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# A novel quantitative real-time PCR test for *Mycobacterium tuberculosis*

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A novel system for real-time PCR is described based on the combined use of a double-labelled hydrolysis probe with a proofreading enzyme, in contrast to current detection systems which require DNA polymerases with 5'-3'nuclease activity. Hydrolysis probe design for the new system is simple, only requiring a double labelled oligonucleotide with a reporter dye and a quenching dye at each terminal end, and a 3´-mismatched region (1-2 base pairs) with the target DNA sequence. In the intact probe the proximity of the reporter dye and the quencher allows the latter to reduce fluorescence intensity. During the amplification process by the proofreading DNA polymerase the probe hybridises to the target sequence and the enzyme releases the 3´ labelled mismatched nucleotides. In this way elongation of the nascent DNA occurs as well as the emission of a fluorescent signal. Using this novel approach we have successfully designed a diagnostic kit which detects and quantifies *Mycobacterium tuberculosis* in clinical samples. The use of proofreading DNA polymerases enhances the copy fidelity and sequence discrimination of the system, thus ensuring accurate real-time PCR results. The test can be used in fields as diverse as pharmacogenomics, clinical diagnostics and biochemical research.

Real-time PCR is becoming an increasing important sector in the overall PCR-based diagnostic market. The methods used for detection in real-time PCR are currently dominated by hydrolysis probe methods such as are used in the Taqman system, but alternative detection methods for real-time PCR have been developed and are being successfully applied in routine applications (e.g. scorpions technology, hybridisation probes, molecular beacons, etc.) Despite these various advances on the technological front, there are still significant non-technical barriers such as intellectual property issues which prevent their practical penetration into the clinical diagnostic market which is nowadays limited to a few large companies. Patent legislation is designed to reward innovation and invention by awarding the holder of a patent the right to have an effective monopoly on the commercialisation of the invention. For several years the basic patents covering the use of PCR have been held by Roche. The expiry of some of these basic PCR patents should enable competitive companies to, at last, enter the PCR diagnostic market, thus signalling the end of one era and the beginning of a new one in which other biotechnology/diagnostic companies will come into their own. The resulting, more competitive, market should bring about more powerful yet cheaper diagnostics products for end users.

To get round existing patents and still provide an efficient and cost-effective assay system, the R&D Team of Biotools B&M Labs has developed a novel technology for real-time PCR detection (Lionprobes). The first diagnostic kit practically based on Lionprobes is now being launched on the European market.

The first kit, BioTub-QT detects and accurately quantifies *Mycobacterium tuberculosis* in clinical samples (blood, sputum, biopsies) on different real-time PCR platforms (e.g. Corbett and Applied Biosystems).

The aim of quantitative real-time PCR is to accurately quantify the initial content of target nucleic acid present in a sample. This is done by monitoring the progress of the PCR reaction by measuring the fluorescence of a reporter system, either a labelled probe or DNA binding dye. Reactions are characterised by the cycle threshold (Ct), the cycle at which fluorescent intensity is greater than background fluorescence. The Ct value is representative of the starting copy number in the original template [1].

Current real-time PCR detection systems ensuring high specificity use hydrolysis and hybridisation probes, which require the 5´-3´exonuclease activity and the strand displacement activity, respectively, of the Taq DNA polymerases. However, in applications requiring high DNA fidelity the use of Taq DNA polymerases may introduce random mutations [2, 3], which may interfere with downstream sample analysis and manipulation.

Proofreading DNA polymerase enzymes mediate high fidelity DNA replication both *in vivo* and *in vitro* [4-8], by removing mismatched bases due to 3´ to 5´ exonuclease activity. The application of such proofreading DNA polymerases to the analysis of single nucleotide polymorphisms (SNP) has long been recognised. Selective amplification dependent upon template nucleotide sequence is achieved by using 3' end-labelled primers. Thus, matched primers yield labelled products, whereas mismatched primers produce unlabelled products.

As for real-time PCR, proofreading DNA polymerases have only been used in combination with SYBR Green I, the non-specific DNA binding agent [9]. To meet the different needs of both the routine market and also those of specific research projects, real-time PCR requires accurate detection technologies. Thus, the more real-time PCR detection systems are available on the market, the more powerful will be the RT PCR technique itself.

We have developed a real-time system mediated by a proofreading DNA polymerase and a hydrolysis probe acting as a primer-probe, in which the DNA polymerase activity is not only used for amplification of the desired sequences but as a system of fluorescence generation on a sequence dependent mechanism.

