8. Sample data - typical results

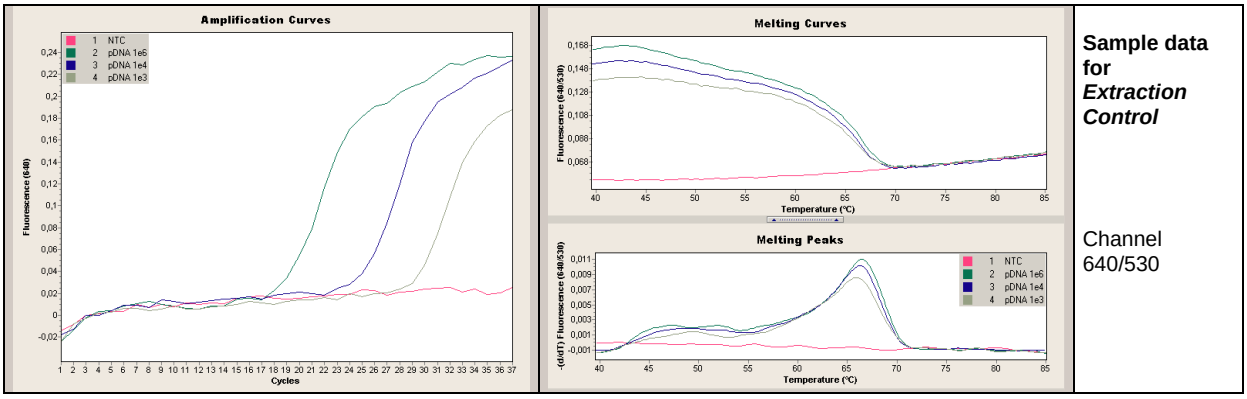

Fig.1. Sample data for the *Extraction Control* system.

Upper panels: Data from channel 640/530. Left panel amplification curves (Second Derivative Maximum) with calibration row. Right panel melting curves for the target.

Interpretation of the results

The amplification of the Reference Solution (stock solution 1:10000) allows to detect any alterations eventually occurred during the extraction procedures.
The crossing point (CP) of the Reference Solution works as the border line between samples successfully extracted and samples with not reliable extraction.

In particular:

Samples showing a CP higher than the CP of the Reference Solution can not be considered reliable samples.

Samples showing a CP lower or equal the CP of the Reference Solution are good samples.

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

LightMix® for the *Extraction Control*

Cat.-No. 40-0259-16

Reagents for the detection of *Extraction Control* DNA using the LightCycler® Instrument 1.x / 2.0.

Lyophilized mix of primers and probes (6 tubes with 16 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):

LightCycler® FastStart DNA Master^{PLUS} HybProbe	Cat.-No. 03 515 575 001
or LightCycler® FastStart DNA Master HybProbe	Cat.-No. 03 003 248 001
High Pure PCR Template Preparation Kit	Cat.-No. 11 796 828 001

1. Introduction

PCR analysis of biological samples may result sometime difficult due to problems related to the extraction process. In order to provide a validation of the nucleic acid preparation step, the Extraction Control has been developed and specifically adapted for PCR in glass capillaries using the LightCycler instrument.

The LightMix® for the detection of DNA from the *Extraction Control* provides a fast, easy and accurate system to identify successful DNA extraction of samples.

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

2. Description

This LightMix® detects a part of the *Extraction Control* DNA indicating the presence of *Extraction Control* DNA in a nucleic acid extract.

A 278 bp fragment of the *Extraction Control* DNA is amplified with specific primers and detected with probes labeled with LightCycler® Red 640 (detected in channel 640/530) The PCR product is identified by running a melting curve with a specific melting point (Tm) of 66,5°C in channel 640.

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.

3. Set contents

6 Vials containing premixed and lyophilized primers and hybridization probes for 16 reactions each
1 Vial Control DNA (10^{10} genomic copies, lyophilized)

4. Programming

The protocol consists of four program steps

• Program 1: Denaturation of sample and activation of the enzyme
• Program 2: PCR-amplification of the target DNA
• Program 3: Melting curve for identification of the *Extraction Control* DNA derived PCR product
• Program 4: Cooling the instrument

When using the Roche FastStart reagents run an initial heating for 10 min at 95°C.

