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THE APPLICATION OF COMPETITIVE PCR TECHNOLOGY TO ASTHMA RESEARCH

A PhD Thesis

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ADDENDUM

I PUBLICATIONS ARISING DIRECTLY FROM THESIS

1. Glare, EM, Divjak, M, Rolland, JM and Walters, EH. (1999) Asthmatic airway biopsy specimens are more likely to express the IL-4 alternative splice variant IL-4δ2. *J Allergy Clin Immunol*, **104**(5), 978-82.

2. Glare, EM, Divjak, M, Bailey, MJ and Walters, EH. (2001) The usefulness of competitive PCR: airway gene expression of IL-5, IL-4, IL-4δ2, IL-2, and IFN-γ in asthma. *Thorax*, **56**(7), 541-8.

3. Glare, EM, Divjak, M, Bailey, MJ and Walters, EH. (2002) β-Actin and GAPDH Housekeeping Gene Expression in Asthmatic Airways is Variable and Not Suitable for Normalising mRNA Levels. *Thorax*, **in press**.

II HYPOTHESES

> "Hypotheses need to be clearly spelt out in the earlier chapters, ie chapters 2-5. Hypotheses don't really appear until chapter 6."

The earlier chapters of this thesis describe subjects, biological samples and their processing (Chapter 2), including RNA extraction (Section 2.3), the development of high-sensitivity PCR (Chapter 3), the development of competitive PCR assays (Chapter 4) and the development of dual mRNA-species competitive PCR for IL-4 splicing variants (Chapter 5). As the work in these chapters is developmental in nature, our hypotheses were essentially that our aims could be realised.

I hypothesised that sufficient RNA could be extracted from airway biopsies to conducted gene expression assays (Section 2.5). Pilot extractions showed that yields of RNA were more consistent when two rather than one biopsy was collected. Some previously published studies suggested that it was reasonable to hypothesise that RNA could be extracted from BAL cells [46, 47]. It was subsequently found that, when starting with more than 1×10^6 cells, RNA could be consistently extracted.

Knowing that only small amounts of sample RNA would be available and that cytokines are expressed transiently, it was hypothesised that the sensitivity of a RT-PCR-based quantitative method would be required to measure the mRNA levels. Whilst PCR was and is fabled to be able to amplified and detect only one target molecule, I found that under standard conditions recommended at that time this hypothesis was not normally achieved (Section 3.6). My work on the factors affecting PCR sensitivity lead to the concept referred to as the Monte Carlo effect. The ensuing hypothesis was that sensitive conditions could be established for each amplicon by looking at the effect of factors on the repeatability of reactions being amplified from below the threshold of the Monte Carlo effect (Section 3.6.1). To quantitate the PCR products, I initially hypothesised that a chemiluminescence-X-ray film method as had been used by others [66] would be appropriate for this study (Section 3.3.1). The response of the film was found not to be directly proportional to the input signal (Figure 3.2). I subsequently hypothesised that direct fluorescence

scanning of stained gels would have suitable quantitative features for measuring competitive PCR assays (Section 3.3.2) and the conditions for achieving this were identified (Figure 3.4).

In the development of competitive PCR assays (Chapter 4), it was hypothesised, as proposed by the founders of the method [8, 109, 133], that comparison of competitor and native PCR products after exponential amplification could lead to the quantitation of the initial input of native target molecules. Validation studies indicated that relative quantitation could be achieved and indicated the best conditions to use (Section 4.4.3). Importantly, these validation studies also showed that some assays were not quantitative using the standard method (Section 4.4.2.3 and Figure 4.3). The basic concept of competitive PCR was extended to IL-4 and its splicing variant IL-4δ2 (Chapter 5). Here the hypothesis was that the two different native targets and the competitor products would compete equally together in PCR amplification (Section 5.3.1). Validation studies indicated that relative quantitation of both mRNA species could be achieved simultaneously from the one reaction (Section 5.3.3).

III QUESTIONS FROM CHAPTER 4: THE DEVELOPMENT OF COMPETITIVE PCR ASSAYS

III.i Relative Input Ratios

"How were input ratios calculated?"

The method for calculating relative input ratios is described on page 71 (last three sentences) and in the legend of Figure 4.3

III.ii Validation of IL-2 RT-Competitive PCR

"Figure 4.4, page 76 Describe the experimental method in the body of the text as well as figure legend."

Consistent with the style of this thesis, the method of conducting the validation study is written in full in the Materials and Methods Used section, Section 4.2.3, paragraph 2.

III.iii Conclusions of the Experiments

"What was the conclusion of these experiments? Page 88: Where are the data on the results of quantification, eg ranges of detection."

The ultimate conclusion of the validation experiments of the various competitive PCR assays was that relative quantitation could be achieved (Section 4.4.3). For IL-2, IL-5, IFN-γ, β-Actin and GAPDH the output ratios followed the theoretical line of direct linear relationship with relative input ratios (Figures 4.4 and 4.5). I subsequently concluded that Equation 4.3 (page 67) could be used in these assays to calculate the input amount of native template (as discussed in Section 4.4.4). The validation studies for TGF-β showed that when either native or competitor templates were in excess they competed more strongly in the amplification (Section 4.4.2.3 and Figures 4.3). I concluded that the former strategy of calculating the input native copies was not appropriate. Instead, the linear relationship between input and output ratios could be utilised to estimate the input ratio and subsequently the input of native template could be calculated (Section 4.4.4.1).

The method that I used to calculate the relative input ratios in the analysis of the native:competitor titrations has the effect of centralising the data around the 1:1 ratio (Section 4.4.2.3, last paragraph). Whilst it makes intuitive sense that the assays are mostly likely to be quantitative at a input ratio of 1:1, the deduction of quantitative ranges from this is still some what subjective. For this reason, quantitative ranges were not formally calculated. In most cases, output ratios conformed to the theoretical line beyond input ratios of 10:1 and 1:10 (Figures 4.4 and 4.5). Pilot studies were conducted to optimise the amount of sample to use and the amount of competitor to add (Section 4.5.1). When the assays were used in the patient studies the resulting output ratios were relatively tightly grouped around ratios of 1:1, showing the benefit of conducting the latter optimisation. For example, for β-Actin and GAPDH, 100 % and 98 %, respectively of the reactions gave ratios greater than 0.1 (Section 6.6).

IV TYPOGRAPHICAL ERRORS

Page	Location		Change	
			From	To
15	Paragraph 3	Line 3	know positive control	known positive control
16	Paragraph 1	Line 4	is assume to be	is assumed to be
16	Section 1.3	Line 3	cytokines though to	cytokine thought to be
22	Section 2.2.3.1	Line 3	experience researcher	experienced researcher
23	Paragraph 1	Line 2	incubation of ice	incubation on ice
31	Footnote	–	calculation assume that	calculation assumes that
35	Paragraph 1	Line 3	(Pharmacia Biotech, Uppsala, Sweden	(Pharmacia Biotech, Uppsala, Sweden)
36	Section 3.2.2.2	Line 3	Molecular dynamics	Molecular Dynamics
37	Paragraph 2	Line 3	(Table 4.2	(For design see Section 4.3 and Table 4.2,
44	Paragraph 1	Line 6	is considerable more	is considerably more
50	Paragraph 2	Line 8	range represents closely	range represent closely
57	Paragraph 1	Line 1	Proceeding chapters	preceding chapters
57	Section 4.2.2	Line 6	(Table 4.2, page 65)	(Section 4.3 and Table 4.2, page 65)
61	Paragraph 2	Line 4	(Section cross ref)	(Section 5.2.3)

Page	Location		Change	
			From	To
63	Paragraph 2	Line 7	that was compatible	that were compatible
65	Table 4.2	Note 2	used is indicated	used are indicated
67	Last paragraph	Line 6	Know amounts of	Known amounts of
69	Footnote 4	–	–	Place on page 68
70	Section 4.4.2.3	Line 1	series of reaction	series of reactions
70	Section 4.4.2.3	Line 2	used by the one of	used by one of
80	Paragraph 2	Line 5	to conducted	to conduct
81	Paragraph 1	Line 3	input and output rations	input and output ratios
91	Section 5.2.2	Line 1	(Section 4.2.1)	(Section 3.2.3, page 57)
92	Section 5.2.4	Line 6	buffer incubating	buffer by incubating
95	Figure 5.3	Legend	bands are indicated	bands in base pairs are indicated
96	Paragraph 2	Line 8	bands to quantitated	bands to be quantitated
105	Table 6.1	Line 3	Age median	Age median in years
109	Figure 6.2	Legend	–	Place on page 108
113	Section 6.5	Line 4	3 months period	3 month period
114	Figure 6.5	Legend line 1	post –pre-intervention	post-intervention minus pre-intervention

Page	Location		Change	
			From	To
114	Figure 6.5	Legend line 4	one of theses latter	one of these latter
131	Table 7.1	Line 3	Age median	Age median in years
133	Section 7.2.1	Line 2	from 70 and BAL cells from 75	from 81 and BAL cells from 89
142	Paragraph 2	Line 3	theses studies	these studies

This thesis uses the more recent fashion of not italicising species names and restriction enzyme names.

May, 2002

LIST OF CHAPTERS

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DECLARATION

This thesis contains no material that has been accepted for the award of any other degree or diploma in any university or other institution. To the best of my knowledge, this thesis contains no material previously published or written by another person except where due reference is made in the text.

Eric M Glare

ACKNOWLEDGMENTS

The saying, "don't fix it if it isn't broken", is often quoted in relation to scientific research. Unfortunately, frequently in research there are also often insufficient funds, time and the will to test whether techniques actually work as they are assumed. I have experienced the privilege of conducting a PhD project with a research group that has believed in thorough optimisation and validation of methodology, with a focus on the future and the search for truth rather than short-term gains.

I am indebt to my PhD supervisor, Prof E Haydn Walters, not only for his guidance but also for his willingness to work with me as a partnership to develop the methodology and its subsequent application to lung samples in this thesis. My project was conducted in parallel with that of my colleague, Maja Divjak (PhD submitted), who has focused on in situ hybridisation techniques. It was a great honour to be Maja's scientific partner and confidant. I am also grateful for the support of my co-workers Dr Chris Ward, Dr Anna Matyi-Toth, Dr Ling Zheng, Dr A Tom C Kotsimbos and Dr Daphne Vogiagis, for their interest in my work and their many discussions of my results. The statistical analyses in this thesis were complex and were conducted in conjunction with Michael J Bailey, Biostatistician and Senior Lecturer, Department of Epidemiology and Preventive Medicine, Monash University. For technical support and sample reception and storage, I am appreciative of Bernadette Orsida, Tiffany Bamford, Michael Pais and Bryce Feltis. I thank Ros Bish, RN, Sally Gollant, RN, Dr Frank Thien, Dr Xun Li and Dr David Reid for patient recruitment, bronchoscopy and clinical assistance in general. Computer support was provided by Dr Ian Sloan.

At a personal level, I am grateful for the full support of my partner, Karl Bryant. This PhD thesis would not have been completed without his emotional and personal support.

STYLE OF THIS THESIS

The work in this thesis followed a linear development of the technology of competitive PCR and its subsequent application to a cross-sectional study. To try and make it easier to read I have not used a traditional thesis style but instead have used more of a typical book chapter format. This means that the review of the literature relevant to the thesis is divided between an introductory chapter and in-context details appearing within the respective chapters. Likewise, each chapter has its own materials and methods section as well as discussion sections relevant to that chapter. Lists of abbreviations, figures and tables are provided in appendices.

