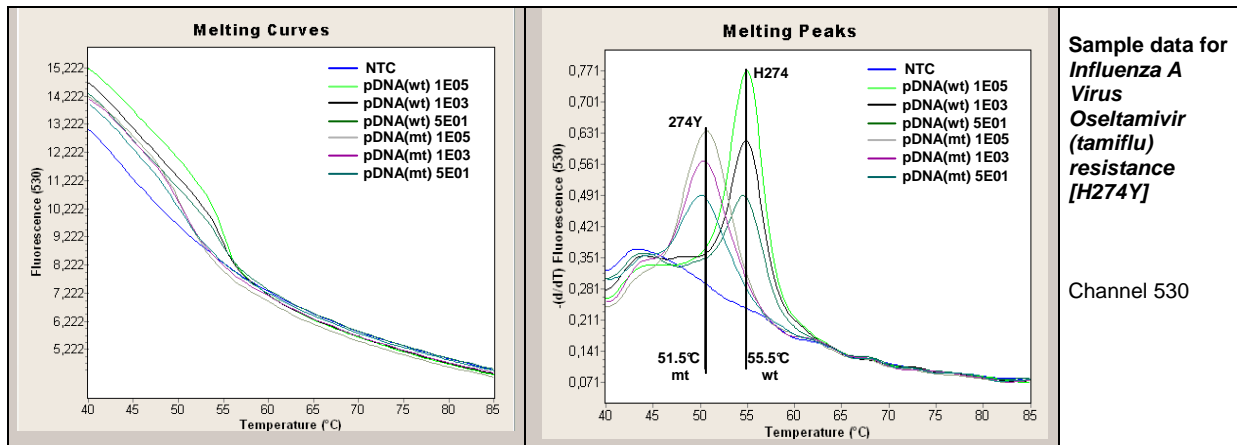


## 8. Sample data - typical results



**Fig.1. Sample data for the *Influenza A Virus Oseltamivir (tamiflu) resistance [H274Y]* detection system**

**Left panel:** Data from channel 530. Melting curves for the target.

**Right panel:** Data from channel 530. Melting peaks for the target.

**Note:** The values of the respective melting temperatures ( $T_M$ ) may vary  $\pm 2.5^\circ\text{C}$  between different experiments. The  $\Delta T$  between the melting peaks for heterozygous genotypes may vary  $\pm 1.5^\circ\text{C}$ .

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These reagents were developed and manufactured by TIB MOLBIOL GmbH, Berlin, Germany. LightCycler<sup>®</sup> hybridization probes produced under license from Roche Diagnostics.



## **LightMix<sup>®</sup> Kit Influenza A Virus HxN1 Tamiflu resistance [H274Y]** **Cat.-No. 40-0582-32**

Reagents for the detection of one C/T variations causing *Influenza A Virus Tamiflu (Oseltamivir) resistance* using the LightCycler<sup>®</sup> 1.x / 2.0 Instruments.

Lyophilized mix of primers and probes (3 tubes with 32 rxns each) 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 NOT freeze!**

### **Additional reagents required (Roche Diagnostics):**

High Pure Viral Nucleic Acid Kit	Cat.-No. 11 858 874 001
Transcriptor First Strand cDNA Synthesis Kit	Cat.-No. 04 379 012 001
LightCycler <sup>®</sup> FastStart DNA Master HybProbe	Cat.-No. 03 003 248 001

## **1. Introduction**

Tamiflu (Oseltamivir) is a neuraminidase inhibitor that provides valuable defenses against seasonal and pandemic Influenza A virus. The resistance to the antiviral drug is related to different mutations in the neuraminidase (NA) gene; N1 and N2 type genes show different mutation patterns. The most frequent cause for the resistance of N1 viruses is a C/T base exchange resulting in a substitution of amino acid Histidin 274 by Tyrosin [H274Y]. Less frequent are variations of Aspartic Acid 119.

Most isolates of seasonal Influenza A H1N1 (Europe, 2009) are Tamiflu resistant while the mutation has been found very sporadically in patients with pandemic H5N1 Asia and more recently in pandemic H1N1/2009 virus infections.

The 2005 version of this kit made use of LightCycler<sup>®</sup> hybridization probes, detecting the mutation in H5N1 Asia virus by running a melting curve analysis. The NA genes from seasonal Influenza A/H1N1 and H1N1/2009 are quite different, affecting also the binding site of the anchor probe and thus preventing to use the previous version of this kit for H1N1 viruses. In order to offer a general test it has been changed to a SimpleProbe<sup>®</sup> oligomer, omitting the use of a second anchor probe and targeting the H274Y region only.

This LightMix<sup>®</sup>-System is tested with the Roche Diagnostics "LightCycler<sup>®</sup> FastStart DNA Master Hybridization Probes" ready-to-use reaction mix in the LightCycler<sup>®</sup> 2.0 Instrument.

