the correct genotype could still be established. Incorrect measurements found in two samples obtained from healthy individuals could indicate possible microconversions that should be further investigated with complementary methods (carrier frequency is estimated to be 1:50 in the general population (Liu et al. 2022)).

In addition, 46 prenatal samples with various genotypes including (mosaic) triploidy, trisomy 13, trisomy 21, F8 intron 22 inversion type 1 or F8 intron 22 inversion type 2 but without CAH were included to verify cut-off values for copy number determination with P050 CAH. The expected FRs for the corresponding copy number were found in all samples tested, with the exception of 20/552 ambiguous or incorrect measurements. Ambiguous measurements were only slightly outside of the expected cut-off range and therefore the correct genotype could still be established. Incorrect measurements found in two prenatal samples indicated possible non-pathogenic microconversions (pseudogene-to-gene).</td></tr>
<tr><td>Limit of Detection</td><td>A study using representative probemixes was conducted to evaluate the minimum and maximum amount of DNA acceptable as the assay input. Results support the use of 50-250 ng of human DNA as the recommend input amount. The use of insufficient or too much sample DNA can affect performance. These lower and higher limits of detection were verified using P050 CAH on three samples with known CNVs and on one sample without any mutation and expected results were obtained using both the lower and upper input amount of DNA.</td></tr>
<tr><td>Interfering substances</td><td>SNVs or other polymorphisms (e.g. indels) in the DNA target sequence and impurities in the DNA sample (e.g. NaCl or KCl, EDTA and hemoglobin) can affect the MLPA reaction.

A study using P050 CAH was performed to assess the potential for interference of endogenous and exogenous substances on genomic DNA on samples with known CNVs and one sample with normal copy numbers. For most probes, expected FRs (FRs within the expected cut-off category) were obtained even in the presence of potential interferents at concentrations shown in the table below.
<table>
<tr><th>Interferent</th><th>Source</th><th>Testing Concentration</th><th>Results*</th></tr>
<tr><td>EDTA</td><td>Exogenous – specimen collection tubes</td><td>1.5 mM</td><td>Copy number: Expected FR for 124/144 measurements</td></tr>
<tr><td>NaCl</td><td>Exogenous – DNA extraction</td><td>40 mM</td><td>Copy number: Expected FR for 143/144 measurements</td></tr>
<tr><td>Fe^{3+} ($FeCl_3$)</td><td>Exogenous – DNA extraction</td><td>1 µM</td><td>Copy number: Expected FR for 143/144 measurements</td></tr>
<tr><td>Heparin</td><td>Exogenous – specimen collection tubes</td><td>0.02 U/mL</td><td>Copy number: Expected FR for 143/144 measurements</td></tr>
<tr><td>Hemoglobin</td><td>Endogenous – blood sample</td><td>0.02 µg/µl</td><td>Copy number: Expected FR for 33/144 measurements</td></tr>
</table>
* Results are summarised for eight CYP21A2 probes and four CYP21A1P probes across all four samples tested in triplicate.

Hemoglobin and EDTA had the largest effect on copy number determination, as FRs within an ambiguous or incorrect range were found in all samples. DNA extraction methods from blood remove hemoglobin and EDTA, and during testing of 56 samples extracted from blood, the expected FRs were found. Therefore, it is only when hemoglobin and EDTA are present in excess that deviating results for these probes can be found. Importantly, Coffalyser.Net issued warnings or errors for the samples in which hemoglobin and EDTA showed an effect, as well as lowered quality scores.

To minimise variability across samples, 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.</td></tr>
<tr><td>Cross-reactivity</td><td>Cross-reactivity is the potential for probes to bind to homologous regions (e.g. pseudogenes) or other cross-reactive sequences. Quality tests were carried out to determine whether probes are specific to their target sequence and all probes met the quality criteria for specificity.</td></tr>
<tr><td>Accuracy</td><td>Results of accuracy are derived from trueness and precision studies. For trueness, six previously genotyped samples were tested using P050 CAH and found to have the expected results. Assay precision was tested by repeatedly testing samples with known copy number status over multiple days, and by multiple operators. Results showed a correct call in 697/702 data points, leading to a precision of >99%.</td></tr>
</table>

Study	Description
Clinical validity*	Large deletions in the *CYP21A2* gene as a result of large rearrangements explain 20-30% of CAH cases (Fernandez et al. 2020, Liu et al. 2022, Xia et al. 2022, Xia et al. 2024, Yuan et al. 2024).