ABSTRACT

The inflammatory process in asthmatic's airways, including infiltration of the mucosa by eosinophils and activated T cells, is thought to be orchestrated by a Th2-type response. Th2 responses are characterised by elevated expression of Interleukin-4 (IL-4) and IL-5 but not Interferon-γ (IFN-γ). IL-2 promotes T cell activation and proliferation and may also be upregulated with asthma. Studies have been conducted in the past decade to quantitate the in vivo expression of these cytokines to confirm the nature of the response and the relative importance of Th2 cytokine expression. Only two such studies have utilised RT-PCR technology and would be characterised as semi-quantitative as they have relied on the use of housekeeping gene expression to normalise the data for variation introduced by sample processing and quantitation. This methodology is reliant on the assumption that housekeeping gene expression is constant in all samples under investigation, even though it has been suggested for a decade that the expression of the two most widely used genes, β-Actin and GAPDH, are likely to be sensitive to cellular activation and proliferation. In addition, quantitation of IL-4 expression is likely to have been confounded by the inadvertent co-detection of an alternatively spliced IL-4 mRNA, IL-4δ2, encoding a receptor antagonist. The aim of this project was to develop competitive RT-PCR assays for IL-2, IL-4, IL-4δ2, IL-5, IFN-γ, β-Actin and GAPDH and to examine their utility by application to a cross-section series of bronchoalveolar lavage (BAL) cells and airway biopsies from asthmatics and control subjects. GAPDH and β-Actin were found not to be useful for conventional normalisation as their expression was down-regulated with asthma and up-regulated with inhaled corticosteroid ICS use. Strong positive correlations between IL-4 and IL-4δ2 suggest coordinated regulation and a role for IL-4δ2 in modulating Th2 responses. Even in the context of no significant BAL eosinophilia, small differences in IL-5 and IL-2 expression were detected, suggesting that competitive RT-PCR is quite sensitive for quantitating small differences in gene expression.

1 ASTHMA AND THE QUANTITATION OF GENE EXPRESSION

1.1 GENE EXPRESSION AND ITS MEASUREMENT

Gene expression involves four stages: 1) transcription, where chromatin decondenses allowing transcription factors and RNA polymerase II to bind to the genomic sequence initiating synthesis of a primary RNA transcript; 2) processing of the primary transcript, where intron sequences are spliced out and the mature mRNA is transported from the nucleus to the cytoplasm; 3) translation from mRNA that is complexed with ribosomes, producing a polypeptide chain; and 4) post-translational folding and modification of the protein producing the final gene product (Figure 1.1) [1]. Each stage has multiple internal steps that are subject to regulation as well as the final removal of RNA or protein by degradation. Rates of gene expression can be determined by measuring the amount of accumulated mRNA and protein. Whilst quantitation of mRNA and protein may allow distinction of regulation before or after translation, further studies are required to determine whether synthesis of mRNA or protein, respectively, have changed or if their removal by degradation has changed.

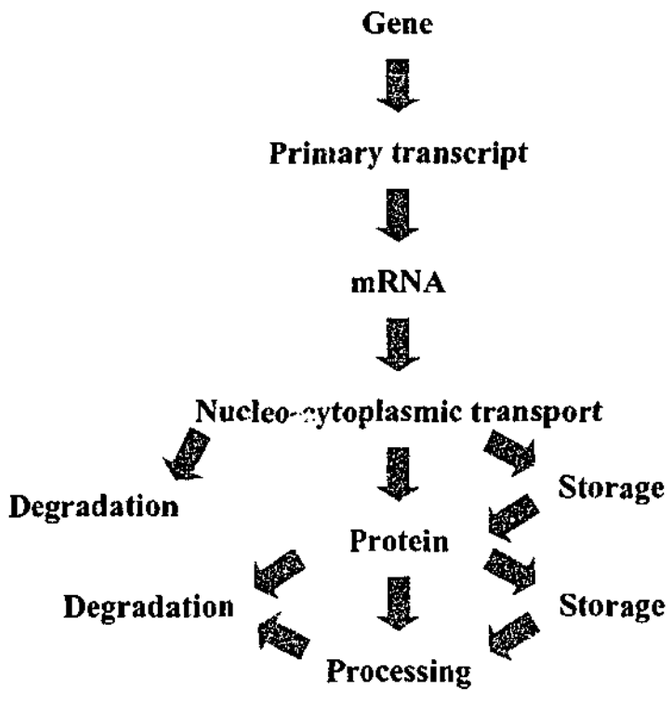

Figure 1.1 The process of gene expression

This thesis focuses on the measurement of mRNA to determine levels of gene expression. mRNA can be assessed by four key strategies: 1) Northern or dot blot hybridisation; 2) nuclease mapping; 3) reverse transcription followed by PCR (RT-PCR); and 4) in situ hybridisation. Blot hybridisations involve binding of RNA, either directly or after electrophoresis, to a membrane support. Specific mRNAs are then detected by hybridisation of nucleic acid probes. The strategy of in situ hybridisation is similar except that probes are applied directly to cells or tissue slices, allowing expression to be associated with morphology. In nuclease mapping strategies, specific probes are hybridised to mRNA forming a homoduplex and then excess probe and RNA are digested with a single strand-specific nuclease allowing the amount of homoduplex to be quantitated. In RT-PCR, RNA is reverse transcribed to cDNA and then gene-specific primers facilitate amplification of a portion of the mRNA sequence by successive cycles of denaturation, annealing and elongation. The exquisite sensitivity of RT-PCR has made the strategy an attractive one where the amount of sample is limiting and/or the level of mRNA expression is low. The challenge has been to harness the exponential amplification of PCR to produce a quantitative assay.

1.1.1 Quantification by PCR

The accumulation of PCR products (N_n) after n cycles of amplification can be described mathematically:

$$N_n = N_0(1+E)^n$$

Equation 1.1

where N_0 is the starting number of molecules of template and E is the efficiency of amplification expressed as a fraction between 0 an 1 [2]. The efficiency of amplification is dependent on the conditions of cycling, the quality of template, the sequence composition of the template and the primer sequences themselves.

The exponential amplification of PCR products is limited, however, with synthesis entering a saturation or plateau phase in later cycles (Figure 1.2). The cycle at which reactions enter the saturation phase is dependent on the initial concentration of template and primer-specific factors of the PCR. The saturation phase is usually thought to be the result of the build up of inhibitors such as pyrophosphate and

excess PCR product, and/or the exhaustion of reaction components including primers, nucleotides, and enzyme. Attempts to confirm any of these factors as being the cause of the saturation phase have failed to do so [3].

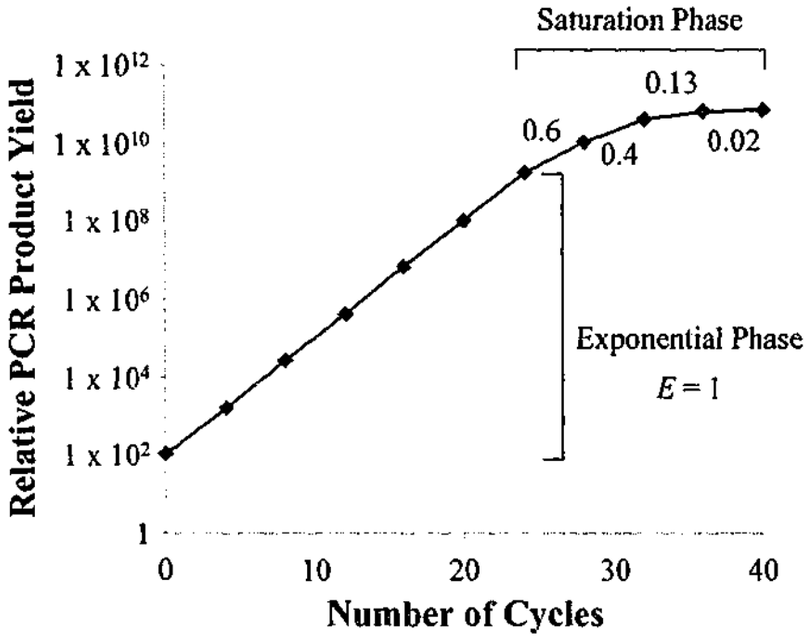

Figure 1.2 Characteristic kinetics of PCR amplification

Simulation of the synthesis of PCR products during exponential amplification and saturation phase using Equation 1.1. The initial input (N_0) was 100 and the efficiency of amplification (E) over the exponential phase was 1. To achieve the flattening of the curve characteristic of the saturation phase, the efficiency was decreased every 4 cycles. The value of E used for each segment of the saturation phase is indicated on the curve.

During the saturation phase, the amplification efficiency drops dramatically. In the simulated example (Figure 1.2), the efficiency of amplification was decreased over the saturation phase from 100 % at 24 cycles to 2 % at 40 cycles (E = 1 to 0.02). PCR amplification into the saturation phase allows reactions commencing with less template to catch up with reactions with larger inputs such that inputs that differ by several logs will have indistinguishable yields of PCR product. The intrinsic variability of PCR amplification results in a degree of uncertainty as to when reactions will enter the saturation phase. This uncertainty prevents the use of the

linear relationship between initial template (N_0) and the amplified PCR product (N_n) predicted by Equation 1.1 for quantitative analysis of nucleic acids. Protocols based on this relationship are semi-quantitative or qualitative in nature.

In attempts to achieve a quantitative assay, two main strategies have been utilised: 1) amplification of an external standard curve and 2) co-amplification of a competitive internal standard control. In both strategies, it is imperative that the same primer sites as the gene of interest are used for the control template so that both templates are amplified with similar efficiency.

Small inherent changes in the amplification efficiency, particularly in early cycles, are amplified exponentially in subsequent cycles, compromising the ability to quantitate. The distinct disadvantage of the external standard curve strategy is that tube-to-tube variations are not controlled for and thus, the technique is often considered semi-quantitative. In internal control strategies, variations in the quality of DNA or cDNA from different test samples, such as concentrations of salt impurities, affect the amplification of both native and competitor templates in an identical and competitive fashion, allowing quantitative detection of the gene of interest.

1.1.2 Competitive PCR

The internal control of PCR amplification by co-amplification of native and exogenous competitor templates requires that the templates contain identical primer sites and that the generated PCR products be distinguishable. Competitors may be DNA fragments, or alternatively, RNA molecules can be used to conduct competitive RT-PCR where both reverse transcription and PCR are controlled (Figure 1.3) [4, 5].

