LightMix® Kit *CYP 2C9*2* and *CYP 2C9*3*

Cat.-No. 40-0298-32

Detection Kit with reagents for the detection of alleles *CYP2C9*2* and *CYP2C9*3* in human DNA using the Roche Diagnostics LightCycler® 1.x / 2.0 / 480 and cobas z 480 Analyzer.

Lyophilized mix of primers and probes for a total of 96 reactions with a final volume of 20 µl each. **Store protected from light at room temperature (18-25°C), do N OT freeze!**

Instructions for use with the LightCycler® 1.x / 2.0 Instruments see pages 4-5
Instructions for use with the LightCycler® 480 and cobas z 480 Instruments see pages 6-7

1. Introduction

Cytochrome P450 (CYP) enzymes are monooxygenases primarily localized in the liver. They are in particular important for oxidation reactions in drug metabolism. *CYP 2C9* is known to metabolize commonly prescribed drugs such as S-warfarin, Ibuprofen, Diclofenac [1]. For warfarin also *VKORC1* is known to be relevant for dose adaption [2]. *CYP2C9* belongs to the CYP2C subfamily, including also the isoenzymes *2C8*, *2C18* and *2C19*.

The *CYP2C9* gene is polymorphic. Within the inactive alleles *2C9* *2, *3, '6, *15 and *25 only **2* and **3* occur more frequently in Caucasians. *2C9*2* (rs1799853) causes an amino acid change p.Arg144Cys while *2C9*3* (rs1057910) causes p.Ile359Leu. See also: www.cypalleles.ki.se/cyp2c9.htm.

Variations in the *CYP 2C9* gene can been detected by Real-Time-PCR using TaqMan probes [3] or probe based melting curve analysis with the LightCycler® instruments [4].

This device identifies the alleles **2* and **3* which exhibit a particular medical relevance. Patients with a slow metabolizer genotype will need most likely a lower dose of S-warfarin and have a higher risk of bleeding. A reliable genotyping assists for an individual and safe pharmacotherapy.

The kit has been tested with 'LightCycler® FastStart DNA Master HybProbe' only.

2. Description

Two 374 bp and 180 bp long fragment of the human *CYP2C9* gene are amplified with specific primers. Allele**2* is detected with a SimpleProbe® probe (detected in channel 530) and allele**3* is detected with LightCycler® Red 640 labeled probes (detected in channel 640).

The genotypes are identified by running a melting curve with specific melting points (Tm) of 58.8°C for the wildtype and 50°C for the mutant for human *CYP 2C9*2* in channel 530 and 48.9°C for the wildtype and 59°C for the mutant for human *CYP 2C9*3* in channel 640 (LightCycler® 1.x / 2.0 Instruments). View data for LightCycler® 480 Instruments, in *8.3. Sample Data – typical results*.

The use of a color compensation file generated with the LightMix® – Assay Color Compensation 530/640/690 is a prerequisite for identifying the genotypes in the corresponding channel.

The supplied control DNAs allow for the accurate comparison with unknown samples.

For use in LightCycler® 480 Instruments use filter combination 483-533 instead of channel 530 and filter combination 483-640 instead of channel 640 for detection.

3. Set Contents

3 Vials with red cap containing lyophilized primers and probes for 32 PCR reactions *CYP 2C9*

1 Vial with colorless cap containing *CYP2C9*2* reaction wild type control DNA (10^5 per reaction)
1 Vial with colorless cap containing *CYP2C9*2* reaction mutant control DNA (10^5 per reaction)
1 Vial with colorless cap containing *CYP2C9*2* reaction hetero control DNA (10^5 per reaction)

1 Vial with colorless cap containing *CYP2C9*3* reaction wild type control DNA (10^5 per reaction)
1 Vial with colorless cap containing *CYP2C9*3* reaction mutant control DNA (10^5 per reaction)
1 Vial with colorless cap containing *CYP2C9*3* reaction hetero control DNA (10^5 per reaction)

4. Additional Reagents and items required

	Roche Diagnostics
ColorCompensation HybProbe order n°40-0318-00	Cat. -No. 05 997 704 001
LightCycler® FastStart DNA Master HybProbe	Cat.-No. 03 003 248 001
LightCycler® Capillaries (20 µl) (LightCycler® 1.x / 2.0 Instruments)	Cat.-No. 04 929 292 001
LightCycler® 480 Multiwell Plate 384, white (LightCycler® 480 Instrument)	Cat.-No. 04 729 749 001
or LightCycler® 480 Multiwell Plate 96, white (LightCycler® 480 Instrument)	Cat.-No. 04 729 692 001

For use in LightCycler® 1.x Instruments with software version 3.5.3 read channel F2 instead of channel 640, channel F3 instead of channel 705 and channel F1 instead of channel 530 for detection. We recommend upgrading LightCycler® 1.x Instruments to software version 4.1.