The two essential components of this novel approach are a double-labelled probe with a 3´mismatch in its sequence and a DNA polymerase with 3´-5´exonuclease proofreading activity (Pfu). The probe is a linear oligonucleotide, possessing a fluorophore and a quencher at each terminal end of its sequence and designed to present a single (or double) 3´ mismatch with the template. In the intact probe the quencher is close to the fluorophore, and thus reduces the reported fluorescence intensity. During Pfu amplification when the probe hybridises to the target DNA, the enzyme excises the labelled 3´mismatched base pair and thus it allows elongation of the nascent DNA and the production of a fluorescence signal. In this way the labelled probe acts simultaneously as an amplification primer and a detection probe for the method.

## Design of probes

The precise position of the fluorophore and the quencher on the oligonucleotide is an important factor in the design of the probe. The emission of fluorescence in the conventional TaqMan assay is due to the release of the 5´-terminal fluorophore of the probe by the 5´-3´exonuclease activity of a Taq DNA polymerase.

As our new system relies on the 3´-5´exonuclease activity of the Pfu instead, we evaluated the effect on fluorescence emission of the fluorophore position (namely either at the 5´or 3´end) within the hydrolysis probe. In general, the position of the flurophore dye does not affect either the fluorescence emission nor the amplification efficiency. However, certain DNA sequences may quench intermolecular fluorescent labelled probes. In such cases the fluorophore marker must be positioned at the 3´end of the oligonucleotide in order to be released by the enzyme. This is the case for the Lionprobe system for *Mycobacterium tuberculosis* which is therefore labelled with the quencher TAMRA at the 5´end, and with the fluorophore FAM at the 3´end.

Degradation of the intact probes due to the 3´-5´ exonuclease activity of proofreading DNA polymerases could cause interference with the results. Therefore, the question of probe degradation was carefully studied under experimental conditions. It was observed that only the hybridised probe was the substrate of Pfu exonuclease activity.

In contrast to other real-time PCR detection technologies e.g. scorpion probes, molecular beacons, etc., which require a complex probe structure, the design and synthesis of the Lionprobes is simple. Moreover it only needs two oligos per gene instead of the three oligos (two primers and one probe) used in the TaqMan assays, molecular beacons or hybridisation probes. Our experience regarding the 3´ mismatch probe region is that single or double mismatches are highly successful.

## Technology behind the kit

The BioTubQT Kit consists of two amplification detection reactions which take place in the same amplification tube: the amplification of the insertion element IS6110 of the *M tuberculosis* complex and the amplification of the internal control included in the kit. For this purpose the kit contains a common reverse primer and two double-labelled Lionprobes.

The target sequence for *Mycobacterium tuberculosis* was selected on the basis of highly conserved regions among the *Mycobacterium tuberculosis* complex sequence. The probe for *Mycobacterium tuberculosis* was labelled with the quencher TAMRA at the 5´end, and the fluorophore FAM at the 3´end, whereas the internal control probe was labelled at the 5´end with the fluorophore CAL FLUOR 610 and at the 3´end with the quencher BHQ-2. Both probes present two mismatches at the 3´end of the hybridisation area with the target DNA. During the amplification reaction, the Pfu recognises the 3´mismatch hybrid and consequently cleaves the mismatched labelled base pair because of its 3´exonuclease activity. Once the 3´end of the probe is corrected, amplification priming is