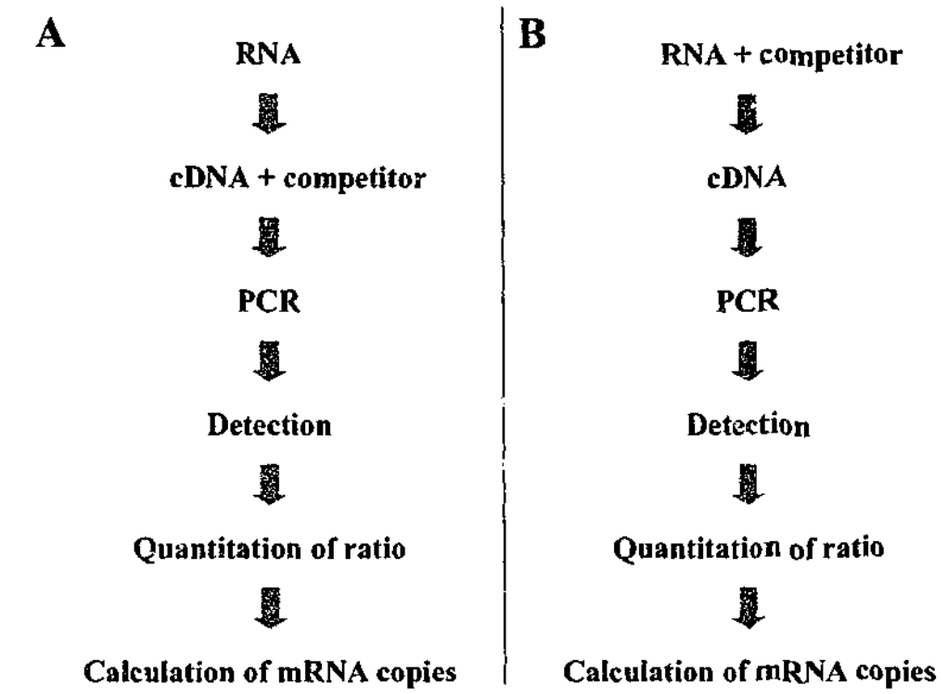

Figure 1.3 Quantification of mRNA by competitive PCR

Flow diagrams showing the process of mRNA quantitation by RT-competitive PCR using a DNA competitor molecule (A) and competitive RT-PCR using a RNA competitor (B).

Both homologous and heterologous templates have been utilised [6]. Homologous competitors are usually obtained by mutagenesis and include fragments that differ by the presence of a restriction enzyme site, fragments that have insertions, such as genomic intron sequences and molecules that contain deletions. Heterologous competitors, where the specific primers flank a completely different intervening sequence, may be obtained by ligation of primer sequences to a DNA fragment or the primers may be added during PCR using composite primers. In the latter strategy, the composite primers may also contain a bacteriophage RNA polymerase promoter site for in vitro transcription of a complementary RNA (cRNA) for competitive RT-PCR [7]. Oligonucleotide synthesis is an alternative approach to obtain a competitor template with larger molecules being produced by overlapping fragments [8].

The disadvantage of homologous competitors is that they may form heteroduplexes with the native template detracting from the ability to quantitate the native and competitor templates separately [5, 9, 10]. The difference in the intervening sequence between heterologous competitor and corresponding native template may cause the two PCR products to be amplified at different rates [5]. These potential problems emphasise the need to conduct validation studies to confirm the quantitative nature of the competition between native and competitor templates in any newly established assay. Well-designed validation studies should also define the best parameters for quantitating the PCR products as well as define the quantitative range of the assay [11]. To achieve quantitative competition between competitor and native templates, it is imperative that input ratios across the quantitative range retain their relative relationship to each other after PCR amplification, detection and quantitation of the products [12].

1.2 GENE EXPRESSION IN ASTHMA

The prominent clinico-physiological characteristics of asthma are typical symptoms of episodic chest tightness, wheeze and cough, accompanied by reversible airflow obstruction and increased bronchial hyperreactivity to non-specific stimuli. Asthma is characterised pathologically by an inflammatory process within the airways. In chronically inflamed asthmatic airways, the mucosa is infiltrated by eosinophils and activated T cells. Tissue damage in asthma is believed to be due, at least in part, to the release of toxic granule proteins from activated eosinophils in response to cytokines liberated from inflammatory cells (Figure 1.4). The cross-linking of surface-bound IgE on mast cells leads to the release of mediators that are also thought to contribute to asthma symptoms, particularly the acute response to exposure to antigen.

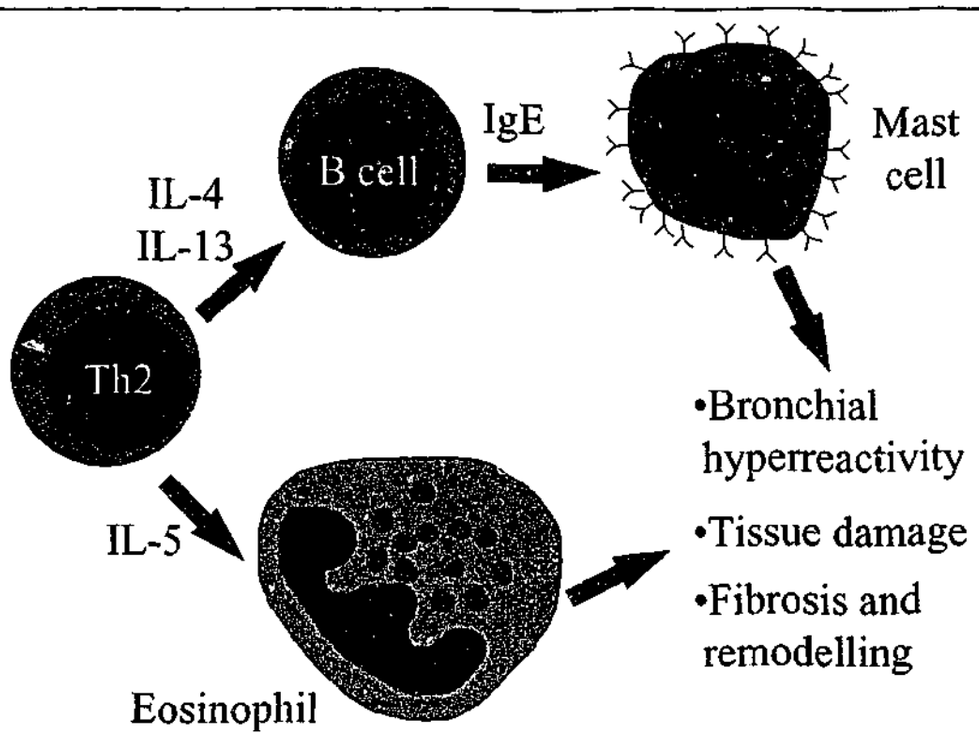

Figure 1.4 The pathophysiology of asthma is the result of a Th2 response

T cells play a central role in controlling immune responses. Th2 cells, characterised by expression of IL-4, IL-5, IL-6 and IL-10, control humoral responses including IgE production and eosinophilia in allergic reactions (Figure 1.5) [13, 14]. In contrast, Th1 cells express IFN-γ and TNF-β and characteristically induce cell-mediated immunity where activated macrophages mediate delayed-type hypersensitivity responses and the killing of intracellular microorganisms. Th1 and Th2 cells cross-regulate the activity of each other. IFN-γ expression, for example, stimulates the expansion of Th1 cells but inhibits Th2 cells whilst IL-4 inhibits cytokine production and proliferation of Th1 cells and promotes Th2 responses [14, 15].

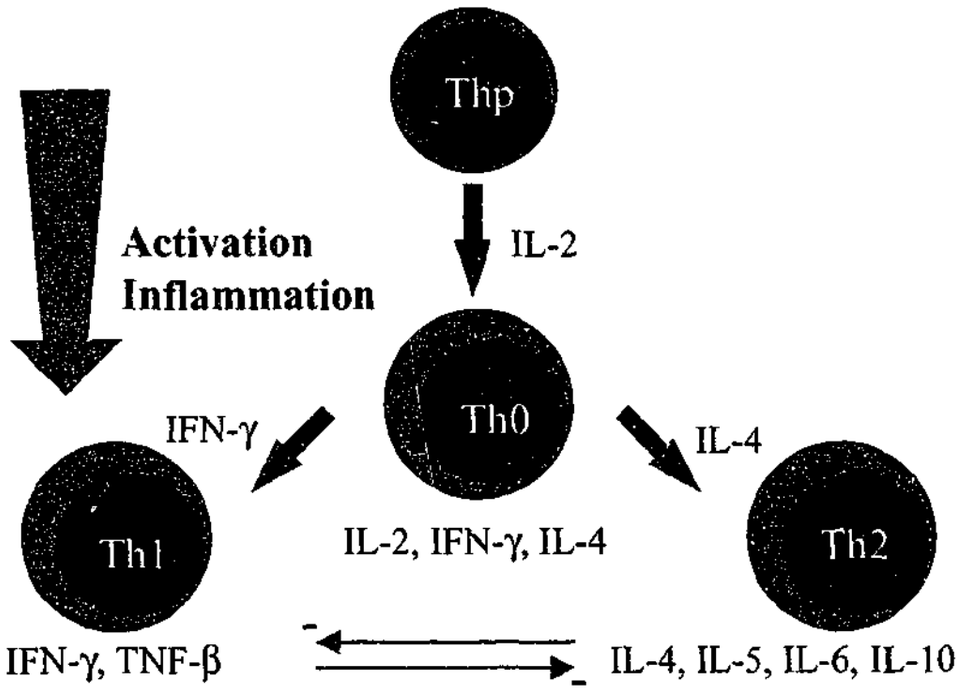

Figure 1.5 Th cell activation and Th1 and Th2 responses

The concept of Th1 and Th2 cells came from studies in mice where resting or precursor T cells, producing only IL-2, appear to pass through an intermediate Th0 stage (with IL-2, IL-4 and IFN-γ production), before becoming polarised into Th1 or Th2 cells (Figure 1.5). The most common difference found in human studies is IL-2 expression concomitant with IL-4 or IL-5, whilst in mice IL-2 production in activated T cells is usually confined to Th1 cells [16]. Additionally, Th2-like T cells may also express significant amounts of IFN-γ with or without IL-4 [17, 18].

Inhaled corticosteroids (ICS) are the mainstay in the clinical management of asthma and are thought to reduce inflammation by acting especially on cytokine expression. The expression of the Th2-type cytokines Interleukin-5 (IL-5) and IL-4 have been particularly implicated as important in the pathogenesis of asthma [reviewed 19]. IL-5 promotes the differentiation of committed eosinophil precursors and prolongs their survival (Figure 1.4) [13]. Eosinophils are selectively activated by IL-5, inducing pro-inflammatory effector functions, including degranulation [20]. IL-4 may also promote eosinophilia by increasing the expression of VCAM-1, an adhesion molecule involved in selective eosinophil recruitment [21]. IL-4 induces isotype

switching in B cells to IgE production [22]. IL-4 expression further regulates atopic responses by stimulating the proliferation and activation of B cells and mast cells [13, 14].

1.2.1 Human in vivo studies of cytokine expression

Animal models and in vitro studies of cellular responses to antigen have implicated Th2 cytokines in the pathogenesis of asthma. A range of strategies have been implemented over the past decade to determine the importance of Th2 cytokine expression in vivo in human asthma. Available samples include bronchoalveolar lavage (BAL) and endobronchial biopsies obtained at bronchoscopy, blood and induced sputum.

The induction of sputum is the least invasive strategy but has suffered from lack of standardisation and variability in the degree of sampling of bronchi and nasopharynx [23-25]. Nevertheless, studies of cytokine gene expression have been conducted. A qualitative RT-PCR technique was able to detect IL-5 mRNA after two successive rounds of 35 cycles of amplification of sputum-derived RNA [26]. Samples from asthmatics were more likely to be positive than atopic controls, who in turn had a higher frequency of detection of IL-5 mRNA than nonatopic subjects. In another study, in situ hybridisation of cytospun aliquots of sputum showed up regulation of IL-4 and IL-5 in asthmatics compared to control patients [27]. In co-localisation studies, IL-4 and IL-5 mRNA was predominantly expressed by $CD3^+$ T cells.

