The scope of quantitative polymerase chain reaction assays in clinical molecular pathology

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Introduction

Clinicians routinely look to a diverse selection of quantitative measures of biochemical parameters in various body fluids as a convenient means of assessing the disease state of patients. Values for electrolytes, metabolites and serological properties all provide information that may be useful in diagnosis, prognosis and in determining treatment of clinical conditions. Increasingly, more is being learned about the molecular basis of a wide range of diseases which, until recently, have been relatively poorly understood. Immunohistochemistry and *in situ* hybridisation have proved to be popular techniques in the qualitative investigation of patterns of gene expression in biopsy specimens. The molecular pathologist's role in clinical diagnostics is expanding and increasingly requires rapid and reliable means of quantifying the levels of various key molecules (for example, amplified genes, viral genomic DNA or specific mRNA species).

In clinical investigations, specific protein concentrations in the serum do not necessarily correlate with concentrations found in diseased organs and do not provide information about the localisation of the main source of the measured gene product. In addition, molecules that may be important indicators of diagnosis or prognosis of neoplastic or inflammatory lesions are often localised to the disease site and may not be measured usefully by a serological test. Molecular analysis of small pieces of biopsy material may therefore provide more useful information.

The methods of choice for monitoring molecular parameters such as viral load, gene copy number and gene expression have been generally regarded to be those that involve some form of "blotting" procedure. Nucleic acids can be quantitated readily by these methods. Additionally, a large number of proteins can be quantitated rapidly by enzyme linked immunosorbent assay (ELISA). However, the requirement by these methods for large or fresh samples limits their use and attractiveness to the molecular pathologist who will probably be presented with only small amounts of material with which to work. In the future, sample size will become more important as minimally invasive biopsy procedures become more popular, resulting in less material becoming available for molecular investigations.

The alternative approach to quantitation of molecules by blotting or ELISA involves the polymerase chain reaction (PCR) which, because of its extreme sensitivity, permits the use of small samples such as those taken from fine needle biopsies or aspirates. Where com-

parisons with hybridisation analyses have been possible, results of quantitative PCR (qPCR) reactions are comparable.^{1,2,3} Less than 10 copies of target sequence are required for a PCR reaction and it is possible to amplify from material extracted from single cells.⁴ In addition, nucleic acid preparation procedures can be modified to permit the use of fixed and paraffin wax embedded material, thus permitting analysis of archival material.^{5,6}

In this article we present an overview molecular quantitation by PCR from practical and clinical viewpoints. Examples are given and some of the factors that affect quantitation of DNA or RNA are discussed.

Quantitative PCR: the technology

When designing a qPCR assay, three major considerations must be taken into account. These considerations arise as a result of features peculiar to the qPCR reaction that generally do not substantially affect the results of qualitative PCR assays. They are: (1) variations in amplification efficiency between reaction tubes; (2) the plateau effect; and (3) loading errors.

AMPLIFICATION EFFICIENCY

Because the number of product molecules increases exponentially with thermal cycle number, small changes in amplification efficiency between sample tubes can have a profound effect on the final concentration of reaction products. One way of dealing with this problem is to coamplify a predetermined quantity of a competitor template with each target template. In parallel reactions, a constant amount of the same sample is spiked with a series of known concentrations of competitor. If the two templates are shown to amplify at similar rates (the slopes of plots of log product concentration against cycle number for each template are similar), then the competitor concentration that produces equimolar competitor and target product concentrations is indicative of the level of target sequence in the sample (competitive PCR^{7,8}) (fig 1). The use of RNA as opposed to DNA competitors can be used to control for reverse transcriptase variations in quantitative reverse transcriptase PCR assays.^{9,10}

The competitor products must be in some way distinguishable from the target products (for example, inclusion of a small intron¹¹ or modifications made by recombinant methods⁹⁻¹³). The most reliable choice of competitor is that in which the sequence difference is as small as possible (for example, modification of a restriction site by end fill-in or by site-dir-

