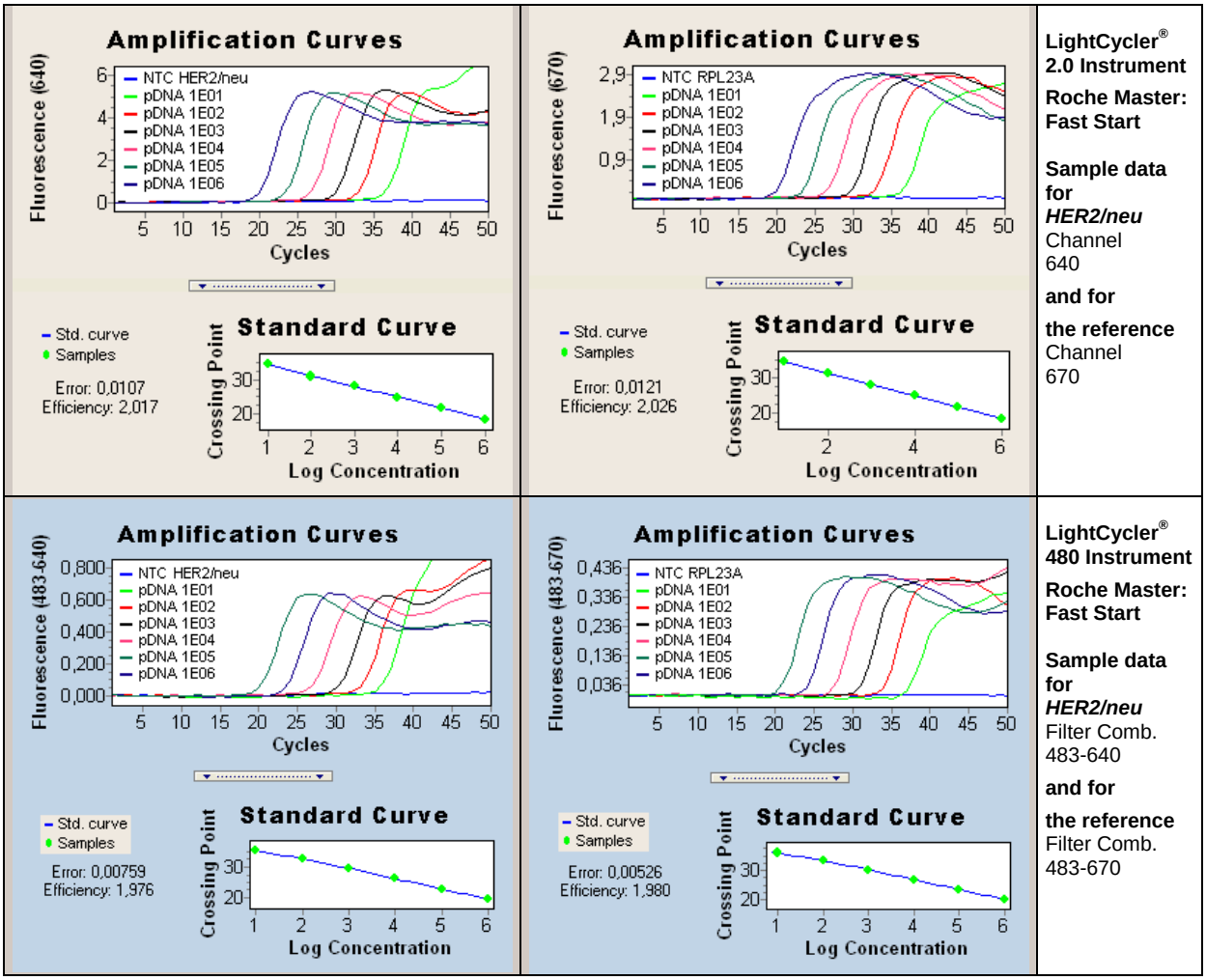
8. Sample data - typical results



LightCycler® 2.0 Instrument
Roche Master: Fast Start
Sample data for *HER2/neu* Channel 640
and for
the reference Channel 670

LightCycler® 480 Instrument
Roche Master: Fast Start
Sample data for *HER2/neu* Filter Comb. 483-640
and for
the reference Filter Comb. 483-670

Fig.1. Sample data for the *HER2/neu* detection system.

