REPRODUCIBILITY AND SOURCES OF ERROR IN REVERSE TRANSCRIPTASE - POLYMERASE CHAIN REACTION METHODS

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Reverse transcriptase - polymerase chain reaction (RT-PCR) has become a popular method for measurement of mRNA levels in tiny samples, including microdissected specimens. Quantitative RT-PCR involves many steps, but there has been no systematic survey of its accuracy, or which steps introduce the greatest variation. It is not clear whether using competitive PCR to give molar measurements of mRNA is more reliable than comparisons made between non-competitive results.

We pooled mRNA from rat glomeruli and performed RT-PCR on multiple aliquots, each equivalent to one glomerulus. We also performed PCR on equivalent aliquots of pooled cDNA following RT of mRNA from rat glomeruli. Comparative and semi-competitive RT-PCR was performed. ELISA was then used to quantify PCR products.

We found that the RT, PCR, and ELISA efficiency is very consistent both within a run and between runs, even with samples the size of one rat glomerulus. For the entire process, standard deviation is less than 20% of the mean of replicate measurements. This confirms that the method is viable for the determination of relative levels of mRNA. Variation is similar with comparative or competitive methods, indicating that although competitive RT-PCR is reproducible, its added complexity may be unnecessary if comparisons between samples are required rather than molar quantitation of mRNA.

Reproducibility is limited principally by human error, the most frequent error being incorrect pipetting. These errors may be exaggerated by the sensitivity of the combined techniques. Errors can be limited by practical measures such as using larger volumes of more dilute reagents, and using pipettes of the correct volume.

PCR DETECTION OF MYCOBACTERIUM TUBERCULOSIS IN FORMALIN FIXED AND PARAFFIN EMBEDDED TISSUES

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Aim: Microbiology is the usual tool available to pathology in the identification of organisms. However, presented specimens are not always suitable for cultures. Often the only available tissue has been fixed and embedded in paraffin. We extended an existing PCR method used to detect mycobacteria in unfixed samples, to identify M. tuberculosis in paraffin embedded tissue.

Method: *The IS6110 insertion sequence is thought to be specific to all* members of the M. tuberculosis family, and is present at 1-15 copies per genome. We used primers which amplified 123bp of this sequence. The paraffin embedded material was dewaxed using xylene and ethanol, and the DNA released into Tris buffer pH 8.5 by treatment at 100°C for 30 minutes. The amplified sequence can be readily detected on 12% polyacrylamide gels.

We found that re-amplifying the products after the first round of PCR increased the sensitivity of the test. The technique was tested on 40 suspected TB cases, 15 of which did not demonstrate AFB using conventional methods.

Results: The method was tested on control specimens of embedded M.tuberculosis, for which the number of bacilli present had been previously determined. The PCR method indicated the presence of M.tuberculosis in all the AFB +ve clinical samples. In addition, M.tuberculosis was demonstrated in all the AFB -ve samples. Non-tuberculosis control samples were PCR negative.

Conclusion: The method promises to be a useful diagnostic test for the rapid and sensitive detection of M. tuberculosis in paraffin embedded tissues.

The Application of a Novel Multi-colour Fluorescence In-situ Hybridisation to the Analysing Gene Expression in Non-Hodgkin's lymphoma

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There has been considerable interest in the in-situ detection of mRNA, since this enables the expression of specific gene to be placed within the context of an individual cell, which not be determined by Northern blotting or reverse transcriptase polymerase chain reaction (PCR). The ability of analysing gene expression in formalin-fixed paraffin wax-embedded material is important because it would make available an enormous resource of archival material. The use of fluorescence-labelled probes has allowed more than one mRNA species to be targeted in cryostat sections and in cultured cells. Paraffin wax-embedded material has been more difficult to work with due to autofluorescence problems, however, we have recently developed a fluorescence in-situ hybridisation (FISH) procedure (Albalwi et al, Trends Genet., 1997, 13, 41<http://www.elsevier.nl/locate/tto>T40062) for the simultaneous detection of multiple mRNAs in formalin-fixed paraffin-wax embedded tissue, using small oligonucleotides (28-30 bases). By use of a CCD camera, which provides a high degree of sensitivity, we have identified expression of both abundant (immunoglobulin) and low abundant (e.g. BCL-2) mRNAs in archival lymph node material. We are currently using this approach to examine potential differences in MYC mRNA versus myc protein expression between low and high grade non-Hodgkin's lymphomas (NHL). Our initial results have revealed a heterogeneous pattern of expression among of low grade NHL, but a homogeneous pattern was observed among high grade NHL. Current studies include an analysis of other oncogenes (e.g. BCL-6, PIM-1) expression in a low grade NHL that subsequently transform to high grade disease.

The Application of Multi-Colour Fluorescence In-situ Hybridisation to the Analysis of Abnormal Chromosomes in Non-Hodgkin's lymphomas.

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Numerical and structural chromosome aberrations are recognised as important diagnostic and prognostic indicators in human diseases, including non-Hodgkin's lymphomas (NHL). Recently, fluorescence in-situ hybridization (FISH) has been used with increasing success to identify and characterise many chromosomal aberrations, leading to the development of multiple chromosome painting methods for simultaneous detection of multiple probes in metaphase target cells. In this study, we have analysed 15 NHL biopsy samples and 5 human lymphoma cell lines (Daudi, HS602, Raji, MC116 and Namalwa). Some of the cell lines were partially analysed cytogenetically in the early 1970s using solid staining techniques, but no chromosome analysis has been previously attempted on the HS602 cell line. The detection of chromosome abnormalities in malignant lymphoma patients and cell lines by conventional cytogenetics is particularly problematic because of difficulty in routinely preparing metaphases spreads of sufficient quality or quantity and the complex nature of many of chromosomal changes. We completed cytogenetic analyses on all of the NHL samples and the cell lines. We then followed a strategy of refining these preliminary findings by applying multicolour FISH with whole chromosome painting probes derived from flow sorted chromosome libraries, using direct and indirect combinatorial labelling with two fluorochromes, to detect up to five different chromosomes on the same target metaphase. The application of multiple chromosome painting has enabled complex rearrangements to be characterised and made the identification of marker chromosomes possible.