The analysis of blood samples, particularly peripheral blood mononuclear cell (PBMC) and $CD4^+$ T cell fractions, have been useful in detecting differences in gene expression between asthmatics and normal controls [for example 28, 29-32]. The levels of cytokines such as IL-5 released spontaneously in culture are quite low and often below the sensitivity of ELISA (enzyme-linked immunosorbent assay), even in atopic asthmatics [28, 29]. Similar sensitivity problems have been encountered in in situ hybridisation [31] and PCR-based studies [32, 33]. Nevertheless, all the quantitative studies conducted from blood samples have found IL-5 gene expression to be upregulated with asthma in comparison to non-asthmatic samples (Table 1.1).

Table 1.1 In vivo gene expression studies of Th cytokines in asthma [1]

Sample Type	Method [2]	Ref	Subject Groups [3]	IL-5	IL-4	IFN-γ	IL-2
PBMC [4]	ELISA, Bioassay	[29]	AA, ANA, Normals	↑ Asthma		↔	↔
PBMC [4]	ELISA	[28]	AA, NAA, ANA, Normals	↑ Asthma		↔	
Blood T cells [4]	ELISA, Bioassay	[30]	AA, NAA, Normals	↑ Asthma	↑ AA [5]	↓ Asthma	↔
Blood $CD4^+$ T cells	ISH	[31]	Asthmatics, Controls	↑ Asthma	↑ Asthma	↔	↔
PBMC	ISH	[34]	Asthmatics, Controls	↑ Asthma	↑ Asthma	↔	↑ Asthma
PBMC	RT-PCR	[32]	Asthmatics, Controls	↑ Asthma	↑ Atopy	↔	↔
BAL fluid [6]	ELISA, Bioassay	[30]	AA, NAA, Normals	↑ Asthma	↑ AA [5]	↔	↑ NAA [7]
BAL cells	ELISA	[28]	AA, NAA, ANA, Normals	↑ Asthma		↓ NAA [7]	
BAL cells	ISH	[35]	AA, Normals	↑ AA	↑ AA	↔	↑ AA
BAL cells	ISH	[36]	AA, Controls	↑ AA	↑ AA	↔	↑ AA
Airway biopsies	ISH	[36]	AA, Controls	↑ AA	↑ AA	↓ AA	↔
Airway biopsies	ISH	[37]	AA, NAA, ANA, Normals	↑ Asthma	↑ Asthma		
Airway biopsies	IHC	[37]	AA, NAA, ANA, Normals	↑ Asthma	↑ Asthma		
Airway biopsies	RT-PCR	[37]	AA, NAA, ANA, Normals	↑ AA	↑ AA ↑ Atopy		
Airway biopsies	ISH	[38]	Asthmatics, Controls [8]	↑ Asthma	↑ Asthma	↔	↔
Airway biopsies	IHC	[39]	AA, Normals	↔	↑ AA		

[1] ↑, significantly increased with; ↓, significantly decreased with; ↔, no significant difference between the subject groups. Only quantitative studies are included in the table. Multiple methods in the same study are entered as separate studies for convenience (refer to reference number (Ref)).

[2] IHC, immunohistochemistry; ISH, in situ hybridisation

[3] AA, atopic asthmatics; NAA, non-atopic asthmatics; ANA, atopic non-asthmatics; Normals, control patients with out a history of clinically-defined atopy or asthma; Asthmatics, asthmatics with or with out atopy, usually mostly atopic; Controls, patients with out asthma but with or with out atopy.

[4] Spontaneous release measured after unstimulated culture

[5] AA, but not NAA, were significantly different from control groups.

[6] Concentrated supernatant of BAL

[7] NAA, but not AA, were significantly different from Normals.

[8] Atopy was not determined in this study.

Due to the sensitivity problems associated with blood samples, most studies have focussed on mitogen- or antigen-stimulated cytokine levels [40-43], with such data reflecting the ability of the stimuli to induce de novo cytokine expression rather than in vivo cytokine levels. However, as cytokines are primarily local messengers, the ability of blood samples to reflect inflammatory events in the lung in situations other than acute episodes of asthma is limited [44]. For these reasons this study, as well as a number by other groups and my own group, have focussed on samples collected at bronchoscopy.

In keeping with the results from blood samples, IL-5 gene expression has been shown to be elevated in BAL fluid, BAL cells and airway biopsies in a total of nine studies (Table 1.1). However, a study of atopic asthmatics failed to show a significant increase in IL-5 immunohistochemistry staining compared to normal patients [39]. The lack of statistical significance in this study was most likely due to the sensitivity of the technique as IL-5 was detected in only about half of the samples.

In vitro studies of the function of IL-4 have predicted that IL-4 might be upregulated in vivo with both atopy and asthma due to its role in mediating IgE responses and stimulating eosinophil adhesion, respectively. The majority of in vivo studies have either only looked at atopic asthmatics or have combined small numbers of both atopic and non-atopic patients in their asthmatic and control groups (Table 1.1), allowing the potential for atopy and asthma to confound the observations. Within this context, all the quantitative studies of IL-4 expression in vivo have found IL-4 upregulation with atopy and/or asthma. Whilst asthma and atopy have been shown to be associated with increased IL-4 independently of each other, three studies have shown significantly increased expression in atopic asthmatics but not non-atopic asthmatics in comparison to control samples, suggesting that atopic asthma is associated with additive amounts of IL-4 expression [30, 37].

In most studies of asthma and atopy, significant differences in IFN-γ and IL-2 expression have not been found (Table 1.1). However, IFN-γ protein, measured by ELISA in a study of blood T cells [30] and IFN-γ mRNA in biopsies, measured by in situ hybridisation [36], have been found to be decreased with asthma. In a third

study, IFN-γ expression was found to be decreased in non-atopic asthmatics compared to both atopic asthmatics and the control groups [28]. Similar inconsistencies have been found with IL-2 expression with six studies not finding any significant differences and four studies finding an increase in expression with asthma (Table 1.1). In common with the IFN-γ measurements, one of these studies found IL-2 in BAL fluid to be increased in non-atopic asthmatics but not atopic asthmatics compared to normal controls [30]. These inconsistencies in detection of differences in IFN-γ and IL-2 expression reflect the interaction between variations in method resolution, disease severity and the intrinsic biological variability of human subjects. In the latter mentioned anomalous result of IL-2 expression, the levels of IL-2 in most of the normal and atopic asthmatic BAL fluid samples were below the detection limit of the assay [30] and so a spurious result was likely.

1.2.2 PCR methodology in asthma

During the course of this PhD project since 1993, the published literature of PCR methodology in asthma has shown a slow process of development. The PCR methods have all been published somewhat earlier even in laboratory manuals [45]. The development has reflected the growing sophistication of journal readers and an increasing appreciation of the short-comings of simpler methodologies.

Qualitative RT-PCR methods, where straight forward reactions are scored for positivity, have shown that cytokine mRNA can be detected with some qualitative differences in sputum samples [26], blood samples [33], BAL cells [46, 47] and airway biopsies [33]. The amount of sample input [46] and the number of cycles of PCR amplification [26, 33] have been manipulated in attempts to reveal more differences between asthmatics and normal controls. The simplicity of this method has allowed a large number of cytokines to be analysed from the same set of samples. For example, Gelder, et al, looked at 13 cytokines in their study of 31 sputum samples from asthmatics, atopics and normal controls [26].

To avoid scoring false negative samples, where there may have been a failure in RNA extraction or cDNA synthesis, the detection of β-Actin housekeeping gene expression has been used as a positive control [26, 33, 47]. The use of such a

positive control does not preclude false negatives where only the cytokine reaction has failed and at best is a crude indicator of the success of RNA extraction and cDNA synthesis.

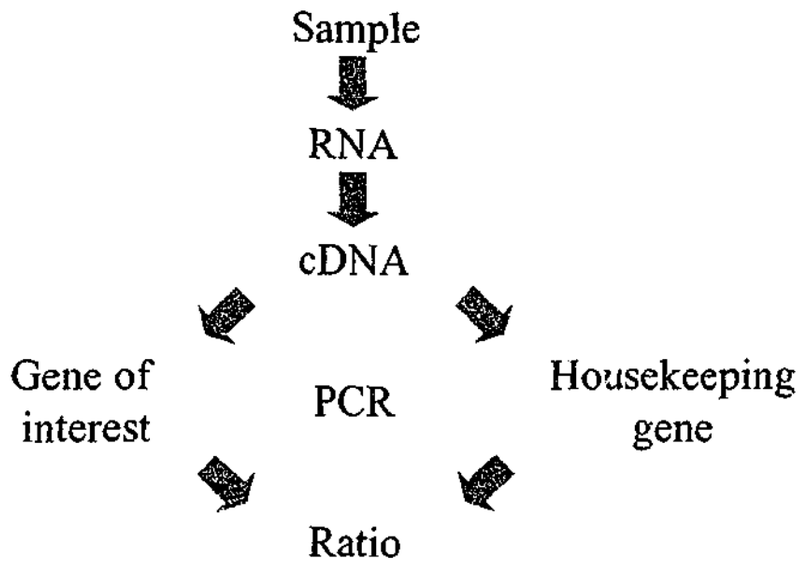

Figure 1.6 Semi-quantitative PCR using housekeeping gene expression.

In this method, PCR amplification of the gene of interest is conducted in parallel with that of a housekeeping gene, such as β-Actin. The products of the two PCR are quantitated and the ratio is calculated to normalise the data of the gene of interest with respect to the housekeeping gene.

To achieve a semi-quantitative assay researchers have quantitated both the cytokine and the housekeeping gene PCR products and expressed the result as a ratio (Figure 1.6) [32, 43, 48, 49]. This type of assay requires that the PCR products be sampled during exponential amplification where the relationship between different amounts in different tubes is maintained. The exponential phase can be difficult to determine (Section 1.1.1) and the appropriate cycle number is most eloquently determined by amplification of a dilution series of positive control template that encompasses the likely range of input cDNA [for example 32]. The use of housekeeping gene expression aims to control for the variation introduced in the steps from sample processing through to PCR amplification. The validity of the semi-quantitative method is dependent on the assumption that housekeeping gene expression remains

constant between all samples under analysis. Whilst housekeeping gene expression is required for the survival of every cell this does not mean that their expression is not regulated. Furthermore, different cell types are likely to have different requirements for housekeeping gene activity (see Section 6).

The strategy of expressing results as ratios with housekeeping genes does not control for batch-to-batch or tube-to-tube variations in PCR amplification detection and band quantitation. In this method, all samples in a given study must be processed in a single batch. Typically, blot hybridisation followed by X-ray film exposure have been used as the detection method. The labelling of probes and the exposure of X-ray film are particularly variable steps [50].

To [illegible]ome batch variations and to allow the results to be related directly to starting amounts, external standard curves have been constructed from serial dilutions of a know positive control for each gene including the housekeeping gene [37, 51-53]. The use of standard curves assumes that the positive control template, usually plasmid DNA, is amplified with the same efficiency as the sample cDNA even though they differ in nature. The chemokine, RANTES (regulated on activation, normal T cell expressed and secreted) [51] and the cytokines, IL-13 [53], IL-4 and IL-5 [37, 52] have been reported to be upregulated in BAL cells and/or airway biopsies from asthmatics by this method.

