Relative Quantification of Cytokeratin 20 on the LightCycler Instrument

An innovation providing new insights into the detection of micrometastases in research samples

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Introduction

Residual tumour cells, currently undetectable by conventional methods, may be responsible for cancer recurrences after apparent tumour clearance. The possibility of detecting these so-called "micrometastases" or "minimal residual disease" by RT-PCR amplification of tissue- or tumour-specific genes in extra-tumoural samples such as blood, bone marrow and lymph nodes has driven a growing amount of research in recent years [1].

A promising candidate marker of micrometastases is cytokeratin 20 (CK20). Expression studies found its expression restricted to gastrointestinal tissue, bladder transitional and Merkel cell carcinoma [2], therefore CK20 detection in extra-tumoural samples may be indicative of metastasis from these tumours. However, whilst some

studies have found associations between CK20 RT-PCR detection and stage, grade and recurrence [3-5], others have reported no significance [6] or non-specific detection [7].

To determine the feasibility of RT-PCR micrometastases marker detection, the reasons for such discrepancies need to be examined. This has been difficult to achieve due to the qualitative nature of the PCR analysis and a lack of standardisation between studies to date. Roche Molecular Biochemicals has developed the new quantitative RT-PCR assay for CK20 RNA detection, the LightCycler–CK20 Quantification Kit, to overcome this limitation.

Materials and Methods

Reverse transcription

cDNA was produced in a 20-µl reaction including 10 µl RNA, 1 µl LightCycler-CK20 Reverse Transcriptase, 4 µl LightCycler-CK20 Reverse Transcriptase Reaction Mix, 2 µl LightCycler-CK20 Deoxynucleotide Triphoshpates and 1 µl LightCycler-CK20 Random Hexamers. The reactions were incubated at 25 °C for 10 minutes, 42 °C for 30 minutes and 94 °C for 5 minutes.

LightCycler PCR

PCR amplification and detection on the LightCycler was performed using the DNA Hybridisation Probe format. Each reaction included 2 µl cDNA, 2 µl LightCycler-CK20 Enzyme Master Mix and 2 µl LightCycler-CK20 Detection Mix (including primer and Hybridisation Probes) or LightCycler-CK20 Reference Detection Mix (including primer and Hybridisation Probes) in a 20 µl volume. For each sample, two reactions, one for CK20 and one for the reference gene porphobilinogen deaminase (PBGD), were performed in separate capillaries. In the LightCycler Instrument, each reaction capillary was incubated for 1 minute at 94 °C before 50 cycles of 94 °C for 0 seconds, 60 °C for 10 seconds and 72 °C for 5 seconds. The PCR run was concluded with a 40 °C incubation for 30 seconds. Fluorescence was monitored at the conclusion of each 60 °C incubation. The fluorescence detected in channel F2/F1 was analysed using the LightCycler Analysis Software. The crossing point for each reaction was determined using the Second Derivative Maximum algorithm and the arithmetic baseline adjustment.

PCR Quantification

To determine CK20 quantities, the ratio of CK20 to PBGD in an unknown sample relative to the CK20 to PBGD ratio in a calibrator sample was determined using the LightCycler Relative Quantification Software. The calibrator is included in each run and set to a value of 1 000 000 – therefore a sample with a ratio of 10 000 would have 100 times less CK20 than the calibrator. Determination of the relative CK20 ratio was a function of the PCR reaction efficiency and the crossing points of CK20 and PBGD of the unknown and calibrator samples. This calculation has been included in the LightCycler Relative Quantification Software.

Results and Discussion

Previous micrometastases marker detection assays often comprised nested, conventional RT-PCR followed by visualisation on agarose gels, providing limited qualitative analyses [1]. Roche Molecular Biochemicals has developed the LightCycler-CK20 Quantification Kit, which offers significant advantages for the detection of micrometastases.