Summary of Safety and Performance (SSP)
The SSP is available in the European database on medical devices (Eudamed), https://ec.europa.eu/tools/eudamed, or upon request.

Content – Probe Details Sorted by Chromosomal Position

Chr. position	Target	Exon	Distance to next probe	Mutation	rsID	Length (nt)	Probe number	Warning
6p21.32	*CYP21A1P*	Upstream (Exon 1)	0.9 kb	-113 SNP	rs1246774295	292	22963-L32401	¥
6p21.32	*CYP21A1P*	Exon 3	0.3 kb	G111fs*21 mut (del8nt)	rs387906510	184	15221-L20262	
6p21.32	*CYP21A1P*	Exon 4	0.8 kb	I173N mut	rs6475	175	22961-L32398	¥
6p21.32	*CYP21A1P*	Exon 7	30.8 kb	L308Ffs*6 mut	rs267606756	220	17261-L21170	
6p21.32	*CYP21A2*	Upstream (Exon 1)	0.8 kb	wt -113 SNP		307	22964-L32402	¥
6p21.32	*CYP21A2*	Exon 3	0.1 kb	wt I2G-C		253	21552-L32321	¥ ∞ Δ
6p21.32	*CYP21A2*	Exon 3		wt I2G-A		258	21552-L20299	¥ ∞ ∑
6p21.32	*CYP21A2*	Exon 3	0.3 kb	wt G111fs*21 (del8nt)		190	15221-L20261	
6p21.32	*CYP21A2*	Exon 4	0.4 kb	wt I173N		157	22959-L32396	¥
6p21.32	*CYP21A2*	Exon 6	0.0 kb	wt V238E		232	17270-L16990	±
6p21.32	*CYP21A2*	Exon 6	0.4 kb	wt M240K		238	17271-L16989	±
6p21.32	*CYP21A2*	Exon 7	3.6 kb	wt L308Ffs*6		214	17261-L21169	
6p21.32	*TNXB*	Exon 35	0.1 kb			148	19037-L14637	¬ ∫
6p21.32	*TNXB*	Exon 35	2.6 kb			318	15230-L14636	¬ ∫
6p21.32	*TNXB*	Exon 31	2.3 kb			355	15232-L01515	¬
6p21.32	*TNXB*	Exon 29	13.8 kb			326	22965-L32568	¬ *
6p21.32	*TNXB*	Exon 20	2.6 kb			202	22962-L32399	¬ *
6p21.32	*TNXB*	Exon 19	63.1 kb			364	15235-L04400	¬
6p21.32	*ATF6B*					346	01979-L20800	¬
1q	Reference					336	09027-L09281	
2p	Reference					373	05953-L30687	*
3q	Reference					135	16316-L21434	
4q	Reference					226	14471-L16191	
5q	Reference					130	00797-L13645	
6q	Reference					208	13384-L25019	*
7q	Reference					166	05721-L31493	¥
8q	Reference					279	04988-L20303	
9q	Reference					267	14758-L32312	*
13q	Reference					244	16307-L19696	
18q	Reference					382	13329-L14755	

Probe lengths may vary slightly depending on capillary electrophoresis instrument settings. Please see the most up to date Coffalyser sheet for exact probe lengths obtained at MRC Holland. The *CYP21A2, CYP21A1P and TNXB* exon numbers are derived from MANE project and are based on MANE Select transcript. For more information, see the probe sequences document available on the product page at www.mrcholland.com. Annotations of several probes with targets at the edge of or slightly outside the coding region, were altered. The exon numbering from the previous version of this product description is disclosed between brackets.
Chromosomal bands are based on: hg18.
Abbreviations: wt, wildtype; mut, mutation.

7. Precautions and Warnings

Probe changes

*	New probe in version D1.
¥	Probe changed in this product version. Minor alteration, no change in sequence detected.

