

LightCycler Instrument. A 112-bp fragment of HER2/neu gene and a 133-bp fragment of the reference gene from human genomic DNA that serves both as control for DNA integrity and as a reference for relative quantification are amplified by polymerase chain reaction (PCR) with specific primers. The amplicons are simultaneously detected in a single capillary by using two specific pairs of Hybridization Probes.

This simultaneous quantification is achieved because the HER2/neu-specific oligonucleotide is labeled at the 5'-end with LightCycler-Red 705, and the reference gene specific oligonucleotide is labeled at the 5'-end with LightCycler-Red 640. The use of a previously stored color compensation file is a prerequisite in this dual color experiment. To determine the HER2/neu DNA amplification level, the new LightCycler Relative Quantification Software is applied.

No post-PCR processing is needed and the risk of contamination is minimized, as quantification and detection are performed simultaneously in the same sealed capillary without any further handling steps. DNA isolated from cell cultures, scientific biopsy material, and other biological

samples (e.g., frozen or formalin-fixed paraffin-embedded tissue) can be used as sample material. For assay characteristics and features and benefits see Table 1.

For an extensive performance description of the LightCycler-HER2/neu DNA Quantification Kit see article "Realtime Quantification of HER2/neu Gene Amplification by LightCycler Polymerase Chain Reaction (PCR) – a New Research Tool" on this page of this Biochemica issue.

<http://biochem.roche.com/lightcycler>



Product	Pack Size	Cat. No.
LightCycler-HER2/neu DNA Quantification Kit	1 kit (32 reactions)	3 113 922
LightCycler-Color Compensation Set	1 set (5 reactions)	2 158 850
LightCycler Relative Quantification Software	1 software package	3 158 527

Real-time Quantification of HER2/neu Gene Amplification by LightCycler Polymerase Chain Reaction (PCR) – a New Research Tool

Kurt Beyser^{1*}, Astrid Reiser², Christof Gross¹, Carmen Möller¹, Karim Tabiti² and Josef Rüschoff¹
¹Institute of Pathology, Klinikum Kassel/Germany; ²Roche Diagnostics GmbH, Penzberg/Germany
 *corresponding author: jruesch-sk@t-online.de

The tyrosine growth factor receptor HER2/neu is frequently overexpressed in breast cancer and other solid tumors, mostly due to gene amplification. A novel PCR method, the LightCycler-HER2/neu DNA Quantification Kit from Roche Molecular Biochemicals, has been evaluated to quantify HER2/neu gene copies in several breast cancer research samples. Unlike other PCR approaches [1-3] this assay uses a reference gene, which is co-localized to HER2/neu on chromosome 17. Consequently, it also corrects for the possible occurrence of polysomy of chromosome 17.

Introduction

The proto-oncogene HER2/neu is located on chromosome 17 (q21) and encodes a 185-kDa transmembrane glycoprotein with tyrosine kinase activity that is a member of the epidermal growth factor receptor family

[4-6]. Gene amplification and overexpression of HER2/neu is frequently observed in various human cancers (e.g., breast, stomach, ovarian, bladder, lung cancer). In breast cancer, for example, 10-34% of all affected individuals show this gene amplification/overexpression [7].

The techniques used to evaluate the HER2/neu gene status have included gene-based assays such as Southern and slot blotting, in-situ hybridization (fluorescent and nonfluorescent) and PCR methods [7]. Each of the techniques mentioned has its advantages and disadvantages. In order to perform a fast pre-screening with high throughput that is able to deal with formalin-fixed archived material, PCR might be the best choice. The PCR approaches published so far have used reference genes which are not localized on chromosome 17. Therefore it is not possible to distinguish whether a small region of the chromosome or the whole chromosome is amplified. But chromosome aneuploidy, including loss and gain of chromosome 17, is seen frequently in breast cancer [8]. In addition, most PCR approaches were only semi-quantitative.