Amenable to large scale analysis

Optimisation of reaction concentrations and the provision of reaction mixes minimise the manipulations

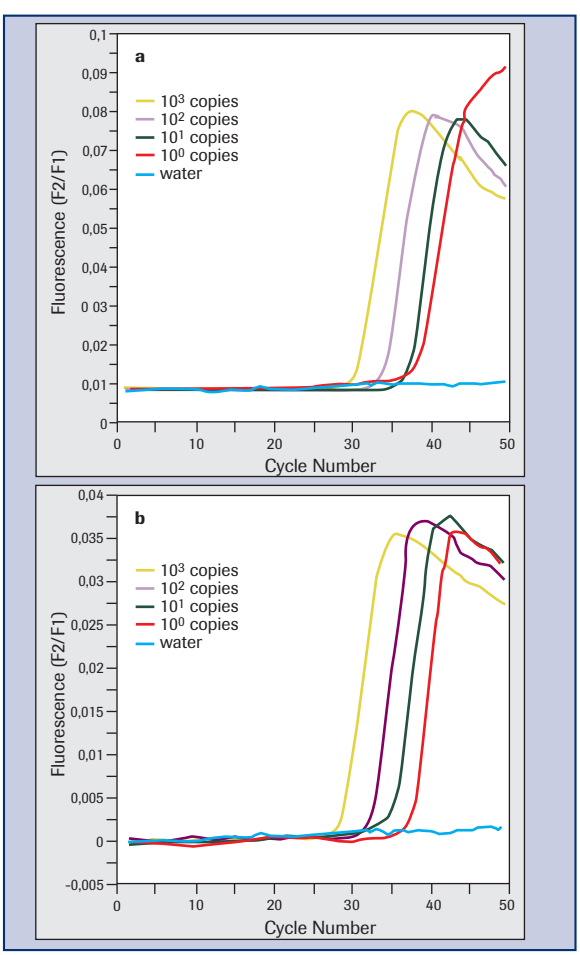

Figure 1: Sensitivity of (a) CK20 and (b) PBGD detection monitored with the LightCycler Instrument using CK20 plasmid DNA as exemplary system. The detection of a single copy of CK20 (red line) is displayed

Table 1: Comparison of the reproducibility of (1) LightCycler Relative Quantification Software derived and (2) standard curve derived methods for quantity calculation. The mean values ± coefficients of variation of the replicates are displayed

1) LightCycler Relative Quantification Software derived

RNA replicates	CK20 Crossing Point	PBGD Crossing Point	CK20 copies per ng RNA	Calibrated CK20 : PBGD
Calibrator	24.10 ± 0.6 %	25.73 ± 0.5 %	NA	1 000 000
1 ng (9)	27.81 ± 0.5 %	30.23 ± 0.9 %	NA	1 769 606 ± 14 %
10 ng (9)	24.99 ± 1.3 %	27.33 ± 1.0 %	NA	1 698 947 ± 12 %
100 ng (9)	22.48 ± 0.4 %	24.45 ± 1.4 %	NA	1 321 202 ± 12 %
All (27)			NA	**1 596 585 ± 17 %**

2) Standard curve derived

RNA replicates	CK20 Copy Number	PBGD Copy Number	CK20 copies per ng RNA	Calibrated CK20 : PBGD
Calibrator	21 560 ± 11 %	2 598 ± 11 %	210 ± 11 %	1 000 000
1 ng (9)	1 729 ± 11 %	141 ± 16 %	1 729 ± 11 %	1 556 000 ± 30 %
10 ng (9)	12 026 ± 22 %	930 ± 22 %	1 203 ± 11 %	1 578 231 ± 17 %
100 ng (9)	65 762 ± 17 %	5 853 ± 11 %	658 ± 11 %	1 350 182 ± 9 %
All (27)			**1 196 ± 40 %**	**1 494 804 ± 22 %**

required and, along with the LightCycler closed capillary system, reduce the risk of cross-contamination. Using the rapid thermocycling capabilities of the LightCycler, the total assay time is 120 minutes including 45 minutes for the PCR, hence providing a rapid assay amenable to large-scale analysis.