For use in LightCycler® 480 Instruments use filter combination 483-533 (LightCycler® 480 II Instrument filter combination 465-510) instead of channel 530 and filter combination 483-640 (LightCycler® 480 II Instrument filter combination 498-640) instead of channel 640 for detection.

4.1. Optional Additional Reagents

High Pure PCR Template Preparation Kit	Cat.-No. 11 796 828 001

5. Product Characteristics

PCR results (activation, 50 cycles and melting curve) are obtained within 50 minutes with the capillary based LightCycler® 1.x / 2.0 Instruments and within 80 minutes with '480' plate based Instruments.

Sensitivity
These reagents detect the variation with 1 ng human DNA using the Roche 'LightCycler® FastStart DNA Master HybProbe' (in an exemplary system, using cloned targets as reference).

Measuring range
Allowed range of target DNA is 10 to 100 ng using 'LightCycler® FastStart DNA Master HybProbe'.

Storage and Stability
- Lyophilized reagents are stable for at least 6 months after shipment when stored protected from light at room temperature (18-25°C). See expiry on the product label.
- **Do not freeze** lyophilized reagents.
- Dissolved reagents are stable for at least 10 days when stored protected from light and refrigerated (4°C).

6. Experimental Protocol

The following procedure was developed for use with the LightCycler® 1.x / 2.0 / 480 Instruments.
Start programming before preparing the solutions. See the Instrument operator's manual for details.

Sample material: Use aqueous nucleic acid preparations (e.g. 'High Pure PCR Template Prep. Kit' for genomic DNA samples).

Negative control: Always run at least one no-template control (NTC) - replace the template DNA with water.

Positive control: Run a positive control - replace the template DNA with the provided control DNA.

6.1. Preparation of parameter-specific reagents (32 reactions):

One reagent vial with a **red** cap contains primers and probes to run 32 rxns for *CYP 2C9*2* and **3*.

Add 66 µl PCR-grade water to each reagent vial, mix the solution (vortex) and spin down.

►**Use2 µl** reagent for a 20 µl PCR reaction.

This solution is stable at least five days when stored refrigerated at 4°C. Avoid prolonged exposure t o light.

6.2. Preparation of the control DNA

Add 80 µl PCR-grade water to each vial (1.6 x 10^6 target molecules) with a colorless cap.
Mix the target DNA by pipetting the solution up and down 10 times.

Note: Control DNA can be dissolved up to 160 µl to achieve 32 control reactions.

►Use 5 µl **control DNA** for a 20 µl PCR reaction.

This solution is stable at least five days when stored refrigerated at 4°C, for long term storage free ze at -20°C. Avoid repeated freezing thawing cycles. For the heterozygote control DNA provided with the kit please note that the relative amounts of wild type DNA and mutant DNA may change during time.
Please note that opening these vials may cause contaminations of the work-space (aerosol).

6.3. Preparation of the LightCycler® reaction mix

In a cooled reaction tube, prepare the reaction mix by multiplying each volume for a single reaction by the number of reactions to be cycled plus one additional reaction.

For use with the Roche FastStart Master	
Single reaction	**Component**
9.8 µl	water, PCR-grade (colorless cap, provided with the Roche Master kit)
1.2 µl	Mg^{2+} solution 25 mM (blue cap, provided with the Roche FastStart kit)
2.0 µl	**reagent** mix (parameter specific reagents containing primers and probes, see **6.1.**)
2.0 µl	Roche Master (red cap, for preparation see Roche manual)
15.0 µl	Volume of reaction mix

Table 1

Mix gently, spin down and **transfer 15 µl** of the reaction mix to a capillary or well.

Add 5 µl of sample or standard to each capillary or well for a final reaction volume of 20 µl.
Close the capillaries / attach a foil to the multiwell plate and seal, and spin down.

Start run.

