CAH RealFast™ CNV Assay

REF 7-410 Σ 100 reactions

-20° C / 2-8°C CE IVD

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1. Intended Use

The CAH RealFast™ CNV Assay is a fast and accurate real-time PCR test to determine copy number variations (CNV) of the *CYP21A2* gene. Partial or complete deletions of *CYP21A2* are known to be associated with defective adrenal steroid metabolism. The kit is designed to identify patients with congenital adrenal hyperplasia (CAH) and should be used in conjunction with the CAH StripAssay® or DNA sequencing. The semi-quantitative assay discriminates between deletions, duplications and normal copy number status in a human genomic DNA extract.
Reference sequence: HGVS: NG_007941.2

2. Introduction

CAH is an autosomal recessive disorder of the adrenal cortex (incidence 1:10,000-15,000) caused in about 95% of cases by genetic defects in the steroid 21-hydroxylase gene *CYP21A2*. The resulting lack of cortisol and aldosterone biosynthesis is ultimately leading to androgen excess. The wide range of clinical manifestations includes classic salt-wasting CAH, classic simple-virilizing CAH as well as mild non-classic forms. The underlying aberrations within the *CYP21A2* gene are (1) point mutations, (2) small deletions/conversions and (3) chromosomal rearrangements like complete *CYP21A2* deletions, duplications or chimeric *CYP21A1P/CYP21A2* genes. Large deletions including *CYP21A1P/CYP21A2* chimeras account for 27% to 39% of mutant alleles in Europe and are in most cases associated with a severe form of the disease.

3. Kit Contents

RealFast™ 2x **Probe Mix**	1 vial	white cap	1.000 µl
CAH CNV **Assay Mix**	1 vial	purple cap	550 µl
CAH CNV **Calibrator**	1 vial	green cap	75 µl

The kit contains reagents for 100 reactions in a final volume of 20 µl each.

The RealFast™ 2x Probe Mix comprises HotStart Taq DNA polymerase and dNTPs in an optimized buffer system. The CAH CNV Assay Mix consists of gene-specific primers as well as dual-labeled hydrolysis probes for *CYP21A2* and the endogenous control (EC) gene. A CAH CNV Calibrator representing the normal status with two functional *CYP21A2* copies is supplied with the kit.

4. Storage and Stability

CAH RealFast™ CNV Assay is shipped on cooling blocks. On arrival, store the kit at -20°C. Alternatively, store at 2 to 8°C for short-term use within one month. The kit withstands up to 20 freeze/thaw cycles with no loss of activity. Avoid prolonged exposure to intense light. If stored correctly, the kit will retain full activity until the expiration date indicated on the label.

5. Product Description

5.1. Principle of the Test

The test is based on the fluorogenic 5' nuclease assay, also known as TaqMan® assay. Each reaction contains gene-specific primer pairs for amplification of *CYP21A2* and endogenous control (EC) gene fragments with 141 bp each. Further components are two dual-labeled, gene-specific hydrolysis probes, the **FAM-labeled CYP21A2 probe** and the **HEX-labeled EC probe,** which hybridize to an internal sequence of the amplified fragments. The proximity of the 5'-fluorescent reporter and 3'-quencher dye on intact probes prevents the reporter from fluorescing. During the extension phase of PCR the 5' - 3' exonuclease activity of Taq DNA polymerase cleaves the 5'-fluorescent reporter from the hybridized probe. The physical separation of the fluorophore from the quencher dye generates a fluorescent signal in real-time, which is proportional to the accumulated PCR product.
The CAH RealFast™ CNV Assay is a relative quantitation assay and compares the amount of both nucleic acid targets (*CYP21A2* and EC) in relation to the CAH CNV Calibrator. The EC gene is used to normalize fluorescence signals between different samples and serves as a PCR positive control.