Highest specificity

For both CK20 and PBGD, PCR detection only occurs after the annealing of four sequence-specific oligonucleotides. These primers and Hybridisation Probes are designed to span exon-intron boundaries excluding the possibility of genomic DNA detection.

Quantification of PCR products at crossing point

The major feature of this assay is the ability to monitor and quantify PCR amounts using the LightCycler. Using the Hybridisation Probes format, the entire PCR reaction process is monitored by fluorescence detection, enabling measurement at the beginning of the detectable exponential phase ("crossing point"), which is considered the most reliable point of quantification [8].

Improved limits of detection

The use of a "hot start" enzyme (FastStart Taq DNA Polymerase) for PCR reduces primer dimer formation [9] and ensures maximum sensitivity, an important feature when considering the low copy numbers associated with micrometastases detection. Sensitivity tests demonstrate comparable or even improved limits of detection to previous conventional assays [3-7]. In spiking experiments, CK20 could be detected from one HT29 colorectal cell in a background of 1 ml blood and 10^5 HL60 promyeloleukemic cells whilst unspiked background cells were negative (data not shown). In plasmid dilution experiments, one copy of CK20 and PBGD could be detected (Figure 1).

Standardisable quantification

In addition to the assay development, the quantification model used has also been an important consideration. Whilst crossing points provide an indication of sample quantity, suitable models for their interpretation into standardisable and comparable values are yet to be established. The model of efficiency-adjusted relative quantification has been implemented in the new LightCycler Relative Quantification Software. It incorporates three important controls for factors potentially affecting quantification thereby maximising reproducibility.

The first element is the inclusion of housekeeping gene PBGD quantities into calculations. This provides a PCR run control for RNA loading and integrity and obviates the need for prior estimation of RNA quantities. This may provide a more relevant representation than absolute copy number quantification methods that rely heavily on spectrophotometric assessment of standard curve copy numbers and RNA loading amounts. The second element is the inclusion of a calibrator with each RT-PCR run and its CK20 to PBGD ratio into each sample calculation. The inclusion of the calibrator ratio helps to adjust for inter-PCR run variations and sets a reference for comparison and standardisation. The third element is the use of individual PCR-defined reaction efficiencies for CK20 and PBGD in each calculation, providing a control in the case of inefficient PCR reactions.

Using a sample which was replicated 27 times involving three different RNA concentrations, three reverse

transcription and six PCR reactions, the relative ratio obtained through the LightCycler Relative Quantification Software was compared to one whereby copy number determinations from standard curves (Table 1) were used. In support of this selection, the coefficient of variations for a calibrated CK20 relative ratio were 17 % and 22 %, respectively, with the larger error of the latter likely to be introduced by the variations in standard curve construction between PCR runs. Without factoring PBGD quantities and a calibrator ratio, the error from a method determining absolute CK20 copy numbers per nanogram RNA via a standard curve was 40 %.

Summary

This study describes the new LightCycler-CK20 Quantification Kit that may prove a valuable tool for micrometastases detection in research applications. The assay is quantitative, rapid, convenient-to-use, flexible, specific, sensitive and amenable to large-scale routine analysis. A PCR quantification model is used that maximises reproducibility and provides a platform for standardisation across laboratories. This assay provides a more informative analysis and may help to unravel reasons behind discrepancies between studies on micrometastases detection. Such a standardised, quantitative assay amenable to large scale analysis potentially opens the possibility to determine thresholds for background expression and investigate the potential significance of measuring tumor load. Further intensive studies are required to clarify this potential.

Order INFO

Product	Pack Size	Cat. No.
LightCycler-CK20 Quantification Kit	1 kit (96 reactions)	3 118 835
LightCycler Relative Quantification Software	1 software package	3 158 527

References

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