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Figure 1 Competitive PCR using an exogenous standard which is a modification of the target template produced by the insertion of four base pairs by filling in an internal restriction enzyme cleavage site with Klenow fragment. The upper panel shows the effect of serial twofold dilutions of the exogenous standard (upper bands) on the relative amplification of the target sequence. The graph is a logarithmic plot of the ratio of band intensity against the concentration of the standard. The gradient of the slope approximates to -1.0 , as would be expected from theory.

ected mutagenesis of the cloned target product). These conservative changes are less likely to cause differences in amplification efficiency between the two templates than large insertions or deletions. However, competitor templates that contain a region of exogenous sequence between the two primer recognition sequences (PCR mimics) have been used successfully and can reduce the problem of heteroduplex formation between template and competitor amplimers. These synthetic competitors can be constructed so that they are multifunctional—that is, they have multiple primer recognition sequences and can be used to quantitate the expression of a number of different genes.⁹

THE PLATEAU EFFECT

The PCR does not continue in its exponential phase indefinitely. Reagents are consumed, there is generation of the polymerase inhibitor

pyrophosphate and a decrease in activity of Taq DNA polymerase during the course of a PCR run. All of these factors affect the graph of the log product concentration against cycle number which becomes non-linear (plateau phase) and reaches a maximum at high cycle number¹⁴ (fig 2). This effect can be eliminated simply by terminating the reaction at a point where the increase in product concentration is still exponential (that is, before the plateau phase). The point at which the reaction becomes saturated must be determined experimentally so that any particular sample has a template concentration that remains within the quantifiable range of the assay. Non-competitive PCR assays rely on the fact that prior to the plateau phase the amount of product molecules varies linearly with amount of template put into the reaction¹⁵ (fig 3). These procedures, however, do not have internal controls for intertube variability or loading.

Figure 2 The effect of cycle number on the generation of PCR product (A). This gives an exponential increase up to 34 cycles before a plateau is reached (B and C).

Owing to the low cycle number required to maintain the assay in the linear range (often 18 to 25 cycles), the amount of PCR product in a quantitative reaction is frequently below the level of detection of ethidium bromide stained agarose gels. A number of solutions to the problem of detection of very low amounts of products is available. Conventional techniques

Figure 3 Non-competitive PCR demonstrating that the amount of product is directly proportional to the initial quantity of target sequence, which was varied by serial dilution of cDNA.

such as Southern blotting of the products¹⁶ and densitometry (or better—phosphor—imaging) of incorporated radiolabel or high pressure liquid chromatography have their drawbacks for quantitative assays but are used widely, especially in semiquantitative assays. Newer techniques are more applicable to the safe, highly sensitive and rapid analysis of qPCR samples in the clinical laboratory. These include detection of fluorescent labels in automated detection systems such as a DNA sequencer or detection of products in solution hybridisation by magnetic bead separation and electrochemiluminescence.¹⁷

LOADING ERRORS

Inaccuracies in the amount of starting material as measured by total RNA or DNA concentrations are important variables that affect quantitation. In northern blot analysis, hybridisation with a second probe specific for a “housekeeping” reporter gene (often β -actin or G3PDH) acts as a control for both loading and RNA degradation. Similarly, in qPCR assays, amplification of a second gene can control for these factors, assuming that both PCR assays remain in the quantitative phase of amplification.^{18,19} The ratio of the target product concentration to the control product concentration is taken as a measure of the initial amount of the target sequence in the sample (differential PCR). Ideally, coamplification of the two sequences should be performed in a single reaction vessel but there should be awareness that the two amplimers may compete in some instances.²⁰

By combining both differential and competitive PCR in a single reaction vessel (two competitive PCRs, one for the gene of interest and one control), substantial enhancements to the reproducibility and accuracy of quantitations can be made.¹²