The latter studies of asthma indicate a fundamental problem with semi-quantitative PCR methods. The published standard curves, whilst linear, show inputs covering a wide range of 10-fold dilutions that result in outputs covering only 1 or 2 Logs [37, 51-53]. For example, the β-Actin standard curve in one study covered eight 10-fold serial dilutions that were detected within 1 Log of arbitrary units from densitometry of X-ray film [37]. The phenomenon appears to be somewhat protocol dependent as the standard curves from another group show far less compaction of the output with respect to the input [51]. A wide semi-quantitative range is being achieved at the expense of resolution. A small variation in the measurement of the density of a band will result in a large change in the predicted input amount.

The wide quantitative range is necessary because the amount of RNA extracted from the patient samples is not usually quantitated and so the input into the assay may vary considerably. In the former mentioned study, the predicted inputs of β-Actin covered a range of approximately 10^5 [37], whils. ß-Actin expression is assume to be constant between their samples. It is likely that the RNA extraction and/or the subsequent reactions of the semi-quantitative RT-PCR essentially failed in some cases and were relatively successful for other samples. Lack of RNA quality and quantity control must increase the variability and reduce the resolution of the method.

Unlike competitive PCR assays, tube-to-tube variation is not controlled in semi-quantitative RT-PCR methods. Recognising the limitations of qualitative RT-PCR, the group of Huang has developed competitive RT-PCR for IL-4, IL-5, IL-13 and IFN-γ mRNA in PBMC and BAL cells [47, 54, 55]. A radioactive tracer was incorporated during PCR amplification allowing bands separated in a gel to be measured after excision. As part of the method development, they measured IL-4, IL-5 and IFN-γ mRNA in BAL cells taken from up to three patients before and after segmental lung allergen challenge [47, 55]. Cytokine mRNA could also be measured in stimulated PBMC (n = 1) and in eosinophilic and mononuclear cell fractions of BAL cells (n = 1). Despite positive indications for the usefulness of the technique and the considerable resources expended to develop such assays, no further studies using competitive PCR technology have been published by this group. Instead, they have published several in vivo and in vitro studies of antigen stimulation models using semi-quantitative RT-PCR [56-58].

1.3 AIMS OF THIS THESIS

The primary aim of this PhD project was to develop and validate methods for quantitating mRNA expression in asthma. Secondarily, I wanted to document the usefulness of the method in quantitating the expression of T cell cytokines though to be central to the control of inflammation characteristic of asthma. The ultimate goal of this endeavour was to establish a tool with which one could determine how important the expression of these cytokines in vivo was to the pathogenesis of

asthma and to subsequently examine the effectiveness of drug treatments.

When this project was commenced in 1993 (part-time), some of the basic PCR quantitative methods had been developed but had yet to be applied to lung samples and the study of asthma. It was perceived that T cell cytokine expression in many asthmatics might be transient and not substantially different from that of atopic and normal control subjects. The difference between asthmatics and controls was likely to be least in those that were clinically mild enough to undergo sampling at bronchoscopy and in those that were using drug treatments, such as inhaled corticosteroids (ICS). This problem was likely to be further compounded by the small amount of biological material that can be obtained from volunteering patients.

Based on these insights, competitive RT-PCR was chosen for the sensitivity that might be possible through PCR amplification and the resolution that could be obtained by the highly controlled nature of the competitive assay. To fully realise the potential sensitivity of competitive PCR it was necessary to establish the conditions for high sensitivity PCR. Validation studies were conducted to determine the best conditions for competitive RT-PCR in order to maximise the quantitative ability of the assays. The assays thus developed were then applied to a cross-sectional analysis of BAL cells and biopsies from asthmatics and control subjects.

2 SUBJECTS AND SAMPLE PROCESSING

2.1 INTRODUCTION

Bronchoalveolar lavage cells and airway biopsies collected at bronchoscopy were chosen for this study of the utility of competitive RT-PCR for the quantification of T cells cytokines in asthma. This work was a sub-project of a larger program of work looking at clinical indices, physiological endpoints and cellular and activation markers of inflammation in asthma. Cytology and immunohistochemical methodologies require preserved cellular morphology and each analysis uses small numbers of cells or 7 μm slices of biopsies. In contrast, RT-PCR-based techniques require relatively large amounts of material mostly to facilitate efficient RNA extraction. Sample reception rates and the efficiency of subsequent processing have a profound impact on the ability to conduct competitive PCR assays. This chapter focuses on the extraction of RNA from BAL cells and biopsies and the effect of this step on the overall utility of competitive PCR technology in asthma research.

2.2 MATERIALS AND METHODS USED

2.2.1 Study Design

Samples for this work were obtained from a cross-sectional study of asthmatics, atopics and normal controls and an attempted longitudinal intervention study of asthmatics.

The longitudinal study was a double-blind, randomised, parallel-group, placebo-controlled study of asthmatics who were using low-dose ICS but were still symptomatic (Figure 2.1) [59]. In addition to their usual dose of ICS of up to 500 μg per day of beclomethasone diproprionate or budesonide plus inhaled salbutamol (200 μg) as needed, patients were randomised to receive placebo, 100 μg per day salmeterol or extra ICS with 200 μg/day fluticasone propionate. Bronchoscopies were conducted at randomisation and after 12 weeks of treatment intervention.

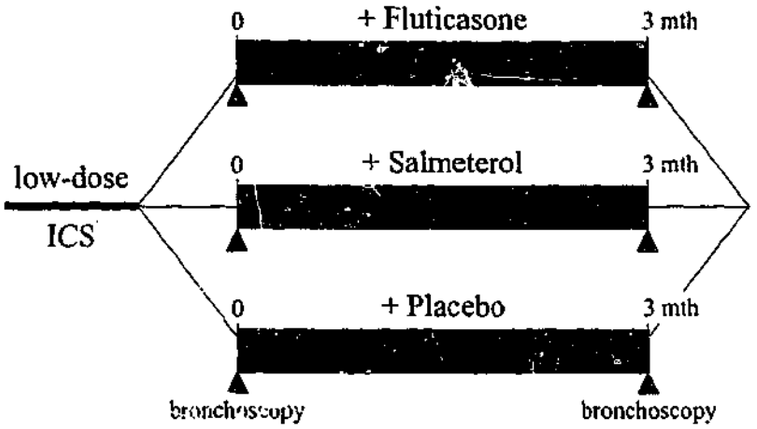

Figure 2.1 Schematic diagram of longitudinal study of supplemental treatments from which samples were obtained.

Asthmatics on low-dose ICS were randomised to receive additional ICS (fluticasone), salmeterol or placebo. Bronchoscopies were conducted at randomisation and after 3 months of intervention.

The rationale for studying patients already on low-dose ICS was to have partially treated asthmatics that had both the potential to improve and to deteriorate. At the time of the design of the study, the main interest was in whether long-acting β_2-agonists, such as salmeterol, have pro-inflammatory effects. Any anti- or pro-inflammatory action could be contrasted with the positive control arm in which the ICS use was essentially doubled with the addition of fluticasone.

2.2.2 Subjects

The demographics of subjects from which data were obtained are given within the chapters that deal with such data (Sections 6.2.1 and 7.2.1) as the numbers varied with assay type.

Asthmatic subjects fulfilled the American Thoracic Society criteria [60] for asthma with documented airway reversibility to inhaled bronchodilator and increased responsiveness to methacholine. They all used salbutamol for relief of symptoms as needed, with the majority of patients (93 of 111) using regular ICS. Salmeterol (50

μg twice daily) was used by 12 patients from whom BAL cells were obtained and in 5 from whom biopsies were taken. No patient was taking any other anti-asthma medication. Volunteers who had suffered from acute respiratory tract infection during the previous 4 weeks, who had any change in asthma medication or hospital admission due to airway disease in the 4 weeks before the study were excluded.

A total of 45 normal volunteer control subjects were also recruited. They had no history of asthma or other respiratory diseases, were using no medication, had no bronchodilator response to salbutamol and had a negative challenge test to 3.2 mg inhaled methacholine. All asthmatic and control subjects were nonsmokers and gave informed, written consent before commencing the study, which was approved by The Alfred Hospital Ethics Committee.

Atopic status was assessed by skin prick testing to a panel of 7 common aero-allergens and was defined by a response of ≥3 mm diameter to one or more. FEV_1 and bronchial responsiveness to methacholine were measured in the morning and after a bronchodilator-free period of at least 8 h (24 h for long-acting beta agonists). A rolling-seal spirometer (SensorMedics Corporation, Yorba Linda, CA, USA) was used for measurement of forced expiratory volume in 1 second (FEV_1) according to American Thoracic Society guidelines [60]. Bronchial responsiveness to methacholine was assessed by a previously established dosimeter technique [61] and expressed as the cumulative dose required to provoke a 20% decrease in FEV_1 (PD_{20}) using linear interpolation from a dose-response plot.

2.2.3 Bronchoscopy

Bronchoscopy was performed within 2 days of methacholine challenge. Subjects were premedicated with 0.4 mg intravenous atropine and 5-15 mg midazolam. Inhaled salbutamol (200 μg) was given to all 15 min before bronchoscopy. Four percent lignocaine spray was applied to the pharynx and larynx and 2% lignocaine below the vocal cords in 2 mL aliquots as required up to a maximum dose of 6 ml. Subjects were monitored using pulse oximetry and administered oxygen at 4 l/min during the procedure. BAL of the right middle lobe was performed by instilling three aliquots of 60 mL phosphate buffered saline with immediate aspiration into

siliconised glass containers. Endobronchial biopsies were taken from the segmental subcarinae of the right lower lobe of each patient using alligator forceps (Olympus, Tokyo, Japan). Samples were transported on ice to the laboratory and 2 biopsies were snap frozen in liquid nitrogen within 15 min and stored at -80 °C. BAL cells in aliquots of 1-4 x 10^6 cells were rapidly pelleted by centrifugation at 1,200 rpm for 15 min, snap frozen and stored at -80 °C.

2.2.3.1 Collection priorities for this work

Both BAL cells and endobronchial biopsies were obtained at bronchoscopy. Up to 8 attempts were made for 6 macroscopically sound biopsies. Four of the biopsies judged by an experience researcher to be most likely to yield good morphology for cytology, immunohistochemistry or in situ hybridisation were frozen in embedding medium. The remaining biopsies and tissue fragments were frozen together for quantitative PCR analysis in this study. From the 3 x 60 mL BAL conducted on the patients in this study, a median of 96 mL return (range 19 – 134 mL) and a median total cell count of 1.1 x 10^7 cells (range 2.3 x 10^6 – 3.7 x 10^7 cells) was obtained. Cytospins for differential cell counts and cell staining were the first priority for the use of BAL cells, using up to approximately 1.4 x 10^6 cells. Secondly, 2.3 x 10^6 cells were allocated for immunophenotyping by FACS before reserving 2 x 10^6 cells for this study. If more cells were available, an additional 2.4 mL or up to 6 x 10^5 cells were used to make more cytospins. Once these priorities were satisfied, more cells to a maximum of 4 x 10^6 cells were stored for quantitative PCR.