7. LightCycler® 1.x / 2.0 Instruments

7.1. Programming

The protocol consists of four program steps

- 1: Denaturation: sample denaturation and enzyme activation
- 2: Cycling: PCR-amplification of the target DNA
- 3: Melting: melting curve analysis for identification of the PCR product derived from the target DNA
- 4: Cooling: cooling the instrument

Program Step:	Denaturation	Cycling			Melting			Cooling
Parameter								
Analysis Mode	None	Quantification mode			Melting Curves mode			None
Cycles	1	45			1			1
Target [°C]	95	95	60	72	95	40	85	40
Hold [hh:mm:ss]	00:10:00	00:00:05	00:00:10	00:00:15	00:00:20	00:00:20	00:00:00	00:00:30
Ramp Rate [°C/s]	20	20	20	20	20	20	0.2	20
Acquisition Mode	None	None	Single	None	None	None	Continuous	None

Table 2

7.2. Data Analysis

For use in LightCycler® 1.x Instruments with software version 3.5.3 read channel F2 instead of channel 640 and channel F1 instead of channel 530 for detection. We recommend upgrading LightCycler® 1.x instruments to software version 4.1.

Switch the color compensation mode on. If this mode is not enabled run the color compensation program. Follow the instructions in the manual of the Color Compensation Kit HybProbe 530/640/690'. Perform data analysis, as described in the LightCycler® Instrument operator's manual.

View *CYP 2C9*3* data in channel 640 and *CYP 2C9*2* data in channel 530, "Tm Calling" Analysis mode (LightCycler® 2.0 Instrument) or Melting Curves mode (LightCycler® 1.x Instrument).

The negative control (NTC) must show no signal.