Program:	Denaturation	Cycling			Melting			Cooling
Parameter								
Analysis Mode	None	Quantification			Melting Curves			None
Cycles	1	37			1			1
Segment	1	1	2	3	1	2	3	1
Target [°C]	95	95	59	72	95	40	85	40
Hold [hh:mm:ss]	00:10:00	00:00:05	00:00:09	00:00:10	00:00:20	00:00:30	00:00:01	00:00:30
Ramp Rate [°C/s]	20	20	10	20	20	20	0.5	20
Acquisition Mode	None	None	Single	None	None	None	Step	None

5. Data analysis

Perform data analysis, as described in the LightCycler® operator's manual. We recommend using the Second Derivative Maximum method (Automated (F'' max)). The cycle number of the Crossing Point (CP) of each sample is calculated automatically. The Fit Points method is more-error prone due to user's influences.

View *Extraction Control* data in channel 640, Quantification mode. The negative control (NTC) should show no signal. For the identification of the PCR product view *Extraction Control* data in channel 640/530, Melting Curves mode.

Typical results (Software Version 4.0)
The provided standard row of cloned and purified DNA with concentrations in the range from 10^6 copies/rxn to 10^1 copies/rxn should have CPs between cycles 18 and 35 (CPs calculated with Second Derivative Maximum method).

6. Product characteristics

PCR results are obtained within 1 hour.

Sensitivity
These reagents detect 10 copies of *Extraction Control* DNA (cloned plasmid).

Measuring range
The linear measuring range of the assay is 10^2 to 10^6 copies of *Extraction Control* DNA.

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® Instrument 1.x / 2.0.
Start programming before preparing the solutions. See the LightCycler® operator's manual for details.

Sample material: For PCR use aqueous nucleic acid preparations (e.g. High Pure Viral Nucleic Acid 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.

(A) Preparation of parameter-specific reagents (16 reactions):

One reagent vial (labeled transparent vial with a blue pellet) contains all primers and probes to run 16 LightCycler® reactions.

Add 66 µl PCR-grade water to the non-colored reagent vial containing the blue pellet, mix the solution (vortex) and spin down.

►Use 4 µl **reagent** for a 20 µl PCR reaction.

This solution is stable for three days or longer if stored refrigerated at 4°C. Avoid prolonged exposu re to light.

(B) Preparation of the Positive Control

(B-1) Preparation of the stock solution:
Add 100 µl PCR-grade water to the vial containing the Control DNA. Mix the target DNA by pipetting the solution up and down 10 times.

(B-2) Preparation of the **Control Solution** (1:1000 dilution of the stock solution):
1. Use 10 µl of the stock solution and add 90 µl PCR-grade water to get stock solution 1:10.
2. Use 10 µl of stock solution 1:10 and add 90 µl PCR-grade water to get stock solution 1:100.
3. Use 10 µl of stock solution 1:100 and add 90 µl PCR-grade water to get the **Control Solution**.

(B-3) Preparation of the **Reference Solution** (1:10000 dilution of the stock solution):
1. Use 10 µl of the Control Solution and add 90 µl PCR-grade water to get the **Reference Solution**.

►Add 20 µl of the Control Solution to 200 µl of biological sample, perform extraction as described in the appropriate protocol.

Notes for extraction:

If the biological sample is viscous (e.g. sputum), it has to be treated with acetylcysteine to render it more fluid before extraction.

If swab contains agarose (known PCR inhibitor), disperse it in 250 µl of saline, let the tube rest for 1-5 min, then transfer 200 µl in a new tube for extraction by carfully pipetting without touching the bottom of the tube.

(C) Preparation of the LightCycler® reaction mix

In a reaction tube cooled below 4°C, prepare the re action 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^{PLUS} kit		**For use with the Roche FastStart kit**
Single reaction	**Component**	**Single reaction**
7.0 µl	water, PCR-grade (colorless cap, provided with the Roche FastStart or FastStart^{PLUS} kit)	7.2 µl
--	Mg^{2+} solution 25 mM (blue cap, provided with the Roche FastStart kit)	1.8 µl
4.0 µl	**reagent** mix (parameter specific reagents containing primers and probes, see **A**)	4.0 µl
4.0 µl	FastStart mix (vial 1 (red cap), combined from vials 1a and 1b, see Roche manual)	2.0 µl
15.0 µl	**Volume of reaction mix**	**15.0 µl**

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