CYP21B MUT ToolSet™ for LightCycler™

For detection of the P30L, I2G, del8bp, I172N, E6 cluster, V281L, F306+T, Q318X, R356W and P453S mutations in the functional CYP21B (21-Hydroxylase) gene

Lyophilized ToolSet for PCR using the LightCycler™ Instrument. Licensed by Roche Diagnostics GmbH

Order#: CYP21B MUT - 16

1 ToolSet for 16 reactions

Store at 4°C, protected from light.
Exposure to light may especially damage
the OligoTool ™ tubes (vials with red caps).

For use with Qiagen HotStar HiFidelity Polymerase Kit (Qiagen Cat.No.: 202605)

1. ToolSet contents

Vial	Label	Content	Quantity
1-11, Red caps	**OligoTools 1-11**	- lyophilized oligos for PCR containing : - primers for specific amplification of the **functional CYP21B gene** - mutation detection and anchor probes	For 16 tests Dissolved: 50 µL
12, Green Cap	**CYP21B Normal Control**	- Wild type human DNA, lyophilized	For 5 uses Dissolved: 500 µL
13-14, Blue cap	**Solvent**	- to dissolve OligoTool / Control	2 x 1000 µL
15, Pink cap	**G Solution**	- Polymerase / Proofreading Enhancer	1000 ul

Additional equipment and reagents required but not supplied : Qiagen HotStar HiFidelity Polymerase Kit (Qiagen Cat.No.: 202605); LightCycler instrument, LightCycler capillaries, DNA extraction materials.

2. Introduction

2.1. Product overview

ToolSet description

The **CYP21B MUT ToolSet** is designed for screening the functional human **CYP21B** gene (**21-Hydroxylase**) for presence of the **P30L, I2G, del8bp, I172N, E6 cluster, V281L, F306+T, Q318X, R356W and P453S** mutations by LightCycler PCR with Melting Curve Analysis within **2.5 hours** including about 30 minutes hands-on time.

Background information

The above mutations except P453S are present in the CYP21A pseudogene from where they may move to the functional CYP21B gene by gene conversion.

Test principle

The functional gene CYP21B is specifically amplified by primer sets selecting against pseudogene sequences yielding amplicons of ≈ 2kb in length requiring the use of a Hot Start high fidelity polymerase with proofreading activity.
For each mutation a separate OligoTool with specific detection probes is used.
For analysis of the 8 bp deletion, two OligoTools are used – one deteting the presence of the 8bp sequence and one detecting the 8 bp deletion.

Note !

In ≈ **30 %** of cases of classical adrenogenital syndrome the functional CYP21B gene is absent or grossly disturbed as a result of a large gene deletion / conversion.
This will result in a failure to amplify long sequences of the functional gene.

It is therefore strongly advised to test a sample for large deletion / conversion before initiating analysis of point mutations and small deletions.
For this we recommend the **CYP21B fl/del ToolSet™ for LightCycler™ .**

Control material **CYP21B Normal Control**, lyophilized.

Storage of ToolSet Store at +4°C when lyophilized, protected from light. The unopened lyophilized ToolSet is stable at +4°C for 12 months from date of manufacture if protected from light. When dissolved store at +4°C for a maximum of 4 weeks, or at -20°C for longer periods (months), protected from light. Avoid freezing and thawing.

Storage of Qiagen HotStar HiFi Kit Store as indicated in the Qiagen HotStar HiFidelity PCR Handbook (-20°C).
Advice : Store the Qiagen HotStar HiFi Polymerase in 10ul aliquots and the Qiagen HotStar HiFi Buffer in 100 ul aliquots both at -20 °C.

3. Preparation for LightCycler PCR

Toolset preparation

1. Before opening tubes, centrifuge them quickly.
2. **Dissolve** the content of the **OligoTool** tubes (Red Caps) with **50 µl of Solvent**.
3. **Dissolve** the content of the **Control** tube (Green Cap) with **500 µl of Solvent**.
4. Recap tubes, vortex gently and centrifuge tubes quickly.
5. Proceed to Reaction Mix preparation.

Reaction Mix Preparation Thaw **HotStar HiFi Buffer** and **HotStar HiFi Polymerase** from the **Qiagen HotStar HiFidelity Polymerase Kit.** Place them and **Genes-4U G solution** and Genes-4U Solvent in a cooled metal block (+ 4 to + 6 °C).

For each DNA sample to be tested, 11 reactions will be set up.
Prepare the Reaction Mix as shown in the following table, mix gently, centrifuge briefly and keep cooled until the next step :

Reagent	µL
Genes-4U Solvent (Blue cap tube)	66
Qiagen HotStar HiFi Buffer	58
Genes-4U G Solution (Pink cap tube)	30
Qiagen HotStar HiFi Polymerase	10
DNA sample	92
Total Reaction Mix	256

More Samples ? **For each DNA sample to be tested prepare a separate reaction mix.**

Reaction Setup **For each sample to be tested, use 11 (eleven) capillaries in the LC carousel. Into each capillary, dispense 2.8 ul of one of the dissolved OligoTools.**
We suggest that you use the order of the mutation positions in the gene :

Capillary #	**OligoTool**
1	P30L
2	I2G
3	Del 8bp
4	With 8bp
5	I172N
6	E6 cluster
7	V281L
8	F306+T
9	Q318X
10	R356W
11	P453S

Into each capillary, dispense 22.2 ul of the Reaction Mix prepared above.