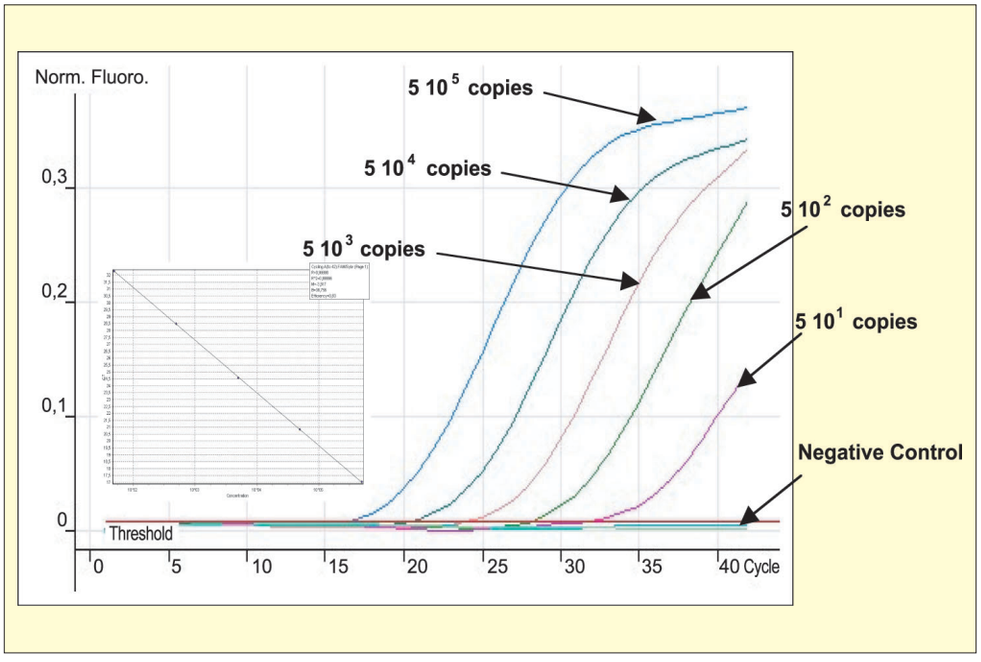


*Figure 1. Real-time PCR results of serial dilutions of the positive control included in the BIOTUB-QT Kit (5x101 to 5x105) using Rotor-Gene 3000 (Corbett Research, Sydney, Australia).*

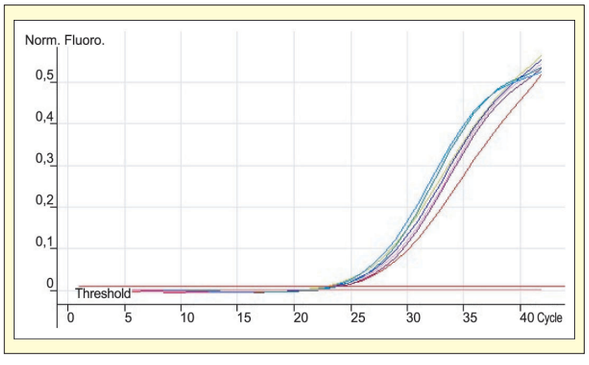


*Figure 2. Fluorescent signal detected for the internal control of the analysed samples shown in figure 1.*

enabled by the probe, and during each amplification cycle the fluorescence emerges as result of the release of the fluorophore (FAM in *Mycobacterium tuberculosis*) or the quencher (BHQ-2 in the internal control) from the probe.

The sensitivity of the *Mycobacterium tuberculosis* probe was evaluated using serial dilutions of a positive control to construct a standard curve. Positive controls consisted of a PCR product containing a non infective generic sequence from *M. tuberculosis* flanked by the common reverse primer and the primer-probe for *M. tuberculosis*. High fluorescent signals and well-shaped standard curves were observed in the range of 5x $10^1$ to 5x $10^5$ copies per reaction [Figure 1]. A linear regression with a correlation coefficient ($R^2$) of 0.99 was obtained when the Ct value was plotted as a function of template concentration [Figure 1 inner]. Underlying the accuracy of this novel technique, it was found that the kit could detect specific targets in a reproducible manner down to 50 copies/reaction To confirm the absence of amplification inhibitors in the reaction the kit includes an internal control which must be analysed at the same time as the target pathogen. The internal control consists of a PCR product containing a sequence from the lectin gene flanked by the internal control probe and the common reverse primer. Since the same quantity is spiked in each amplification reaction, the Ct value is similar for all the samples giving a reproducible signal around Ct 25 [Figure 2]. A negative result in the internal control indicates an inhibition of the amplification reaction.

## Conclusions

The Lionprobe system correctly identifies samples containing the target DNA, and yields negative results with negative control samples. Since Pfu DNA polymerase has neither any activity towards the intact probe, nor any 5´-3´exonuclease activity, the emission of fluorescence in each cycle is proportional to the rate of Pfu cleavage of the hybridised probe. Results obtained with the BioTub kit suggest that the new system is well suited for quantitative analysis of nucleic acid targets.

Lionprobes have the advantage of both an easy probe design and simple synthetic procedure. Because of the proofreading activity of Pfu DNA polymerase, the new system is particularly useful in projects where high amplification and replication fidelity is required.

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