Taking this into consideration we have tested this new approach taking advantage of the LightCycler Instrument and its features combining rapid amplification and real-time fluorescence detection with two different fluorophores (LightCycler-Red 640 and LightCycler-Red 705) in a single capillary.

Materials and Methods

Sample material

Research samples of 57 invasive breast carcinomas were included in this study. The tissue was routinely fixed (12–18 hours) in neutral buffered formalin and embedded in paraffin.

DNA isolation

Sections (5 µm) were mounted on slides. The tissue was deparaffinized in Xylool and rehydrated in ethanol (100% down to 70%). The invasive part of the tumor area was scratched from the slide and DNA was extracted using the High Pure PCR Template Preparation Kit from Roche Molecular Biochemicals according to the pack insert with the following modifications: Proteinase K digestion was performed overnight at 55 °C and the final elution volume was 100 µl. DNA was stored at –20 °C until use.

Quantification using the LightCycler technology

PCR was performed with the LightCycler-HER2/neu DNA Quantification Kit. A 112-bp fragment of HER2/neu gene and a 133-bp fragment of the reference gene were amplified by PCR with specific primers. Both genes are localized on chromosome 17. As two sets of Hybridization Probes are used, one labeled with LightCycler-Fluorescein and LightCycler-Red 640, the other with LightCycler-Fluorescein and LightCycler-Red 705, both amplicons can be quantified simultaneously in one capillary.

A maximum of 30 samples, one positive reaction (LightCycler-HER2/neu Calibrator DNA provided with the kit) and one negative reaction can be analyzed during one LightCycler PCR run. PCR was set up according to the supplier's instructions. In brief, for each reaction 2 µl of the LightCycler-HER2/neu Detection Mix, 2 µl of the LightCycler-HER2/neu Reference Gene Detection Mix, 2 µl of the LightCycler-HER2/neu Enzyme Master Mix and 12 µl of the PCR grade water supplied with the kit were combined, mixed and aliquoted in the capillaries. 2 µl of DNA (LightCycler-HER2/neu Calibrator DNA, which is provided with the kit, or PCR grade water as negative control or the human DNA extracted from the tissue, respectively) were added. The capillaries were sealed, placed in the rotor, centrifuged and placed in the LightCycler Instrument.

PCR conditions were as follows: After an initial 6 minutes pre-incubation step ("activation" of the FastStart Taq DNA polymerase) at 95 °C, 45 amplification cycles were performed each consisting of 95 °C for 10 seconds, 58 °C for 10 seconds and 72 °C for 10 seconds. The fluorescence signals were measured after each primer annealing step (58 °C).

Calculation of the amounts of HER2/neu DNA

The calculation of the relative amounts of HER2/neu DNA compared to the reference gene DNA was performed by the LightCycler Relative Quantification Software from Roche Molecular Biochemicals.

The final results were expressed as a ratio of HER2/neu : reference gene copies in the sample, normalized with the ratio of HER2/neu : reference gene copies in the Calibrator DNA. The ratio HER2/neu : reference gene copies in the Calibrator DNA was set to one.

A ratio of <2.0 is assumed to be negative for HER2/neu overamplification, a ratio of ≥2.0 is assumed to be positive for HER2/neu overamplification.

Results

Typical amplification curves are shown in Figure 1 and Figure 2. For clarity, the amplification curves for HER2/neu and for the reference gene are shown in different screens, as they are measured in different channels (F3 and F2).

LightCycler PCR of HER2/neu negative samples according to immunohistochemistry (IHC)

DNA was extracted from 50 IHC negative samples (DAKO-score of 0 or 1+). The DNA was amplified on two