2.2.4 RNA Extraction

The work of this thesis saw the transition from a traditional phenol-guanidinium isothiocyanate solution RNA extraction to a kit-based spin-column method. Whilst the former was used in preliminary work, essentially all the data discussed in this thesis utilised the spin-column method of RNA extraction.

2.2.4.1 Traditional Method using RNAzol B Solution

Airway biopsies were homogenised with 700 µL RNAzol B (Tel-Test, Friendswood, TX, USA) and a rotor-stator homogeniser (Figure 2.2). BAL cells were lysed with 700 µL RNAzol B by aspiration 3 times through syringe and 23 gauge needle. Phase

separation was induced by the addition of 70 µL chloroform followed by incubation of ice for 15 minutes and centrifugation for 15 minutes at 12,000 g and 4 °C. RNA was precipitated from the aqueous phase with an equal volume of isopropanol, chilling on ice for 30 minutes and centrifuging for 10 minutes at 10,000 g and 4 °C. RNA pellets were washed twice with 70 % ethanol chilled to -20 °C. Excess ethanol was removed by drying for 15 minutes in a centrifugal vacuum drier. RNA was resuspended in 80 µL 10 mM Tris-HCl, pH 7.5 and stored at –80 °C.

2.2.4.2 Qiagen RNeasy Spin Columns

RNA from BAL cells and endobronchial biopsies were extracted with RNeasy Total RNA Kit (Qiagen, Hilden, Germany) (Figure 2.3). Tissue was homogenised in 700 µl lysis buffer RLT using a Polytron rotor-stator (Kinematica, Luzern, Switzerland) followed by aspiration 10 times through a 21 gauge syringe needle to shear chromosomal DNA. Cell pellets were resuspended in 700 µl lysis buffer RLT by aspiration 10 times through a 21-gauge syringe needle. RNA was eluted from spin columns with 40 µl diethylpyrocarbonate-treated water.

2.2.5 Capillary Spectrophotometry

Aliquots of 4 µl of RNA were quantitated by capillary spectrophotometry using an Absorbance Capillary Adaptor Cell (Helix, San Diego, USA) and a dual-beam Cary 1 spectrophotometer (Varian, Melbourne, VIC, Australia). The spectrophotometer was fitted with a rear beam attenuator, which allowed the rear control beam to be blocked by a similar amount as the attenuation of the reading beam by the capillary cuvette. Measurements were taken with Varian RNA/DNA Application Version 3 software with background correction at 320 nm (A_{320}), a slit band width of 2 nm and dwell time of 2 seconds. Samples with $(A_{260}-A_{320})/(A_{280}-A_{320})$ ratios less than 1.7 and/or yields less than 0.5 µg total RNA were excluded from subsequent analysis.

2.3 RNA EXTRACTION

RNA is notoriously susceptible to degradation due to both its chemical structure and the ubiquitous presence of endogenous ribonucleases (RNases). The purity and integrity of isolated RNA is critical for its effective use in subsequent procedures such as quantitative RT-PCR. Extraction of RNA from samples requires four steps be performed: 1) cellular lysis and disruption; 2) inactivation of endogenous RNases; 3) denaturation of nucleoprotein complexes; and 4) purification of RNA away from contaminating cellular components, particularly DNA and protein. Decompartmentalisation of cells during lysis potentially exposes RNA to cellular RNases. RNases, in contrast to deoxyribonucleases (DNases), are difficult to inactivate, do not require cofactors and are heat stable [62]. The majority of RNA extraction protocols utilise chaotrophic salts (chaos-causing denaturants) of guanidinium, including guanidinium hydrochloride and guanidinium isothiocyanate, as they are some of the strongest inhibitors of RNases. Guanidinium isothiocyanate and phenol pose considerable health risks to laboratory workers through skin contact and inhalation. In addition to consideration of prevention of infection from patient samples whilst extracting RNA, aerosol and fume protection from the salts and solvents is also required.

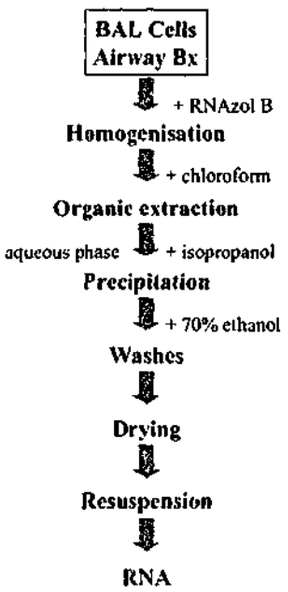

Figure 2.2 Extraction of RNA with RNAzol B Solution

The work of this thesis began with the use of a standard RNA extraction protocol utilising a guanidinium isothiocyanate-phenol solution for cellular lysis and denaturation [63]. Both in house and commercial solutions (RNAzol B, Tel-Test, Friendswood, Tx, USA) were used. The guanidinium isothiocyanate-phenol solution facilitates cell lysis and denaturation of proteins including RNases (Figure 2.2). Chloroform was then added to induce phase separation between the organic solvents and the salt solution. RNA selectively partitions into the aqueous phase free from most proteins and cellular DNA. The RNA was then concentrated by alcohol precipitation and the resulting pellet is washed to remove co-precipitating salts.

In 1995, a RNA extraction kit based on a silica gel matrix which has an affinity for nucleic acids became available. The RNeasy Mini Kits (Qiagen, Clifton Hill, Vic, Australia) are designed to isolate up to 100 µg of total RNA from small quantities of animal cells (up to 1 x 10^7 cells) and tissues (up to 30 mg), bacteria and yeast. Tissue or cells are lysed and homogenised in a guanidinium isothiocyanate high-salt buffer (Figure 2.3). Ethanol is added before applying the lysate to a spin column containing a silica-gel-based matrix. RNA molecules greater than 200 nucleotides bind quantitatively to the column allowing contaminants to be washed away. After the washes, the column is spun dry allowing the purified RNA to be eluted with 40 µL of water.

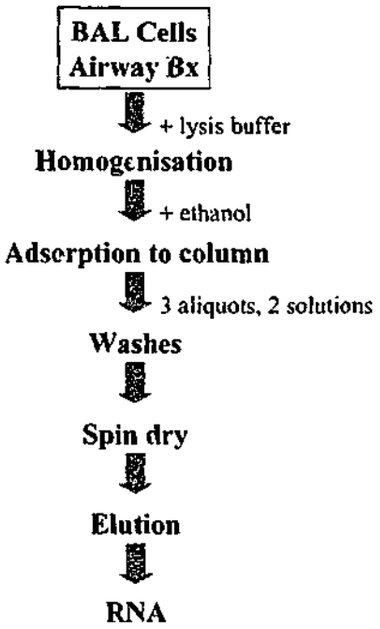

Figure 2.3 Extraction of RNA with Qiagen RNeasy Spin Columns

A comparison of the two RNA extraction techniques is given in Table 2.1. The use of a kit for RNA extraction had the clear advantages of reduced RNase and PCR product contamination potential and superior protocol development and support. The relatively low skill level required and the higher throughput of the affinity matrix spin columns directly contribute to the quality of RNA extractions which in turn determines the ability to conduct patient studies (Section 2.5).

Table 2.1 Comparison of RNA Extraction Methods

	RNeasy Mini Kit	RNAzol B
Provided	Solutions, columns, tubes	1 key solution
Not supplied	Ethanol	Tubes, chloroform, isopropanol, ethanol, water
Lysing and denaturing agents	Guanidinium isothiocyanate, β-mercaptoethanol	Guanidinium isothiocyanate, phenol
Purification by	Washing of affinity matrix	Phenol-chloroform extraction, alcohol precipitation, washing pellet
RNA concentrated by	Binding to affinity matrix	Alcohol precipitation
Scale[1]	1 microfuge tube; multiple loadings of column possible	At least 2 microfuge tubes per sample
Temperatures used	All at room temperature	Room temperature; 4 °C centrifugations; 4 °C and –20 °C incubations
Skill required	Low	High
Throughput[2]	≤ 24	≤ 12
Genomic DNA contamination	Problematic in biopsies (Section 6.3)[3]	Low; acidic lysis shears genomic DNA
Largest factor limiting success rate	Sample dilution of lysate reduces RNA binding to column	Washing RNA pellet will retain salts or discard RNA in a proportion of extractions
Solution for large sample volume[4]	Replace 70 % ethanol with 100 % before loading column	Increase scale of extraction
Dilute lysates	Concentrated by multiple loading to column	Reduced efficiency of RNA precipitation

[1] Minimum size limited by sample volume and/or minimum homogeniser volume.

[2] Recommended maximum number of samples if all samples are of the same size and type and the operator has a recent history of having done the procedure.

[3] Recently introduced optional DNase digestion steps should eliminate this problem.

[4] For example, from variable volume of PBS retained with biopsies or cell pellets at storage.

2.4 CAPILLARY SPECTROPHOTOMETRY OF RNA

Spectrophotometric quantification of nucleic acids is based on the amount of light of specific wavelengths absorbed by pure nucleic acids in solution [62]. Since the quantitation is based on the characteristics of pure nucleic acids, the method also includes an assessment of purity. For both DNA and RNA, maximal absorption occurs at 260 nm. Over a wide range of concentrations, a linear relationship exists between the absorbance of light of 260 nm (A_{260}) and the nucleic acid concentration. This correlation is described by the Lambert-Beer Law:

$$A_{260} = e \cdot c \cdot l$$

Where c is the nucleic acid concentration in µg/mL measured across a lightpath length of l in cm and e is the extinction coefficient of the nucleic acid being analysed. The extinction coefficients of singie stranded RNA and double stranded DNA are 0.025 and 0.020 $(\mu g/mL)^{-1}cm^{-1}$, respectively.

Spectrophotometry is unable to distinguish between RNA, DNA or individual nucleotides [62]. Furthermore, proteins, salts, solvents and buffers also absorb light of 260 nm. Proteins absorb maximally at 280 nm. The A_{260}/A_{280} ratio is used to determine the purity of nucleic acid solutions, validating the nucleic acid concentration calculated from the A_{260} reading. Generally, ratios greater than 1.8 are accepted as indicative of relatively pure nucleic acids. However, the absorbance ratio of nucleic acids is higher in Tris-HCl buffer than in water despite the spectrophotometer being zeroed with the respective solutions [64]. The ratios also vary with ionic strength and pH. The solvation of CO_2 in water over time may change the pH of water and subsequently alter spectrophotometric readings. With this in mind, RNA in this study was eluted from the spin columns with 10 mM Tris-HCl, pH 7.5 and an aliquot of the solution taken at the same time (extraction negative control) was used to calibrate the instrument.

The extraction of RNA from airway biopsies and BAL cells results in small amounts of RNA in a volume of 40 µL. The low concentration of RNA precludes dilution, making spectrophotometry difficult. Traditional methods of spectrophotometry utilise cuvettes with a pathlength of 1 cm with a microcuvette type having minimum

volumes of 400 µL. Sub-microcuvettes are available with minimum loading volumes down to 2 µL. A cuvette of this type with a minimum loading volume of 50 µL was tested for our application but major problems were experienced with air bubbles in the lightpath. Capillary spectrophotometry was subsequently developed to be a reliable method of quantitating small quantities of sample RNA.