Preparation of sample DNA or cDNA template

In addition to the general precautions taken to ensure that all samples should not be cross-contaminated or contaminated with products before amplification by PCR, protocols should be adopted that ensure that the preparation of sample template is performed in a reliable and reasonably consistent fashion. The protocols used routinely for the extraction of DNA and RNA from formaldehyde fixed and paraffin wax embedded tissue samples in our laboratory are outlined below. Further, if mRNA expression is being analysed, the protocol for reverse transcription of mRNA to cDNA should be efficient and reasonably consistent. We use synthetic oligo [dT] or random hexamers to prime reverse transcription with RNase H⁻ MoMLV Reverse transcriptase (Superscript II from Life Technologies). In order to limit mispriming in the first thermal cycle of quantitative PCR reactions, which can affect the reproducibility and accuracy of quantitation, a “hot start” technique²¹ is used.

Protocol for extraction of DNA from paraffin wax embedded tissue sections⁵

A 10 μ m section of paraffin wax embedded tissue is placed in a microcentrifuge tube and incubated at 55°C with buffer A (50 mM KCl, 2.5 mM MgCl₂, 20 mM Tris-HCl (pH 8.0), 0.45% NP-40, 0.45% Tween-20) and 200 μ g/ml proteinase K. Samples are then boiled for 20 minutes before storage at 4°C. DNA concentrations of sample are estimated by spectrophotometry and 100–300 ng of DNA (made up to 15 μ l in buffer A) is used per 100 μ l PCR reaction. There should be enough DNA yielded using this technique for 30–50 PCR assays. Best results are obtained when samples are subjected to PCR within one to two weeks of extraction.

*Protocol for extraction of RNA from paraffin wax embedded tissue sections (based on Miens)**

A 20 μ m section is deparaffinised twice in 400 μ l of xylene for 15 minutes and pelleted. The section is then washed in 400 μ l absolute ethanol and RNA is extracted from the tissue pellet with 300 μ l TRIOL (Life Technologies) (dissolved by vortexing and incubated on ice for five minutes) and 30 μ l chloroform and precipitated with one volume of isopropanol on ice for one to three hours. The RNA is then collected by centrifugation at 4°C for 15 minutes, washed with 200 μ l 75% ethanol, briefly vacuum dried (no more than five minutes) and then redissolved in 50 μ l diethyl pyrocarbonate treated deionised distilled water (DEPC/DDW). Total RNA concentration is determined by spectrophotometry and 1 μ g RNA diluted to 5 μ l with DEPC/DDW is used in a 20 μ l reverse transcription reaction prior to PCR.

Quantitative PCR: clinical applications

Quantitative PCR has a wide range of applications in the clinical context. It has proven especially valuable in evaluating certain viral infections as PCR enables rapid and highly specific diagnosis and titration. Many other applications of qPCR are in use or are being evaluated and promise to be valuable tools in diagnosis and in the direction of treatment strategies. For example, quantitative analysis of viral genomic RNA or proviral DNA provides a good indication of the disease stage in HIV infection^{13,22–24} and can mark the transition from clinical latency to the active replication stage.²⁵

Used in conjunction with minimally invasive sample collection procedures, molecular parameters can be monitored throughout the course of a disease and its treatment (for example, in monitoring of bone marrow transplants for molecular markers of transplant rejection²⁶ or opportunistic infections in immunosuppressed patients²⁷).

It is not necessary in every application of qPCR to provide absolute quantitation. In cases where allele loss determinations, percentage residual cell content or relative levels of cytokines are required, it may be sufficient to perform a simple differential PCR assay

A summary of some of the reports published in the literature detailing the use of PCR in clinical practice