Upper panels: Data from LightCycler® 2.0 Instrument. Left panel channel 640 quantification mode (Second Derivative Maximum) with calibration curve for *HER2/neu*. Right panel channel 670 quantification mode (Second Derivative Maximum) with calibration curve for the reference.
Lower panels: Data from LightCycler® 480 Instrument. Left panel channel 640 quantification mode (Second Derivative Maximum) with calibration curve for *HER2/neu*. Right panel channel 670 quantification mode (Second Derivative Maximum) with calibration curve for the reference.

Copies Ratio *HER2/neu*:reference	<2.0	>2.0
Assumed result for overamplification of *HER2/neu*	negative	**positive**

Tab. 3. Typical analysis results
The copies ratio *HER2/neu*:reference of samples is calculated by means of the created standard rows of *HER2/neu* and the reference.

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

LightMix® Kit *HER2/neu*

Cat.-No. 40-0333-16

Kit with reagents for the quantitative detection of *HER2/neu* DNA or cDNA using the LightCycler® 2.0 / 480 Instruments.

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
or LightCycler® 480 Probes Master (LC 480 Instrument only)	Cat.-No. 04 707 494 001
LightCycler® Multicolor Demo Set	Cat.-No. 03 624 854 001
HighPure PCR Template Preparation Kit	Cat.-No. 11 796 828 001
High Pure RNA Isolation Kit (for cDNA detection only)	Cat.-No. 11 828 665 001
Transcriptor First Strand cDNA Synthesis Kit (for cDNA detection only)	Cat.-No. 04 379 012 001

1. Introduction

The proto-oncogene *HER2/neu* (c-erbB2) on chromosome 17q21 codes for an EGF-like growth factor receptor with tyrosine kinase activity. Gene amplification and overexpression is frequently observed in breast cancer and other human tumors. This can be determined by relative PCR quantification, using a reference gene (DNA) or comparing the expression with that of a housekeeping gene (cDNA).

The LightMix® Kit *HER2/neu* provides a fast, easy and accurate system to identify and quantify this target in a nucleic acid extract. The reference PCR fragment is located within one exon of the RPL23 gene, allowing to use the reagent with genomic DNA samples or with cDNA samples. The reference gene is located on the same chromosome as the *HER2/neu* gene.

This LightMix® Kit is tested with the Roche Diagnostics 'LightCycler® FastStart DNA Master Hybridization Probe' and with Roche Diagnostics 'LightCycler® FastStart^{PLUS} DNA Master Hybridization Probe' in the LightCycler® 2.0 /480 Instruments (96 well and 384 well formats) and with the Roche Diagnostics 'LightCycler® 480 Probes Master' in the LightCycler® 480 Instrument (96 well and 384 well formats).
An 1-step RT PCR procedure was not tested.

2. Description

A 101 bp fragment of the *HER2/neu* DNA is amplified with specific primers. *HER2/neu* DNA is analyzed with hybridization probes labeled with LightCycler® Red 640 (detected in channel 640).
An additional PCR product of 119 bp is formed from the reference system. The hybridization probes are labeled with LightCycler® Red 670. Detection is recorded in channel 670. The reactions do not interfere with each other. The reference is supplied separately to allow running the assay in the presence or absence of the reference.

The use of a color compensation file generated with the Roche Diagnostics 'LightCycler® Multicolor Compensation Set' is a prerequisite to run the duplex reaction.

The supplied standard row allows to determine the linear range of both reactions and to estimate the quantity of the target sequence in unknown samples.

For use in LightCycler® 480 Instruments use filter combination 483-640 instead of channel 640 and filter combination 483-670 instead of channel 670 for detection.

[1]Quantification of HER2/neu Gene Amplification by Competitive PCR Using Fluorescent Melting Curve Analysis. Lyon E., Millson A., Lowery M.C., Woods R. and Wittwer C.T. Clin Chem 47 (2001) 844-851.

3. Set contents

6 Vials with red caps containing premixed lyophilized primers and probes for 16 reactions each
6 Vials with white caps containing the reference system
1 Row with 6 lyophilized standards from 10^1 to 10^6 target equivalents per reaction of *HER2/neu* DNA and of *reference* DNA
1 Sealing foil for the standard row