Capillary spectrophotometry utilises a capillary adaptor cell of the standard 1 cm^2 dimensions (Helix, San Diego, CA, USA). Lenses on either side of the adaptor cell focus the beam of the spectrophotometer onto a disposable quartz capillary. The capillaries have a lightpath of 0.5 mm and require 3 - 4 µL of RNA sample.

2.4.1 Variability of Spectrophotometry

The capillaries are approximately round and the micro-facetted surface produces variable light scatter dependent on its orientation in the lightpath. This artefact produces large variances in A_{260} and calculated RNA concentrations. The variable light scatter can be observed as vertical shifts in wavelength-absorbance curves when a capillary is removed and replaced in the capillary adaptor cell (Figure 2.4). The light scatter can be quantified at wavelengths such as 320 nm, which nucleic acids do not absorb [62]. The adjustment of A_{260} and A_{280} readings by the A_{320} leads to consistent measurements. Surprisingly, correction for variable light scatter was not included in the manufacturer's instructions for the use of the capillary adaptor cell.

In testing spectrophotometers, it was found that the majority of instruments commonly used for nucleic acid quantitation gave unacceptable coefficients of variation. Furthermore, these machines lacked the sensitivity required for airway samples. High quality optics usually reserved for enzyme kinetics were required to achieve coefficients of variation below 1 % for repeat measures between removing and replacing the capillary from the instrument. As spectrophotometry was one of several steps in the competitive PCR assays, it was thought that the coefficient of variation of this step should be below 1 %.

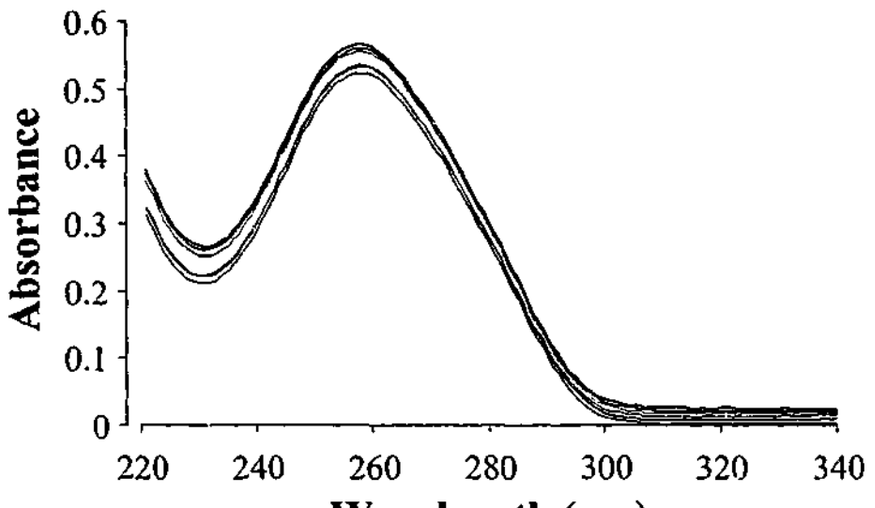

Figure 2.4 Effect of Repeat Measure by Capillary Spectrophotometry

For the Varian spectrophotometer and the parameters used in this study, coefficients of variation of 0.23 and 0.41 % were obtained (n = 6) for the (A_{260}-A_{320}) measurement and the (A_{260}-A_{320})/(A_{260}-A_{320}) ratio respectively[1]. It was noted, however, that the coefficient of variation rose sharply as the concentration of RNA decreased and the absorbance approached the sensitivity of the instrument. Whilst a cut-off yield of 500 ng of RNA was initially chosen on practical grounds for subsequent steps, it was observed that most RNA extractions below this concentration also had poor absorbance ratios, suggesting the absence of sufficient RNA to be accurately measured by the spectrophotometer. The minimum yield of 500 ng RNA corresponds to a concentration of 12.5 µg/mL and adjusted A_{260} readings greater than 0.015.

[1] This testing was conducted in conjunction with Dean C Logan, Varian Australia.

2.5 YIELD OF RNA FROM STUDY SAMPLES

BAL cells gave a success rate of 90 % with a median yield[2] of 2.9 µg (range 0.6 – 12.2), whilst biopsies were extracted at a success rate of 70 % with a median yield of 1.0 µg (range 0.5 – 6.5) (Table 2.2). The success rate was the same between controls and asthmatics and was independent of treatment. The rate of successful RNA extraction of biopsies is similar to that obtained for successful use of biopsies for histological analysis by our group. Biopsies, which macroscopically look useful, frequently can not be used because they are inadequate.

Table 2.2 Yield and Success Rate of RNA Extractions

	BAL Cells	Biopsies
Success Rate (%)	89.2	68.5
Median RNA Yield (µg) (range)	3.1 (0.6 – 12.2)	1.1 (0.5 – 6.5)
Median Absorbance Ratio (range)[1]	2.1 (2.0 – 2.8)	2.1 (1.7 – 2.5)
Longitudinal sample reception rate[2]	76/90	84/90
Proportion of paired samples[3]	22/45	15/45

[1] Adjusted absorbance ratio of $(A_{260}-A_{320})/(A_{260}-A_{320})$.

[2] Three arms of 15 asthmatics with 2 bronchoscopies each.

[3] Divided between 3 arms of the longitudinal study

Of a possible 90 samples in the longitudinal study 76 BAL cell aliquots and 84 biopsy were received for RNA extraction. This sample reception rate compounded with the subsequent failure rate of RNA extraction resulted in only a third of the paired biopsy samples and half of the BAL cell pairs of the intervention study being available for analysis (Table 2.2). These paired samples were spread over the three arms of the study and the number of RNA samples in any one arm precluded

[2] Yield calculation assume that 100 % of the 40 µL elution buffer was recovered. For the vast majority of samples more than 30 µL was recovered.

longitudinal analysis. All longitudinal samples were therefore included in subsequent cross-sectional analyses, with multivariant statistics taking the pairing into account.

2.6 CONCLUDING COMMENTS

The priority of sample collection for RT-PCR analysis and the ensuing rate of RNA extraction are clearly determining factors in the ability to conduct bronchoscopic studies of gene expression in BAL cells and biopsies. We planned the intervention study based upon what we thought was a reasonable prioritisation between the needs of multiple endpoints being measured in biopsy and BAL samples. Collection rates of the number of cells in BAL, sampling rates of macroscopically-intact biopsies and RNA extraction failure rates are factors that need to be considered when calculating the sample size of bronchoscopic studies in order for quantitative RT-PCR to be viable. Alternatively, more of each sample type needs to be dedicated to RT-PCR assays. For longitudinal studies of biopsy samples, it may be necessary to reserve all tissue obtained for RNA extraction for the study to be viable.

Bronchoscopic studies are expensive to carry out and the ethics of obtaining samples from volunteer subjects require that these be used efficiently. In this context, PCR-based techniques need to have a distinct advantage over other methodologies to gain high sample collection priority at bronchoscopy. Significant improvement in the efficiency of RNA extraction was seen in the course of this study. It is possible that further technical advances, such as RNA extraction using automated robotics [65], may improve the efficiency of RNA extraction and increase the viability of PCR-based analysis.

3 DEVELOPMENT OF HIGH-SENSITIVITY PCR

3.1 INTRODUCTION

Early in the developmental phase of this study it was realised that the cytokines we wished to quantitate were expressed at low levels even in mitogen-stimulated peripheral blood mononuclear cells (PBMC). Furthermore, in the relatively mild and/or clinically stable asthmatics we were ethically restricted to study, it was perceived that the expression of theses cytokines may not differ dramatically between patient groups or normal controls. These factors, coupled with the availability of only suboptimal amounts of biological samples (as discussed in Section 2.5), necessitated the development of PCRs that were highly sensitive and quantitative. This chapter follows the development of the methods of analysing PCR products and the conditions for achieving PCR amplification from low copy numbers of template.

3.2 MATERIALS AND METHODS USED

3.2.1 Electrophoresis

3.2.1.1 Agarose Gel Electrophoresis

Agarose gel electrophoresis was conducted with 40 mM Tris-acetate, 2 mM Na_2EDTA, pH 8.5 buffer (1x TAE). When ethidium bromide was used, it was added at a concentration of 0.1 µg/mL so that it was present in both gel and running buffer. Molecular biology grade agarose (Progen Industries, Darra, QLD, Australia) was added to buffer at concentrations of 2 to 3.5 % dependent on the resolution required. The agarose was melted in a microwave, the weight loss was adjusted and then cooled under running water before pouring into a gel former. Generally, 10 µL aliquots of PCR products were mixed with loading dye consisting of a final concentration of 0.025 % bromophenol blue, 0.025 % xylene cyanol, 5 % glycerol, 1x TAE.

3.2.1.2 Polyacrylamide Gel Electrophoresis (PAGE)

PAGE gels were prepared using commercial premixed stocks of acrylamide and N,N'-methylenebisacrylamide (bisacrylamide for short) at ratios of 19:1 or 37.5:1 (Pharmacia Biotech, Uppsala, Sweden. Acrylamide/bisacrylamide stock(s) was diluted into a final concentration of 2x TAE buffer usually forming 4 % 19:1 or 8 % 29:1 acrylamide:bisacrylamide gels. The mix was degassed and then 750 µL 10 % ammonium persulfate and 75 µL N,N,N',N'-tetramethylethylenediamine (TEMED) (Pharmacia Biotech) was added per 100 mL of mix before pouring into gel former. The ratio of ammonium persulfate to TEMED was kept constant but the optimum amounts were determined empirically from time to time, so that polymerisation occurred in 10 to 20 minutes.

PAGE was conducted in a SE660 Hoefer Scientific Instruments (San Francisco, CA, USA) apparatus. The gel format was 20 tracks across a width of 18 cm and either 16 or 24 cm long dependent on the resolution required. Double 'sandwich' gels separated by a recessed divider plate were made so that a total of 4 gels could be electrophoresed in one run (Figure 3.5, page 44). To facilitate rapid electrophoresis, the lower buffer reservoir, into which the gels were submerged, was actively cooled using 50 % ethylene glycol circulation through the heat exchanger and a refrigerated bath (Hoefer Scientific Instruments). A power supply with a temperature probe that modulated the power output (BioRad Laboratories, Hercules, CA, USA) was used allowing temperatures of 15-20 °C to be maintained on the outside of the chamber (Figure 3.5). The temperature probe modulation of the power supply also served as an important safety feature, preventing damage to the gels or apparatus in case of failure of the cooling system.

PCR products were diluted 1:10 into loading dye at a final concentration of 2 % ficoll, 0.025 % bromophenol blue, 0.025 % xylene cyanol, 2x TAE. Aliquots of 4 µL were loaded and ran into the gel at 10 W for 10 min and then electrophoresed at 80 W for 100-120 min. After dismantling, the gels were overlayed with 1:10,000 SYBR Green I in 2x TAE buffer and stained for 30-60 min before fluorescence scanning (Section 3.2.2.2).

3.2.2 Detection System

3.2.2.1 Detection of PCR Products by Chemiluminescence

This procedure was conducted with PCR products synthesised with 25 μM digoxigenin (DIG)-dUTP (Boehringer Mannheim, Mannheim, Germany). PCR products electrophoresed in agarose were Southern transferred using positively charged nylon membrane (Boehringer Mannheim) and 0.4 M NaOH for 4-7 hours and then baked at 120 °C for 20-30 min (Figure 3.1, page 38).