Probe warnings

∞	The *CYP21A2* gene has a polymorphism at the I2G locus. The 253 and 258 nt probes both detect the wildtype sequences. **The copy number detected by these two probes should be combined, as shown in Section 5 – Interpretation of I2G probes. Many 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.**
¬	Probes targeting *TNXB* and *ATF6B* are flanking probes, included to help determine the extent of a deletion/duplication/recombination. Copy number alterations of flanking probes alone are unlikely to be related to the condition tested.
±	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 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, V238E and I237N mutations are often present together.
∫	The 148 and 318 nt *TNXB* exon 35 probes are located within the 120 base pair sequence that is absent in the *TNXA* pseudogene.
Δ	This probe may be sensitive to certain experimental variations. Aberrant results should be treated with caution.
∑	In the absence of the I2G A-allele, a small background signal of the 258 nt probe can be visible. This background signal might be displayed as an intra ratio percentage instead of a Final ratio (see our support portal for more details).

Probemix-specific precautions

1. This product is not known to contain any harmful agents. Based on the concentrations present, none of the ingredients are hazardous as defined by the Hazard Communication Standard. **A Safety Data Sheet (SDS) is not required for this product**: none of the ingredients contain

dangerous substances at concentrations requiring distribution of an SDS (as per Regulation (EC) No 1272/2008 [EU-GHS/CLP] and 1907/2006 [REACH] and amendments).
2. Sample or technical artefacts may appear as a (mosaic) copy number change of the whole/partial gene. Whole/partial gene deletions or duplications should therefore be confirmed by analysis of an independent DNA sample, to exclude false positive results.
3. Small changes (e.g. SNVs, small indels) in the sequence targeted by a probe can cause false positive results, even when >20 nt from the probe ligation site. Sequence changes 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. Deviations detected by this product should be confirmed, and single-probe deviations always require confirmation. Sequencing of the target region is recommended. Please contact MRC Holland for more information: info@mrcholland.com.
4. SNVs rs6476, rs12530380 and rs111647200 mentioned in this document require additional caution. Please note that any probe can be affected by known or novel SNVs.
5. Copy number alterations of reference probes are unlikely to be related to the condition tested.
6. **Before testing patient samples, this product requires the identification of suitable reference samples for proper data analysis.** Selecting suitable reference samples for the P050 CAH probemix is complicated due to the presence of the highly homologous and inactive *CYP21A1P* pseudogene and the two I2G wildtype alleles. In our experience, approximately 1 in 5 (~20%) DNA samples from the general European population are suitable for use as reference sample. SALSA Reference Selection DNA SD039 can be used in initial experiments to facilitate the selection of suitable reference samples.
7. Signals of the probes detecting the *CYP21A2* wildtype sequence should be the main focus. These probes detect the copy number of clinically relevant genomic targets. Probes for the *CYP21A1P* sequence are included to aid in results interpretation. For a detailed interpretation guide, see the **Appendix**.
8. In some cases, parental analysis might be required for proper interpretation of results.
9. In the great majority of cases, an increased copy number detected by one or more of the *CYP21A2* probes will not be pathogenic. Samples from healthy individuals may 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.
10. P050 CAH includes two Y-specific fragments to aid in the evaluation of cases presenting with virilization or ambiguous genitalia. When discordant results are obtained with the Y-fragments, further testing is needed.

Technique-specific precautions
See the MLPA General Protocol.

8. Limitations

Probemix-specific limitations
1. All *CYP21A2* specific probes have a homologous sequence in *CYP21A1P*, and vice versa, due to the high homology between the gene and pseudogene, making this region prone to gene conversions and recombination events. As a result, deletions, duplications and gene conversion events are often observed in the *CYP21A1P* pseudogene, thereby complicating analysis.
2. Gene conversions can be obscured due to reciprocal sequence changes in the pseudogene. Please note that the *CYP21A1P* pseudogene very frequently contains the wildtype *CYP21A2* sequence at certain locations, which may mask the loss of *CYP21A2* sequences. Reciprocal exchanges between *CYP21A2* and its pseudogene will not be detected by MLPA. Therefore, 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.
3. Smaller gene conversion events will usually result in a decreased probe signal of one or a few of the CYP21A2 probes. In most cases, the corresponding pseudogene-specific sequence will show an increased copy number. 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.
4. In case two or more CYP21A2 probes are affected by a deletion, P050 CAH results cannot indicate whether these mutations are located in cis or in trans. P050 CAH can only detect the total number of copies of the target sequence and not the precise composition of the alleles. Therefore, if more than one probe is affected, additional methods (e.g. allele-specific PCR) are needed to determine whether this copy number change occurs on one allele or affects both alleles.
5. The *CYP21A1P* gene has a highly variable copy number in healthy individuals.
6. Please note that PCR amplification for sequence analysis of exons 1-3 of the *CYP21A2* gene may produce inaccurate results due to allele dropout, which can 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).
7. Clinically relevant wildtype and mutation sequences on exon 1, 8 and 10 of *CYP21A2* are not covered in this probemix. For additional coverage, NXtec™ D028 Carrier Panel 1 is available. Please note that D028 Carrier Panel 1 is for **research use only**.
8. For use on (un)cultured chorionic villi or amniocytes, maternal cell contamination may lead to wrong conclusions (Nykel et al. 2021, Rebello et al. 1994, van den Berg et al. 2006).