## **2. Description**

A 277 bp fragment of the *Influenza A Virus neuraminidase* N1 type gene is amplified with H1N1/2009 specific primers which also bind to H5N1 Asia and H1N1 sequences. The mutation is detected with an H274 specific SimpleProbe<sup>®</sup> oligomer (detected in channel 530). Since the sequence is not fully conserved the probe contains bases corresponding to both H1N1 seasonal and pandemic virus, resulting in one mismatch for any virus and a consistent melting temperature of 55°C for any Oseltamivir sensitive virus. A decreased melting temperature is an indication for a base variation in the region between amino acids Pro 271 and Cys 280 and is a strong indication for the presence of the resistant 274Y variant. We strongly recommend to sequence such samples to verify the presence of the supposed mutation.

Virus displaying a melting temperature of 55°C is probably Oseltamivir-sensitive and patients should be treated with the drug, if the clinical situation requires it.

The supplied control DNA allows for the accurate comparison with the unknown samples.

For use in LightCycler<sup>®</sup> 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<sup>®</sup> 1.x Instruments to software version 4.1.

### 3. Set contents

- 3 Vials with blue cap containing premixed and lyophilized primers, hybridization probes and competitor for 32 reactions each
- 1 Vial with colorless cap containing control DNA (wt) [H274], 10<sup>5</sup> target equivalents per reaction
- 1 Vial with colorless cap containing control DNA (mt) resistance [274Y], 10<sup>5</sup> target equivalents per reaction

### 4. Programming

The protocol consists of four program steps

- 1: Denaturation: samples 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
<b>Parameter</b>								
Analysis Mode	None	Quantification mode			Melting Curves mode			None
Cycles	1	45			1			1
Target [°C]	95	95	62	72	95	40	85	40
Hold [hh:mm:ss]	00:10:00	00:00:05	00:00:05	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
Sec Target [°C]	-	-	55	-	-	-	-	-
Step Size [°C]	-	-	0.5	-	-	-	-	-
Step Delay (Cycles)	-	-	1	-	-	-	-	-
Acquisition Mode	None	None	Single	None	None	None	Cont	None

### 5. Data analysis

Perform data analysis, as described in the LightCycler<sup>®</sup> operator's manual. We recommend using the Polynomial Calculation Method for analyzing the melting peaks. The melting temperature is determined with the manual T<sub>m</sub> setting.

View *Influenza virus A Oseltamivir (tamiflu) resistance [H274Y]* data in channel 530, Melting Curves mode. The negative control (NTC) should show no signal.

### 6. Product characteristics

PCR results are obtained within 1 hour.

#### Sensitivity

These reagents detect 50 copies of Influenza A virus *Oseltamivir (tamiflu) resistance [H274Y]* using the Roche 'LightCycler<sup>®</sup> FastStart DNA Master HybProbe' with the LightCycler<sup>®</sup> 1x / 2.0 Instruments (in an exemplary system using cloned targets as reference)

#### Storage and Stability

- Lyophilized reagents are stable for at least 3 months after shipment if stored protected from light at room temperature (18-25°C).
- **Do not freeze** lyophilized reagents.
- Dissolved reagents are stable for at least 5 days if stored protected from light and refrigerated (4°C).

## 7. Experimental protocol

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

**Sample material:** Use aqueous nucleic acid preparations (e.g. High Pure RNA Isolation Kit combined with Transcriptor First Strand cDNA Synthesis Kit).

**Negative control:** Always run at least one negative control - replace the template DNA with water.

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

### 7.1 Preparation of parameter-specific reagents (32 reactions):

One reagent vial with a **blue** cap contains all primers and probes to run 32 LightCycler® reactions for *Influenza virus A Oseltamivir (tamiflu) resistance [H274Y]*.

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

► Use 2 µl reagent for a 20 µl PCR reaction.

| This solution is stable for at least three days when stored refrigerated at 4°C. Avoid prolonged exposure to light.

### 7.2 Preparation of the control DNA

Add 40 µl PCR-grade water to each vial ( $8 \times 10^5$  target molecules) with colorless cap (wt) or with colorless cap (mt). Mix the target DNA by pipetting the solution up and down 10 times. (final concentration:  $10^5$  target molecules in 5 µl).

► 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 freeze at -20°C. Avoid repeated freezing thawing cycles.

| Please note that opening these vials may cause contaminations of the work-space (aerosol).

### 7.3 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
8.6 µl	water, PCR-grade (colorless cap, provided with the Roche Master kit)
2.4 µl	Mg <sup>2+</sup> solution 25 mM (blue cap, provided with the Roche FastStart kit)
2.0 µl	<b>reagent</b> 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

Mix gently, spin down and transfer 15 µl each of the reaction mix to a LightCycler® capillary. Add 5 µl of sample or control (see 7.2) to each capillary to give a final reaction volume of **20 µl**.

Start run.