7.3. Sample Data – Typical Results

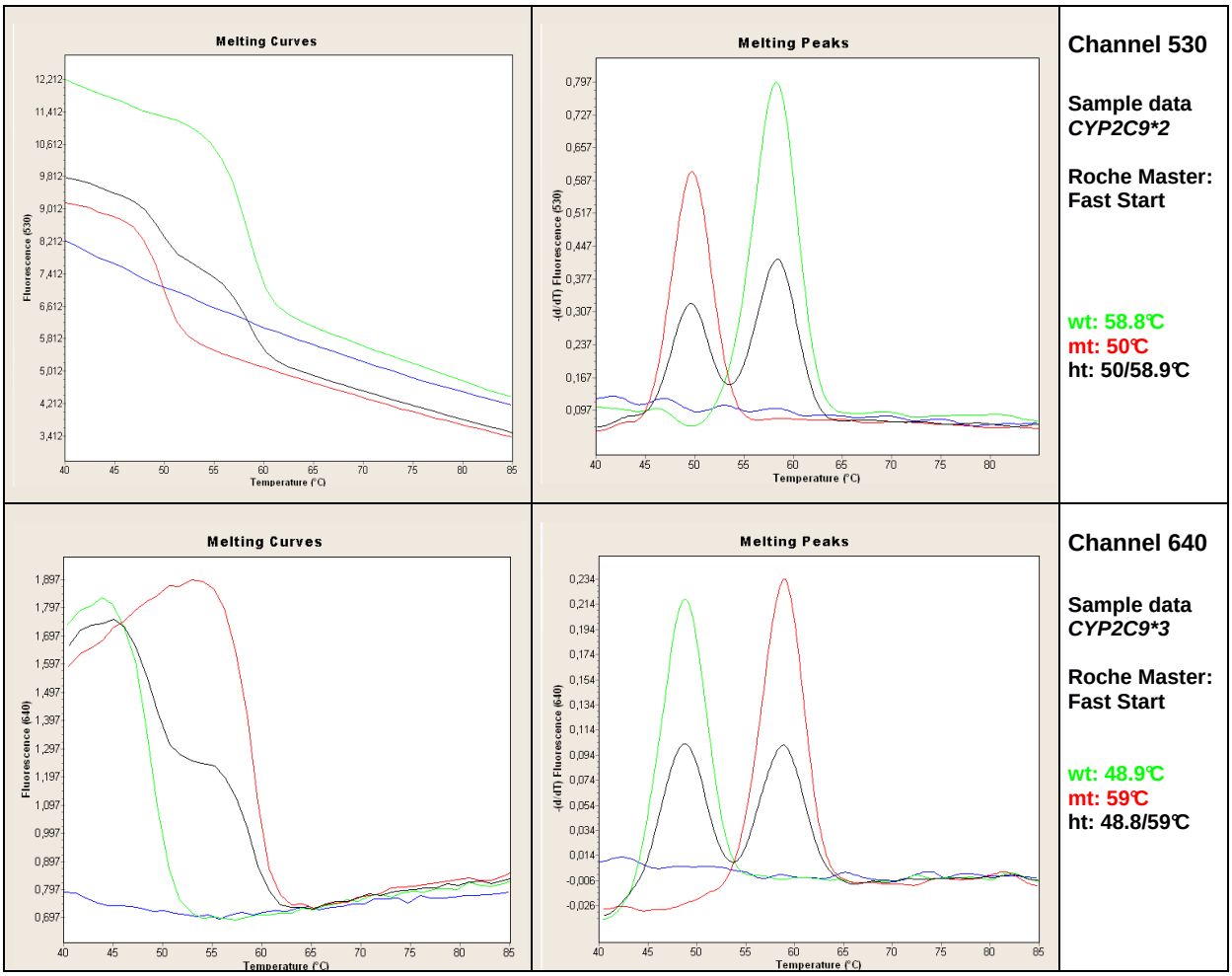

Fig.1. LightCycler® 2.0 sample data for the *CYP 2C9*2 und CYP 2C9*3* detection system.

Upper panels: Data from channel 530. Left panel melting curves for *CYP 2C9*2*. Right panel melting peaks for *CYP 2C9*2*. Wildtype (wt) corresponds with *CYP 2C9 *1/*1*, heterozygous (hetero) corresponds with **1/*2* and mutant (mt) with **2/*2*.
Lower panels: Data from channel 640. Left panel melting curves for *CYP 2C9*3*. Right panel melting peaks for CYP 2C9*3. Wildtype (wt) corresponds with *CYP2C9*1/*1*, heterozygous (ht) with **1/*3* and mutant (mt) corresponds with **3/*3*.

7.4. Interpretation of Data

*2 Channel 530 Melting point(s)		*3 Channel 640 Melting point(s)		CYP 2C9 alleles	Metabolizers Phenotype
-	58.1℃	48.9℃		*1/*1 (wild type)	Extensive
49.7℃	58.1℃	48.9℃	-	*1/*2	Intermediate
	58.1℃	48.9℃	58.7	*1/*3	Intermediate
49.7℃	-	48.9℃	-	*2/*2	slow (inactive)
	58.1℃	-	58.7	*3/*3	slow (inactive)
49.7℃	58.1℃	48.9℃	58.7	*2/*3	slow (inactive)
49.7℃			58.7	never observed	slow (inactive)
-	-	-	-	PCR failure	**Repeat test**
ΔTm 8.4℃		ΔTm 9.8℃			

Table 3 Typical analysis results (LightCycler® 2.0 Instrument, Roche Master: Fast Start)

***Notes**:* The values of the respective melting temperatures (T_M) may vary ±2.5℃ between different experiments. Th e ΔT between the melting peaks for heterozygote genotypes may vary ±1.5℃. Samples with deviating melting curves sh ould be subject to further investigations; sequence analysis can be provided by TIB MOLBIOL Berlin (contact service@tib-molbiol.de).

8. LightCycler® 480 Instruments and cobas z 480 Analyzer

8.1. Programming

The protocol consists of four program steps

- 1: Denaturation: sample denaturation and enzyme activation
- 2: Cycling: PCR-amplification of the target DNA
- 3: Melting: melting curve analysis for identification of the PCR product derived from the target DNA
- 4: Cooling: cooling the instrument

Detection Format:
LightCycler® 480 Instrument: 483-533, 483-640
LightCycler® 480 II Instrument: 465-510, 498-640
Cobas® Z480 Instrument: 465-510, 498-645

Program Step:	Denaturation	Cycling			Melting			Cooling
Parameter								
Analysis Mode	None	Quantification mode			Melting Curves mode			None
Cycles	1	45			1			1
Target [°C]	95	95	60	72	95	40	85	40
Hold [hh:mm:ss]	00:10:00	00:00:05	00:00:10	00:00:15	00:00:30	00:02:00	00:00:00	00:00:30
Ramp Rate [°C/s] **96**	4.4	4.4	2.2	4.4	4.4	1.5	-	1.5
Ramp Rate [°C/s] **384**	4.6	4.6	2.4	4.6	4.6	2.0	-	2.0
Acquisition Mode	None	None	Single	None	None	None	Continuous	None
Acquisitions [per °C]	-	-	-	-	-	-	**3**	-

Table 4

8.2. Data Analysis

Note: cobas z 480 analyzer signal levels are about 50% compared to LightCycler® 480 II results.