5.2. Real-time PCR Instrument Compatibility

The CAH RealFast™ CNV Assay is validated for use with the AB 7500 Fast instrument.
The kit is compatible with various common real-time PCR instruments capable of recording FAM and HEX fluorescence:

- ✓ AB 7500 Fast (Applied Biosystems®)
- ✓ AB StepOne™ (Applied Biosystems®)
- ✓ CFX96™ (Bio-Rad)
- ✓ LightCycler® 480 (Roche)
- ✓ Mx3005P (Agilent Technologies)
- ✓ Rotor-Gene® 6000 (Qiagen)

*» **Note:** RealFast™ CNV QuickGuides for setting up and analyzing experiments on different types of instruments can be downloaded from www.viennalab.com.*

When using AB 7500 Fast, StepOne™ or Mx3005P set passive reference dye to "ROX" ! «

The kit is supplied with **low ROX**. For use with real-time PCR instruments requiring high ROX for normalization of data (e.g. Applied Biosystems® instruments: StepOne™, 7300, 7900/7900HT), add ROX at a final concentration of 1 µM to the 2x Probe Mix.

5.3. Assay Performance Specifications

Determination of **sensitivity** was performed on 107 alleles testing positive for a *CYP21A2* deletion / duplication with a reference kit. The CAH RealFast™ CNV Assay determined all 107 alleles as positive, which equaled a true positive rate of 100%.

Determination of **specificity** was performed on 435 alleles testing negative for a *CYP21A2* deletion / duplication with a reference kit. The CAH RealFast™ CNV Assay determined all 435 alleles as negative, which equaled a true negative rate of 100%.

Limit of detection: 0.2 ng genomic DNA (per reaction)
Recommended DNA concentration: 2 to 20 ng/µl genomic DNA

6. Materials Required but not Supplied

Real-time PCR instrument with FAM (520 nm) and HEX (556 nm) filters, instrument-compatible reaction vessels, disposable powder-free gloves, vortexer, mini-centrifuge for 2.0 ml tubes, tube racks, set of calibrated micropipettes (0.5 – 1000 µl), sterile tips with aerosol-barrier filter, molecular grade water, DNA extraction system, freezer, biohazard waste container.

7. Experimental Protocol

7.1. DNA Extraction

DNA extraction reagents are **not supplied** with the kit. DNA isolated from various specimens (e.g. whole peripheral blood or dried blood spots) can be used. Ensure extracted DNA is suitable for amplification in terms of concentration, purity and integrity. For accurate analysis the DNA amount per reaction should be within the range of 10 to 100 ng for all samples.

7.2. No Template Control

Always include a **No Template Control** (NTC) in each experiment to confirm absence of potential contaminations. Use PCR-grade water instead of DNA.

7.3 CAH CNV Calibrator

Always include the CAH CNV **Calibrator**. The Calibrator (also "reference sample" or "control") has to be defined in the real-time PCR software.

*» **Note**: Control samples like the CAH CNV Calibrator are potential sources of contamination. Make sure to handle them carefully«.*

7.4. Replicates

In order to obtain the desired precision of measurements it is necessary to run the NTC, all samples and the Calibrator in **triplicate**.

7.5. Preparation of **Master Mix**

Gently vortex and briefly centrifuge all solutions after thawing. Set up PCR at room temperature. Prepare sufficient **Master Mix** for all replicates (3 x N samples + 3 x Calibrator + 3 x NTC) plus four to six additional reactions to compensate for pipetting inaccuracies:

Component	per reaction	e.g. 36 reactions
2x RealFast™ Probe Mix	10 µl	360 µl
CAH CNV Assay Mix	5 µl	180 µl
Master Mix	**15 µl**	540 µl

7.6. Preparation of reactions in triplicate

Prepare the reactions for the NTC, all samples and the CAH CNV Calibrator. To appropriately sized tubes, add the volumes of master mix and sample listed below:

Tube	Master Mix Volume	Sample Name	Sample Volume
1	49.5 µl	NTC	16.5 µl
2	49.5 µl	Sample	16.5 µl
3	49.5 µl	Calibrator	16.5 µl

Mix gently and centrifuge the tubes briefly. Run your reactions in **triplicate** and dispense **20 µl** into the appropriate wells of the reaction vessels. To minimize risk of contaminations always pipette templates in the following order: first NTC, then samples, last Calibrator. Immediately close reaction vessels.