Technique-specific limitations
See the MLPA General Protocol.

9. References Cited in this IFU

1. Fernandez CS et al. (2020). Genetic characterization of a large cohort of Argentine 21-hydroxylase Deficiency. Clin Endocrinol (Oxf). 93:19-27.
2. Liu Y et al. (2022). Comprehensive Analysis of Congenital Adrenal Hyperplasia Using Long-Read Sequencing. Clin Chem. 68:927-939.
3. Nykel A et al. (2021). The Influence of Maternal Cell Contamination on Fetal Aneuploidy Detection Using Chip-Based Digital PCR Testing. Diagnostics. 11:1607.
4. Rebello MT et al. (1994). Maternal contamination of amniotic fluid demonstrated by DNA analysis. Prenat Diagn. 14:109-112.
5. van den Berg C et al. (2006). (Potential) false-negative diagnoses in chorionic villi and a review of the literature. Prenat Diagn. 26:401-408.
6. Xia Y et al. (2022). Genetic analysis and novel variation identification in Chinese patients with congenital adrenal hyperplasia due to 21-hydroxylase deficiency. The Journal of Steroid Biochemistry and Molecular Biology. 222:106156.
7. Xia Y et al. (2024). Novel rapid molecular diagnosis methods for comprehensive genetic analysis of 21-hydroxylase deficiency. Orphanet Journal of Rare Diseases. 19:397.
8. Yuan D et al. (2024). Improved Genetic Characterization of Congenital Adrenal Hyperplasia by Long-Read Sequencing Compared with Multiplex Ligation-Dependent Probe Amplification Plus Sanger Sequencing. The Journal of Molecular Diagnostics. 26:770-780.

Implemented changes in the product description
Version D1-04 – 11 May 2026 (03S) - Updated to new template. - Intended purpose updated, specifying that the assay is manual. In addition, it was specified that this assay can detect large deletions in the *CYP21A2* gene, and that copy number variations of the surrounding region can aid in interpretation. It is also specified that the assay can be used for carrier testing. Moreover, it was added that most defects in *CYP21A2* are point mutations, most of which will not be detected by MLPA. Not all exons of *CYP21A2/1P* are covered in this probemix. - SNVs rs544417036 and rs573835051 can affect the probe signal. However, the warning for these SNVs present in previous product description versions has been replaced by a general warning for small sequence changes. - Mutation nomenclature updated in the probe content table for probes targeting sequences on exon 3 and exon 7 of *CYP21A2/1P*. - An additional column for the rsID was added to the probe content table. - CYP21A1P probe warnings for highly variable copy number in normal individuals were removed. This information is now disclosed in section 8. Limitations. - For the TNXB 19037-L14637 and TNXB 15230-L14636 probes, a warning was added regarding the target location of these probes. - For the CYP21A2 21552-L32321 probe, a warning for sensitivity to experimental variation was added. - Probe warning "∞" was slightly adjusted to refer back to the interpretation table for the I2G probes. - A precaution was included for the inclusion of two Y-fragments. - A precaution was added for the need of parental evaluation in some cases for correct results interpretation. - Precaution 7 was slightly adjusted to include a reference to the Appendix. - Limitation 4 was updated to specify that additional methods are needed to determine whether aberrations detected by P050 CAH occur on the same or different alleles. - A limitation regarding the coverage of the *CYP21A2* gene in this probemix was added. - A limitation for maternal cell contamination was added. - Examples on how to select suitable reference samples were included in the Appendix. - An interpretation guide was included in the Appendix. - Probemix is now IVDR certified.