Note: For use on LightCycler® 480 II Instruments select Filter Combination 465-510 instead of Filter Combination 483-533 and Filter Combination 498-640 instead of Filter Combination 483-640 for detection.

Switch the color compensation mode on. If this mode is not enabled run the color compensation program. Follow the instructions in the manual of the Color Compensation Kit HybProbe 530/640/690'.

Perform data analysis, as described in the LightCycler® Instrument operator's manual.

View *CYP 2C9*2* data with Filter Combination 483-533 and *CYP 2C9*3* data with Filter Combination 483-640, "Tm Calling" Analysis mode.

The negative control (NTC) must show no signal.

8.3. Sample Data – Typical Results

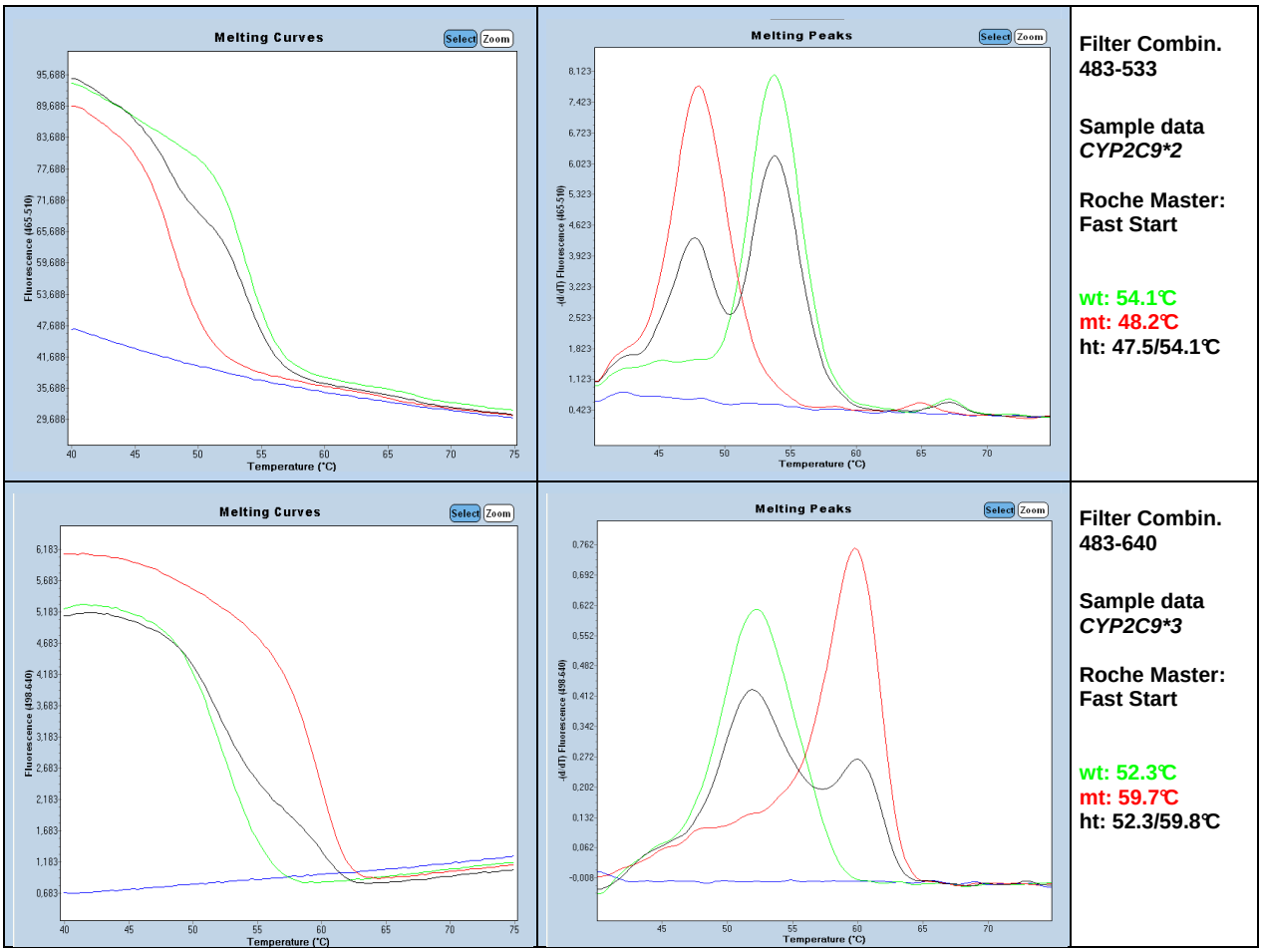

Fig.2. LightCycler® 480 sample data for the *CYP 2C9*2 und CYP 2C9*3* detection system.