*» **Note:** Avoid creating bubbles in the final reaction mix and avoid touching the optical surface of the cap or sealing film without gloves. Both may interfere with fluorescence measurements. Centrifuge briefly if needed. «*

7.7. PCR Program

Program the real-time PCR instrument according to the manufacturer's instructions for relative quantitation experiments.
Place the samples into the thermal cycler and run the following program:

AB 7500 Fast, StepOne™, **CFX96™, LightCycler® 480, Mx3005P** and **other** Peltier heating block-based **instruments:**

Cycles	Temp	Time	Steps
1	95°C	10 min	Initial denaturation
40	95°C	15 sec	Denaturation
	60°C	1 min	Annealing/Extension – **Data acquisition** on FAM- and HEX-channel

Rotor-Gene® 6000:

Cycles	Temp	Time	Steps
1	95°C	10 min	Initial denaturation
40	95°C	15 sec	Denaturation
	36-well rotor: **56°C**	1 min	Annealing/Extension – **Data acquisition** on Green and Yellow channel
	72-well rotor: **60°C**		

8. Data Analysis / Interpretation of Results

The **copy number variation** (CNV) of each sample is determined by calculating the relative quantity of ***CYP21A2*** **(FAM-channel)** by means of the normalizer **EC (HEX-channel)** and comparing it to the CAH CNV **Calibrator.** Most real-time PCR software automatically resolve data of both channels into a bar chart of relative quantities which is normally used for gene expression experiments. The calibrator is set to one and normal samples will have relative quantities close to one, whereas duplications result in significantly higher values and deletions (as well as most *CYP21A1P/CYP21A2* chimeric genes) in significantly lower values. Due to potential measuring errors it is advisable to repeat test reactions which show relative quantities close (± 0.05) to the minimum and maximum for normal samples. A list containing instrument-specific terminology, threshold settings (Cq) and measuring range in relation to the CNV status is given below.

*» **Note**: The 8bp deletion in exon 3 (c.329_336del GAGACTAC) leads to the same results as a complete CYP21A2 deletion or most CYP21A1P/CYP21A2 chimeric genes. Only CYP21A1P/CYP21A2 chimeras which do not include the 8bp deletion will appear normal.*

Homozygous deletions, as well as homozygous chimeric genes and 8bp deletions result in a drop-out of signal for CYP21A2. Only a signal for the EC can be detected.«

Real-time PCR Instrument	Threshold	Relative Quantities			Terminology
		Deletion	Normal	Duplication	
AB 7500 Fast, StepOne™	0.1	< 0.61	0.61 – 1.24	> 1.24	Relative Quantities (RQ)
CFX96™ (Bio-Rad)	automatic	< 0.69	0.69 – 1.19	> 1.19	Relative Normalized Expression
LightCycler® 480 (Roche)	---	< 0.66	0.66 – 1.09	> 1.09	Normalized Ratio
Mx3005P (Agilent Technologies)	0.2	< 0.74	0.74 – 1.27	> 1.27	Rel. Quant. to Cal. (dRn)
Rotor-Gene® 6000 (Qiagen)	0.05	< 0.63	0.63 – 1.20	> 1.20	Relative Concentration

9. Warnings and Precautions

- For *in vitro* diagnostics use only.
- Always use disposable powder-free gloves and wear suitable lab coat when handling specimens and reagents.
- Perform reaction setup in an area separate from nucleic acid preparation and PCR product analysis.
- Use pipettes dedicated for PCR setup only, use aerosol-guarded pipette tips.
- Use instrument-compatible reaction vessels with optically clear caps or sealers.
- Do not mix reagents from different lots.
- Do not use expired kits or kit components.