Disease type	Situation	Sequence amplified	Use(s)	Reference(s)
Infections	viral	CMV	CMV glycoprotein H (gH) DNA	Monitoring CMV infection in immunosuppression
		EBV	EBV EBNA-1 DNA	EBV associated lymphoproliferation in immunosuppression
		HBV	HBV S gene DNA	Monitoring HBV infection
		HCV	HCV genomic RNA	Viral load/response to interferon therapy
		HDV	HDV viral DNA	HDV viraemia
		HIV-1	HIV-1 genomic RNA	Viral load correlates with disease stage
				Relative infected cell burden
				Transition to replicative phase
		HPV	HPV16 DNA	Cervical invasive carcinoma
		HSV, (genital)	HSV viral DNA	Vertical transmission to infant
	bacterial	<i>Salmonella enteritidis</i>	Virulence plasmid DNA	Specific identification and quantification in broth cultures
		<i>Meningitis</i>	TNF α , LT, IL-1	Monitoring cytokine response in CSF
		<i>Mycobacterium leprae</i>	IL-2, INF γ	T _H 1 (tuberculoid) versus
			IL-4,5,10	T _H 2 (lepromatous) response
Cancer	parasitic	<i>Trypanosoma cruzi</i>	Kinetoplast (k) DNA	Estimation of parasitaemia
		Amplified oncogenes	c-erbB2	Breast cancer
			N-myc	Neuroblastoma
			EGFR	Glioma
			HER-2	Ovarian cancer
		Translocated oncogenes	Bcl2 (t[14:18])	Non-Hodgkin's lymphoma (residual disease)
			Bcr-Abl	Chronic myelogenous leukaemia
		Mutations	Ki-ras	Premalignant lesion detection
		Allele loss at oncosuppressor loci	DCC, p53, Rbl, NF-1	Colorectal carcinoma
		Multidrug resistance	APC/MCC	
			MDR-1 mRNA (P-glycoprotein)	Prediction of resistance/susceptibility to certain drugs
	Inflammation	inflammatory bowel disease	Cytokine expression	MCP-1 mRNA
	glomerulonephritis	Renin-angiotensin system function	Renin mRNA	Early decrease in renin expression
Transplantation	transplant rejection/tolerance	Bone marrow transplant	Microsatellites	Evaluation of mixed chimerism
Genetic disease	thalassaemia	Down's syndrome	Hb β A v Hb β S mRNA	Relative expression levels in heterozygotes
Genetic disease	muscular dystrophy	Trisomy 21	Dystrophin mRNA	Antenatal screening of fetal cells in maternal blood
Genetic disease	Down's syndrome	Trisomy 21	microsatellites	Antenatal screening of fetal cells in maternal blood

CMV = cytomegalovirus; EBV = Epstein-Barr virus; HBV = hepatitis B virus; HCV = hepatitis C virus; HDV = hepatitis D virus; HPV = human papillomavirus; HSV = herpes simplex virus; MDR = multidrug resistance; TNF = tumour necrosis factor; IL = interleukin; EBNA = EBV associated nuclear antigen; LT = heat labile toxin; EGFR = epidermal growth factor receptor; INF = interferon; CSF = cerebrospinal fluid.

with an endogenous non-competitive standard. These situations lend themselves to the use of multiplex PCR reactions. In these multiplex PCR reactions multiple genes are amplified with primer sets that are often individually labelled with different coloured fluorescent markers. This technique permits the rapid and cost-effective assay of multiple parameters from a given sample (for example, allele loss at a range of oncosuppressor loci in colorectal cancer²⁸).

Absolute quantitation—that is, expression of the level of target sequence per unit of a sample (for example, numbers of molecules per 10⁶ cells, per ml of blood, per mg of tissue, etc.), is required in virological and other situations. In these cases some form of competitive PCR assay is often used.

There are now many reports of the use of qPCR in all aspects of clinical practice. A summary of some of these reports is presented in the table.

Conclusion

Advances in PCR technology and the application of internal and exogenous amplification standards, together with the novel amplimer detection methods have revolutionised molecular quantitation in the clinic. The power of the PCR as a quantitative measure of molecular parameters in clinical disease has been recognised and qPCR technology is poised to be a powerful diagnostic tool.

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