10. Appendix

Guide on selecting suitable reference samples

In order to detect large *CYP21A2* deletions and CNVs in the surrounding region in patient samples using P050 CAH, it is required to select suitable reference samples. An initial experiment should be performed for the identification of suitable reference samples prior to testing patient samples. SALSA Reference Selection DNA SD039 is provided with this probemix and can be used to identify suitable reference samples. The requirements of reference samples are listed in **section 2. Sample requirements**. By testing 20-25 different DNA samples from unrelated healthy individuals with P050 CAH, and including three reactions of SALSA Reference Selection SD039, there is a good chance of finding at least three different suitable reference samples. Below is **an example** of four sample results from an initial experiment using SD039.

Probe		SD039	Hypothetical sample 1	Hypothetical sample 2	Hypothetical sample 3	Hypothetical sample 4
Length (nt)	Gene, exon, & target	FR	FR	FR	FR	FR
292	*CYP21A1P* upstream region (-113 SNP)	1.0	0.5	1.0	1.0	1.0
184	*CYP21A1P* exon 3 (G111fs*21)	1.0	0.5	1.0	1.0	1.0
175	*CYP21A1P* exon 4 (I173N)	1.0	0.5	1.0	1.0	1.0
220	*CYP21A1P* exon 7 (L308Ffs*6)	1.0	0.5	1.0	1.0	1.0
307	*CYP21A2* upstream region (-113 SNP wildtype)	1.0	1.0	1.0	1.0	1.0
253▲	*CYP21A2* exon 3 I2G-C	1.0	1.0	0	2.0	1.0
258▲	*CYP21A2* exon 3 I2G-A	1.0	1.0	1.0	0	1.0
190	*CYP21A2* exon 3 (G111fs*21 wildtype)	1.0	1.0	1.0	1.0	1.0
157	*CYP21A2* exon 4 (I173N wildtype)	1.0	1.0	1.0	1.0	1.0
232	*CYP21A2* exon 6 (V238E wildtype)	1.0	1.0	1.0	1.0	1.0
238	*CYP21A2* exon 6 (M240K wildtype)	1.0	1.0	1.0	1.0	1.0
214	*CYP21A2* exon 7 (L308Ffs*6 wildtype)	1.0	1.0	1.0	1.0	1.0
148	*TNXB* exon 35	1.0	1.5	1.0	1.0	1.0
318	*TNXB* exon 35	1.0	1.5	1.0	1.0	1.0
355	*TNXB* exon 31	1.0	1.0	1.0	1.0	1.0
326	*TNXB* exon 29	1.0	1.0	1.0	1.0	1.0
202	*TNXB* exon 20	1.0	1.0	1.0	1.0	1.0
364	*TNXB* exon 19	1.0	1.0	1.0	1.0	1.0

▲ A FR of 1.0 for the 253 nt and 258 nt probes corresponds to a copy number of 1. For all other probes a FR of 1.0 represents a copy number of 2.

SD039 contains one copy of the wildtype I2G-**A** sequence, one copy of the wildtype I2G-**C** sequence, and two copies of the other autosomal sequences detected by P050 CAH. Suitable reference samples should have the same composition.

1. Hypothetical sample 1 is not considered a suitable reference sample. While this sample has one copy of each of the wildtype I2G sequences, this sample also has one copy of the *CYP21A1P* pseudogene-specific probes, and three copies of the *TNXB* exon 35 probes, and thus do not fulfil the requirement of having two copies for other autosomal sequences targeted by P050 CAH.
2. Hypothetical sample 2 cannot be used as a reference sample, as this sample does not fulfil the requirement of having one copy of each wildtype I2G sequence, and thus may be a carrier.
3. Hypothetical sample 3 cannot be used as a reference sample as they have two copies of the I2G-**C** sequence and zero copies of the I2G-**A** sequence, thereby not fulfilling the requirement of having one copy of each wildtype I2G sequence.
4. Hypothetical sample 4 shows the same FR as the SD039 for all P050 probes, and therefore can be used as a reference sample in an experiment using patient samples.