4. 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 of the instrument

Program:		**Denaturation**	**Cycling**			**Melting**			**Cooling**
	Parameter								
	Analysis Mode	None	Quantification mode			Melting Curves mode			None
	Cycles	1	50			1			1
	Segment	1	1	2	3	1	2	3	1
	Target [℃]	95	95	60	72	95	40	85	40
	Hold [hh:mm:ss]	00:10:00	00:00:10	00:00:10	00:00:10	00:00:20	00:00:20	00:00:00	00:00:30
LC 2.0:	*Ramp Rate [℃/s]*	*20*	*20*	*20*	*20*	*20*	*20*	*0.2*	*20*
LC 480:	*Ramp Rate [℃/s]* ***96***	*4.4*	*4.4*	*2.2*	*4.4*	*4.4*	*1.5*	-	*1.5*
LC 480:	*Ramp Rate [℃/s]* ***384***	*4.6*	*4.6*	*2.4*	*4.6*	*4.6*	*2.0*	-	*2.0*
	Acquisition Mode	None	None	Single	None	None	None	Continu.	None
LC 480:	*Acquisitions [per ℃]*							*1*	

(Melting not relevant for detection)

5. Data analysis

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 Roche Diagnostics 'LightCycler® Multicolor Compensation Set'.

Perform data analysis, as described in the LightCycler® Instrument 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 influence.

View *HER2/neu* data in channel 640 and data of the reference in channel 670, Quantification mode. The negative control (NTC) should show no signal.

The provided standard row of cloned and purified DNA with concentrations in the range from 10^6 copies/rxn to 10^1 copies/rxn of *Her2/neu* and of the reference 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 *HER2/neu* DNA and of reference DNA (in an exemplary system, using cloned targets as reference).

Measuring range
The linear measuring range of the assay is 10^2 to 10^6 copies of *HER2/neu* DNA and of reference 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℃).
- **Do not freeze** lyophilized reagents.
- Dissolved reagents are stable for at least 5 days if stored refrigerated (4℃).

7. Experimental protocol

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

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

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.
For quantification always use a fresh solution prepared from the provided standard row (single use only).

7.1. Preparation of parameter-specific reagents (16 reactions):

One reagent vial with a **red** cap contains all primers and probes to run 16 LightCycler® reactions for *HER2/neu* DNA.
One reagent vial with a **white** cap contains all primers and probes to run 16 LightCycler® reactions for reference DNA.

Add 66 µl (**33µl!** for use with LightCycler® 480 Probes Master) PCR-grade water to each reagent vial, mix the solution (vortex) and spin down.

►Use 4 µl (**2µl!** for use with LightCycler® 480 Probes Master) **reagent** for a 20 µl PCR reaction.

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

7.2. Preparation of the standard row

The target DNA is provided in 6 different quantities to yield from 10^1 to 10^6 target molecules in 5 µl once resuspended. Start with the lowest concentrated standard (first tube from the extended lip). Use the pipette tip to punch a hole through the sealing foil. Add 40 µl PCR-grade water to each vial of the row. Mix the target DNA by pipetting the solution up and down 10 times.

►Use 5 µl standard for a 20 µl PCR reaction.

This standard solution is not long-term stable and will lose sensitivity under prolonged storage. Use only fresh prepared solutions as quantification references. Older solutions can be used as a qualitative standard (positive control).
After adding the target DNA to the LightCycler® reaction mix use the provided sealing foil to close the vials in order to avoid changes in the concentration due to evaporation.
Please note that opening of 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 480 Probes Master		**For use with the Roche FastStart Master**
Single reaction	**Component**	**Single reaction**
1.0 µl	water, PCR-grade (colorless cap, provided with the Roche Master kit)	2.6 µl
--	Mg^{2+} solution 25 mM (blue cap, provided with the Roche FastStart kit)	2.4 µl
2.0** µl	**reagent** mix (parameter specific reagents containing primers and probes, see **7.1.**)	4.0 µl
2.0** µl	**reference** mix (calibration reagents containing primers and probes see **7.1.**)	4.0 µl
10.0 µl	Roche Master (red cap, FastStart kit: combined from vials 1a and 1b, see Roche manual)	2.0 µl
15.0 µl	**Volume of reaction mix**	**15.0 µl**

**Note: For use with LightCycler® 480 Probes Master on the LightCycler® 480 Instrument please resolve the reagents (see 7.1.) in 33 µl and use 2 µl in Preparation of the LightCycler® reaction mix (see 7.3.) for a final volume of 15 µl!

Mix gently, spin down and transfer 15 µl each of the reaction mix to a LightCycler® capillary (LightCycler® 2.0 Instrument) or to a multiwell plate (LightCycler® 480 Instrument).

Add 5 µl of sample or standard to each capillary or multiwell plate for a final reaction volume of **20 µl.**

Start run.