Upper panels: Left panel Filter Combination 465-510 melting curves for *CYP 2C9*2*, right 465-510 melting peaks for *2. Wildtype (wt) corresponds with *CYP 2C9 *1/*1*, heterozygous (hetero) corresponds with **1/*2* and mutant (mt) with **2/*2*.
Lower panels: Left panel Filter Combination 498-640 melting curves for *CYP 2C9*3*, right 498-640 melting peaks for **3.* Wildtype (wt) corresponds with *CYP2C9*1/*1*, heterozygous (ht) with **1/*3* and mutant (mt) corresponds with **3/*3*.

8.4. Interpretation of Data

*2 Filter 483-533 Melting point(s)		*3 Filter 483-640 Melting point(s)		CYP 2C9 alleles	Metabolizers Phenotype
-	54.1℃	52.3		*1/*1 (wild type)	Extensive
48.2℃	54.1℃	52.3	-	*1/*2	Intermediate
-	54.1℃	52.3	59.7℃	*1/*3	Intermediate
48.2℃	-	52.3	-	*2/*2	slow (inactive)
-	54.1℃	-	59.7℃	*3/*3	slow (inactive)
48.2℃	54.1℃	52.3	59.7℃	*2/*3	slow (inactive)
48.2℃			59.7℃	never observed	slow (inactive)
-	-	-	-	PCR failure	**Repeat test**
ΔTm 5.9℃		ΔTm 7.4℃			

Table 5. Typical analysis results (LightCycler® 480 Instrument, Roche Master: Fast Start)

***Notes**:* The values of the respective melting temperatures ™ may vary ±2.5℃ between different experiments. The ΔT between the melting peaks for heterozygote genotypes may vary ±1.5℃. Samples with deviating melting curves sh ould be subject to further investigations; sequence analysis can be provided by TIB MOLBIOL Berlin (contact service@tib-molbiol.de)

9. References

[1] CYP2C9 gene variants, drug dose, and bleeding risk in warfarin-treated patients: A HuGENet™ systematic review and meta-analysis. Sanderson et al., (2005)

[2] VKORC1 and CYP2C9 genotypes and acenocoumarol anticoagulation status: interaction between both genotypes affects overanticoagulation. Schalekamp T, Brasse BP, Roijers JF, Chahid Y, van Geest-Daalderop JH, de Vries-Goldschmeding H, van Wijk EM, Egberts AC, de Boer A. Clin Pharmacol Ther. 2006

[3] Association of Vitamin K epoxide reductase complex 1 (VKORC1) variants with warfarin dose in a Hong Kong Chinese patient population. Veenstra et al., Pharmacogenetics & Genomics (2005)

[4] Validation of a new fluorogenic real-time PCR assay for detection of CYP2C9 allelic variants and CYP2C9 allelic distribution in a German population, Burian M, Grösch S, Tegeder I, Geisslinger G, J Clin Pharmacol 54 (2002) 518–521

10. Material Safety Data

According to OSHA 29CFR1910.1200, Australia [NOHSC:1005, 1008 (1999)] and the European Union Directives 67/548/EC and 1999/45/EC any products which not contain more than 1% of a component classified as hazardous or classified as carcinogenic do not require a MSDS.

Product is not hazardous, not toxic, not IATA-restricted. Product is not from human, animal or plant origin. Product contains synthetic oligonucleotide primers and probes.

10. Version History

Notes in red mark events require to change procedures

V070111	Original version for LightCycler® 1.x and 2.0 Instruments
V100819	Inclusion of LightCycler® 480 Instruments
V110413	Change to 32rxn/vial and implementation of figures for LC 480
V130822	z 480 included, expiry extended, Roche Color Compensation discontinued
V140414	Editorial changes

Roche SAP order n° 05945712001

Notice to Purchaser

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