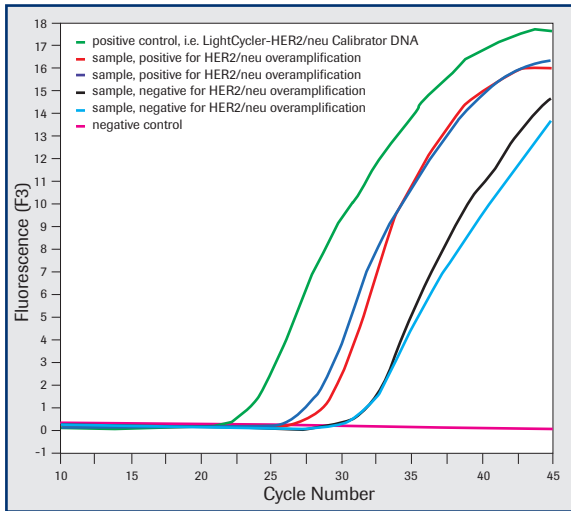


Figure 1:
Fluorescence data
for HER2/neu in
channel 3 (F3)

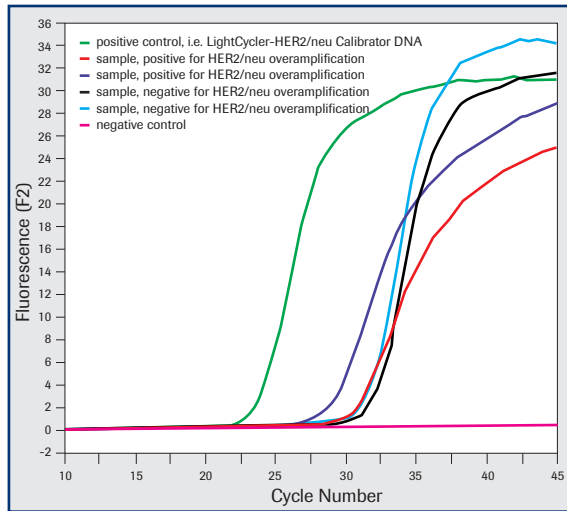


Figure 2:
Fluorescence data
for the reference
gene in channel 2
(F2)

different days. None of the cases had a ratio of HER2/neu to reference gene with the LightCycler-HER2/neu DNA Quantification Kit higher than 1.4, most of them were around 1.0. Thus, it was confirmed that all 50 samples were negative for HER2/neu overamplification (Table 1).

LightCycler PCR of HER2/neu amplified and non-amplified samples according to fluorescence in-situ hybridization (FISH)

DNA was extracted three times and PCR was run in triplicates from seven amplified and five non-amplified cases (according to FISH). All FISH-results were reconfirmed with the LightCycler-HER2/neu DNA Quantification Kit (Table 2).

As indicated in the tables, each DNA extraction from a particular sample results in a distinct ratio of HER2/neu to reference gene, leading to a range of ratios for each

sample. This range of ratios is due to the varying populations of tumor cells in the tissue section the DNA was extracted from. Nevertheless, even the single result from each DNA extraction is always unambiguous and consistent with the pooled finding showing the power of the LightCycler-HER2/neu DNA Quantification Kit.

Discussion

The LightCycler-HER2/neu DNA Quantification Kit is a new research tool for measuring HER2/neu gene amplifi-

Table 1: Ratio HER2/neu to reference gene for 50 IHC-negative (0 or 1+, DAKO score) samples

ratio HER2/neu : reference gene achieved with the LightCycler-HER2/neu DNA Quantification Kit	0.5-0.8	0.8-1.0	1.0-1.2	1.2-1.4
total number of samples	17	20	7	6

Table 2: Ratio HER2/neu to reference gene of seven amplified and five non-amplified cases according to FISH. Ratios were determined in triplicate for each DNA isolated. For each sample at least three independent DNA extractions were performed. The highest and the lowest value of these nine measurements are given