Interpretation of results: detection of large *CYP21A2* deletions and rearrangements

This MLPA probemix detects specific loci that represent the limited sequence differences between *CYP21A2* and *CYP21A1P*. MLPA does not determine the chromosomal location of these sequences.

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. Large deletions of the *CYP21A2* gene may also occur but are very rare. In the table below, hypothetical sample results obtained with P050 CAH are presented.

1. Hypothetical sample 1 shows a deletion of the *CYP21A2* upstream region extending to exon 7. No aberrations are detected in the *CYP21A1P* pseudogene. Please note that in samples with a large *CYP21A2* deletion, some probes may show a normal copy number (not shown in this example). 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.
2. MLPA analysis of hypothetical sample 2 shows three copies of the upstream region, exon 3 and exon 4 of the *CYP21A1P* pseudogene. In addition, one copy of the upstream region, exon 3 and exon 4 of *CYP21A2*, and zero copies for the I2G-**A** sequence are detected.
3. MLPA analysis of hypothetical sample 3 shows three copies of *CYP21A1P* exon 3 and 7 and one copy of *CYP21A2* exon 3 and 7.

In all three cases, additional methods are required for complete delineation of the underlying genotype due to the high sequence homology between *CYP21A2* and the *CYP21A1P* pseudogene. Such methods can uncover whether a gene conversion event has occurred or whether they are isolated events (single -or multi-exon deletions not caused by a conversion event), and whether the aberrations occur on the same allele or different alleles.

Overview of results obtained for hypothetical samples

Probe		Suitable reference sample	Hypothetical patient sample 1	Hypothetical patient sample 2§	Hypothetical patient sample 3
Length (nt)	**Gene, exon, & target**	**FR**	**FR**	**FR**	**FR**
292	*CYP21A1P* upstream region (-113 SNP)	**1.0**	**1.0**	**1.5**	**1.0**
184	*CYP21A1P* exon 3 (G111fs*21)	**1.0**	**1.0**	**1.5**	**1.5**
175	*CYP21A1P* exon 4 (I173N)	**1.0**	**1.0**	**1.5**	**1.0**
220	*CYP21A1P* exon 7 (L308Ffs*6)	**1.0**	**1.0**	**1.0**	**1.5**
307	*CYP21A2* upstream region (-113 SNP wildtype)	**1.0**	**0.5**	**0.5**	**1.0**
253▲	*CYP21A2* exon 3 I2G-C	**1.0**	**0**	**1.0**	**1.0**
258▲	*CYP21A2* exon 3 I2G-A	**1.0**	**1.0**	**0**	**1.0**
190	*CYP21A2* exon 3 (G111fs*21 wildtype)	**1.0**	**0.5**	**0.5**	**0.5**
157	*CYP21A2* exon 4 (I173N wildtype)	**1.0**	**0.5**	**0.5**	**1.0**
232	*CYP21A2* exon 6 (V238E wildtype)	**1.0**	**0.5**	**1.0**	**1.0**
238	*CYP21A2* exon 6 (M240K wildtype)	**1.0**	**0.5**	**1.0**	**1.0**
214	*CYP21A2* exon 7 (L308Ffs*6 wildtype)	**1.0**	**0.5**	**1.0**	**0.5**
148	*TNXB* exon 35	**1.0**	**1.0**	**1.0**	**1.0**
318	*TNXB* exon 35	**1.0**	**1.0**	**1.0**	**1.0**
355	*TNXB* exon 31	**1.0**	**1.0**	**1.0**	**1.0**
326	*TNXB* exon 29	**1.0**	**1.0**	**1.0**	**1.0**
202	*TNXB* exon 20	**1.0**	**1.0**	**1.0**	**1.0**
364	*TNXB* exon 19	**1.0**	**1.0**	**1.0**	**1.0**

▲ A FR of 1.0 for the 253 nt and 258 nt probes corresponds to a copy number of 1. For all other probes a FR of 1.0 represents a copy number of 2.

§ Results indicative of a conversion event P050 CAH require the use of complementary methods, such as allele/locus-specific long-range PCR and/or sequencing for full delineation of the genotype, due to the high homology between *CYP21A2* and its pseudogene and potential reciprocal exchanges, as discussed in section 8. Limitations.