Cap and centrifuge capillaries and run the LightCycler program.

Positive Control Always run a normal DNA control with the samples.
Use the dissolved **CYP21B Normal Control** DNA (Green Cap).

Negative control To prepare a negative control, replace the template DNA with Solvent (Blue Cap).

Extraction of genomic DNA You can use different Kits for DNA isolation, either with a manual method or with an automated system. The elution buffers should be salt-free.
Example : Roche High Pure PCR Template Preparation Kit (Cat.No. 1 796 828)

Application The **CYP21B ToolSet™ for LightCycler™** has been specifically designed for genotyping the functional CYP21B gene for presence of the P30L, I2G, del8bp, I172N, E6 cluster, V281L, F306+T, Q318X, R356W and P453S mutations. Together with large deletions / conversions of the CYP21B gene detectable by the **CYP21B fl/del ToolSet™ for LightCycler™**, about 95 % of mutations underlying CYP21 deficiency / Adrenogenital Syndrome can be detected.
More information is available in the *Genes* section at www.Genes-4U.com

Note : This ToolSet was developed for use in life science research only.

4. LightCycler Settings and Experimental Protocol (Developed with LC Program Version 3.5)

Denaturation	**Value**
Cycles	1
Analysis Mode	None
Temperature Targets	**Segment 1**
Target Temperature (°C)	95
Incubation time (s)	300
Temperature Transition Rate (°/s)	20
Acquisition Mode	None

Amplification	**Value**		
Cycles	**50**		
Analysis Mode	None		
Temperature Targets	**Segment 1**	**Segment 2**	**Segment 3**
Target Temperature (°C)	95	63	72
Incubation time (s)	15	**60**	**120**
Temperature Transition Rate (°/s)	20	20	**3**
Acquisition Mode	None	Single	None

Melting Curve Analysis	**Value**		
Cycles	1		
Analysis Mode	Melting Curves		
Temperature Targets	**Segment 1**	**Segment 2**	**Segment 3**
Target Temperature (°C)	95	36	90
Incubation time (s)	60	60	0
Temperature Transition Rate (°/s)	20	20	0.2
Acquisition Mode	None	None	Continuous

Cooling	**Value**
Cycles	1
Analysis Mode	None
Temperature Targets	**Segment 1**
Target Temperature (°C)	40
Incubation time (s)	30
Temperature Transition Rate (°/s)	20
Acquisition Mode	None

Fluorescence display mode : Use F2/F1. For LC Program Versions 3.3 or lower gains F1=1; F2=15. For LC Program Versions 3.5 and higher use automatic gain control.

5. Typical results

Introduction

Use the Melting Curve program to genotype the human genomic DNA research samples. Melting peaks allow discrimination between possible genotypes of the different mutation sites in the functional human CYP21B (21-Hydroxylase) gene.

The following **table** summarizes the **wild type / mutant Tm** values for each OligoTool.

Note : For the del 8bp position, 2 different OligoTools - "Del 8bp" and "With 8bp" - are used.

OligoTool	**Wild type Tm** (°C)	**Mutant Tm** (°C)	**Probe fits to :**
P30L	63.5	52.5	Wild type
I2G	52.5	58.5	**Mutant**
Del 8bp	No peak	59.5	**Del 8bp**
With 8bp	59	No peak	With 8bp (Wild type)
I172N	62.5	57	Wild type
E6 cluster	62.8	52-54 *	Wild type
V281L	62.5	53	Wild type
F306+T	57	51.5	Wild type
Q318X	63	53	Wild type
R356W	65.8	52.5	Wild type
P453S	59	47.5	Wild type

* Depending on the number of mutations in the E6 cluster

Note : The values for the respective melting temperatures may vary for +/- 2.5 °C between different experiments. The CYP21B ToolSet™ has been developed for and validated with the LightCycler™ and its original accessory materials and reagents. Genes-4U has not validated the ToolSet with other instruments, accessories or reagents.

Examples of Melting Curves : see next page

Figures on the next page show typicals result obtained with the CYP21B ToolSet™ for LightCycler™ using **F2/F1** as fluorescence readout.
BLUE curves indicate Wild type, **RED** curves indicate Mutant Allels.
Conditions : Program version 3.5 with automatic gain setting, No Color compensation, Digital Filter enabled, Calculation Method : Polynomial, Degrees to average : 12-14.

Note : DO NOT USE COLOR COMPENSATION as it may cause spurious peaks.

7. Notices to Purchaser

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CYP21 B Mutation Analysis by LightCycler : Melting Curve Examples

Wild type DNA **Synthetic All Mutant DNA**