Sample	LightCycler-HER2/neu DNA Quantification Kit		Fluorescence in-situ hybridization (FISH)
	ratio HER2/neu: reference gene	Interpretation of the result (HER2/neu overamplification)	
1	10.6 – 25.8	Positive	4.4-5.1
2	3.3 – 7.7	Positive	4.8-5.5
3	2.8 – 4.3	Positive	2.3
4	2.5 – 5.6	Positive	3.2
5	3.0 – 4.3	Positive	4.8
6	2.5 – 5.3	Positive	5.2-5.9
7	2.7 – 3.4	Positive	3.5
8	0.7 – 1.2	Negative	0.7
9	0.4 – 0.8	Negative	0.9
10	0.5 – 1.1	Negative	0.9
11	0.5 – 0.9	Negative	0.8
12	0.6 – 1.3	Negative	0.9

cation in human tumor tissue. Samples negative and positive for HER2/neu overamplification can easily be discriminated. The results achieved are unambiguous and due to the kit set up comparable between runs and individual samples. The kit offers a fast detection method

for HER2/neu DNA overamplification as 30 samples can be tested per LightCycler PCR run in less than one hour. Further intensive research is required to determine the possible clinical usefulness of the real-time quantification of the HER2/neu gene amplification in tumor tissues.



Product	Pack Size	Cat. No.
LightCycler Instrument	1 instrument	2 011 468
LightCycler-HER2/neu DNA Quantification Kit	1 kit (32 reactions)	3 113 922
LightCycler-Color Compensation Set	1 set (5 reactions)	2 158 850
LightCycler Relative Quantification Software	1 software package	3 158 527
High Pure PCR Template Preparation Kit	1 kit (100 purifications)	1 796 828

References

1. Millson, A. et al. (2000), *J. Mol. Diagnost.* 2(4): 233.
2. Gelmini, S. et al. (1997), *Clin. Chem.* 43(5): 752-758.
3. Sestini, R. et al. (1995), *Clin. Chem.* 41(6): 826-832.
4. Coussens, L. et al. (1985), *Science* 230: 1132-1139.
5. Downward, J. et al. (1984), *Nature* 307: 521-527.
6. Bargmann, C. I. et al. (1986), *Nature* 319: 226-230.
7. Ross, J. S. and Fletcher, J. A. (1998), *The Oncologist* 3: 237-252.
8. Jennings, B. A. et al. (1997), *Mol Pathol.* 50: 254-256.

<http://biochem.roche.com/lightcycler>



LightCycler-CK20 Quantification Kit

The most sensitive way to quantify RNA encoding for human cytokeratin 20

The new LightCycler-CK20 Quantification Kit from Roche Molecular Biochemicals expands the parameter-specific quantification reagents in the field of oncology research. This dedicated kit enables the quantitative detection of one CK20 positive cell in 1×10^6 peripheral blood cells.

Background Information

It is believed that many tumor recurrences, despite metastasis free intervals, occur due to the presence of micrometastases that are currently undetectable by conventional methods. Due to their increased sensitivity, PCR-based methods are being investigated as a possible tool for micrometastases detection. For these purposes, many markers are being evaluated for their suitability, with the prime candidates being those with restricted expression. Expression of these genes in extraneous environments is considered a potential indicator of tumor cell dissemination. The human cytokeratin 20 (CK20) belongs to the epithelial subgroup of the intermediate filament protein family that is involved in cytoskeletal structure. Like all other cytokeratins, its expression varies according to tissue type. Due to its restricted expression, CK20 has been studied as a candidate marker of micrometastases, primarily for colorectal and urothelial

cancer. CK20 detection has been correlated with increased recurrence risk and tumor stage.

Product Description

The LightCycler-CK20 Quantification Kit is specifically adapted for PCR in glass capillaries using the LightCycler Instrument. Detection of cytokeratin 20 (CK20) RNA is conducted by a two-step procedure. In the first step, cDNA is reverse-transcribed from RNA using AMV reverse transcriptase and random hexamer priming. In the second step, a 124-bp fragment of CK20-encoding mRNA is amplified from the cDNA by polymerase chain reaction (PCR) using specific primers. The amplicon is detected by fluorescence using a specific pair of Hybridization Probes. Using the same cDNA preparation but in a separate PCR reaction, mRNA encoding for porphobilinogen deaminase (PBGD) is processed as a reference gene. The reaction product serves both as a