The membrane was processed with the DIG Luminescent Detection Kit (Boehringer Mannheim) [66] omitting the hybridisation steps. Briefly, the membrane was blocked with 1 % blocking reagent in 0.1 M maleic acid, 150 mM NaCl, pH 7.5 for 80 min. Anti-DIG-alkaline phosphatase(AP) conjugate was applied at 75 mU/mL in blocking solution for 45 min. The membrane was washed twice with 0.3 % Tween-20, 0.1 M maleic acid, 150 mM NaCl, pH 7.5 over 40 min. The blot was then equilibrated in 0.1 M Tris-HCl, 0.1 M NaCl, 50 mM MgCl2, pH 9.5 before adding Lumigen PPD to 0.1 mg/mL for 5 min.

The membrane was blotted, sealed between plastic sheets and incubated for 15 min at 37 °C before taking various exposures with Hyperfilm-ECL (Amersham, Melbourne, VIC, Australia). A flashgun (Sensitize, Amersham) was used to sensitise autoradiographic film and was calibrated to induce a background absorbance of 0.07 to 0.1. Bands were quantitated by track scanning in a densitometer (LKB Ultrascan II, Pharmacia Biotech).

3.2.2.2 Detection of PCR Products by Scanning Fluorescence

Agarose gels electrophoresed in the presence of ethidium bromide and PAGE gels post-stained with SYBR Green I were scanned directly in a laser-based fluorescence scanner (FluorImager-575, Molecular dynamics, Sunnyvale, CA, USA) (Figure 3.3). The photomultiplier tube voltage was adjusted so that the signal of the brightest band on a given gel was maximised but not saturated ensuring the highest signal-to-noise ratio. The volume of fluorescence in each band was quantitated with ImageQuaNT software (Molecular Dynamics) using local average background correction originating from the perimeter of each object.

3.2.3 IL-1-β PCR

3.2.3.1 Initial Protocol

cDNA synthesis from GeneAmplimer pAW109 RNA (Applied Biosystems), a cRNA from plasmid pAW109, was conducted with the PCR-related Reverse Transcription System (Promega). A 20 µL reaction was made consisting of 10^6 copies of pAW109 cRNA, 1x reverse transcription buffer, 5 mM $MgCl_2$, 1 mM deoxyribonucleotide triphosphates (dNTPs), 25 ng/µL oligo$(dT)_{15}$ primer, 1 U/µL RNasin reverse transcriptase inhibitor and 15 U AMV reverse transcriptase. The reaction was incubated for 1 min at 15 °C and then the temperature was increased to 42 °C over 9 min, followed by 1 h at 42 °C, 5 min at 85 °C and 1 min at 4 °C. The cDNA was diluted to working concentrations with 50 µg/mL nuclease-free glycogen (Roche Diagnostics Australia, Castle Hill, NSW, Australia) in 10 mM Tris-HCl, pH 7.5 and labelled with the RNA copy numbers from which they were derived.

PCR was conducted in 50 µl reactions consisting of 1 U of Taq DNA polymerase (Promega), 1 x Reaction buffer, 2 mM $MgCl_2$, 200 µM deoxyribonucleotide triphosphates (all from Promega), 0.5 µM upper and lower primers (Table 4.2, page 65). Thermal cycling was conducted in a MJ Research PTC-200 (Watertown, MA, USA), with the temperature profile consisting of 40 cycles of 94 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min, concluding with 7 min at 72 °C and 1 min at 4 °C.

3.2.3.2 Hot-Start with a Neutralising Antibody

Hot-start of PCR was facilitated with a neutralising monoclonal antibody (TaqStart Antibody, Clontech Laboratories, Palo Alto, CA, USA). TaqStart Antibody (1.1 µg/µL) was combined in equal amounts with Taq DNA polymerase (5 U/µL), incubated at room temperature for 5 min and then added directly to a PCR master mix.

3.3 CHOOSING A DETECTION SYSTEM

3.3.1 Chemiluminescence

The detection of PCR products in studies to date using respiratory tract samples have largely used blot hybridisation methodology. When this project was commenced, a similar detection system was trialed but the hybridisation was avoided by the incorporation of the hapten, digoxigenin (DIG), into PCR products with the use of DIG-dUTP. The labelled PCR products were then Southern blotted and the hapten immobilised on the membrane was detected by chemiluminescence (Figure 3.1) [66]. An anti-DIG antibody conjugated with alkaline phosphatase was captured on the membrane and dephosphorylation of the substrate Lumigen PPD results in an unstable intermediate which emits light. Chemiluminescence was quantitated by densitometry of exposed X-ray film as well as films such as Hyperfilm-ECL optimised for chemiluminescence.

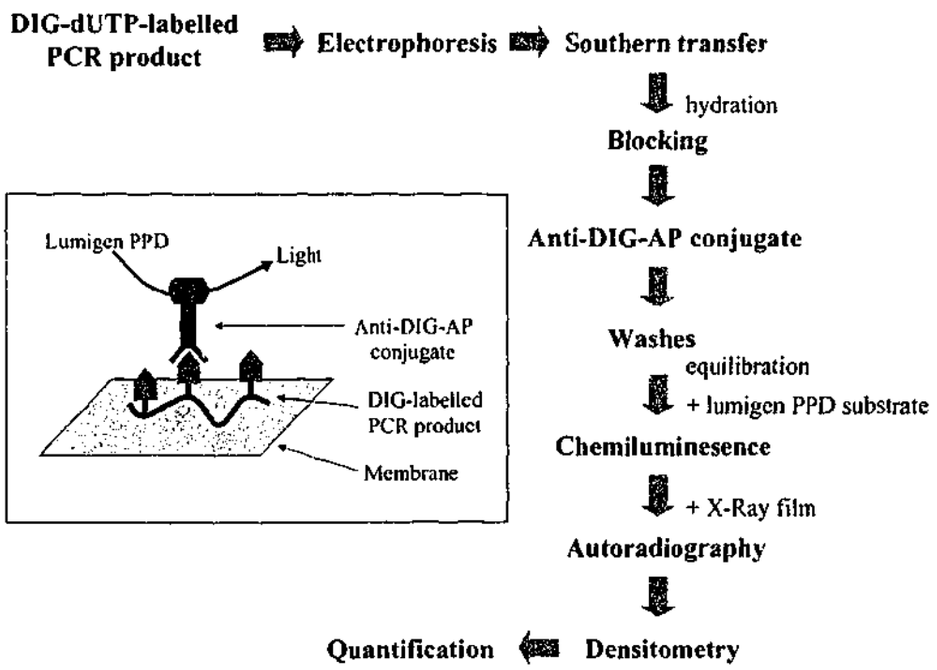

Figure 3.1 Quantitation of PCR Products by Chemiluminescence

To examine the quantitative nature of chemiluminescence detection of PCR products, a labelled PCR product was loaded in various quantities on a 2.5 % agarose gel and the relative band intensities analysed. Hyperfilm-ECL was used without pre-treatment and with pre-flashing. Exposure of the film to a flash of light before autoradiography sensitises the film and has been reported to overcome the non-linear response of film to low intensities of light [67]. The non-linear response of film was seen with short exposures irrespective of the use of pre-flash treatment. However, pre-flashing did increase the sensitivity of the film, with stronger exposures exhibiting linearity (Figure 3.2). The response of the film whilst linear was not directly proportional to the input (compare to the theoretical line), a feature which decreased the quantitative resolution of the method. At stronger exposures, it was noted that increased specific band size and the detection of non-specific bands were beginning to detract from the linearity and this was reflected in large standard errors. In the example shown (Figure 3.2), only intra-band ratios less than 1:5 could be distinguished with accuracy.

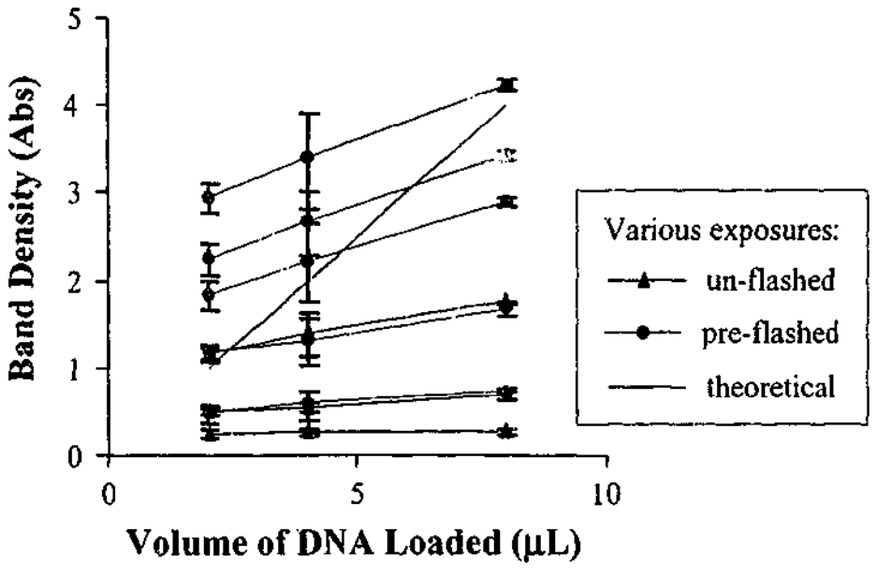

Figure 3.2 Effect of Gel Loading on Quantitation by Chemiluminescence

3.3.2 Scanning Fluorescence

In 1994, a laser-based fluorescence scanner, the FluorImager, became available. The FluorImager can scan gels, microtitre trays or membranes carrying fluorochromes excitable by a 488 nm argon laser. Fluorochromes detected efficiently by the instrument include fluoroscein and its derivatives, ethidium bromide and the new-age intercalating dyes SYBR Green I and Pico Green (Molecular Probes). Agarose gels electrophoresed in the presence of ethidium bromide or similar intercalating dyes can be placed on the carrier plate of the instrument and a scan obtained within 5 min. Similarly, PAGE gels were stained after electrophoresis and then scanned in the FluorImager (Figure 3.3). The image analysis software of the instrument, ImageQuaNT, allows rapid quantitation of band volumes with the generated reports being exported automatically to Microsoft Excel for further analysis.

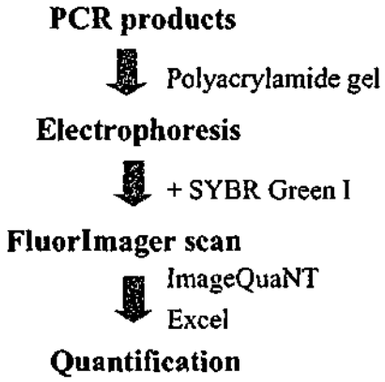

Figure 3.3 Quantification of PCR Products by Scanning Fluorescence

The quantitative range of fluorescence scanning was tested in a number of ways including the variable loading approach used above (Section 3.3.1). However, such approaches are easily confounded by track-to-track variability and pipetting errors. PAGE gels are particularly difficult to load quantitatively. To overcome these confounders, single band PCR products of different sizes were combined in various ratios so that band ratios in each track could be determined independently of the volume loaded. Pipetting errors in setting up a series of changing ratios were

reduced by serially diluting one single band PCR product (the variable product) with a diluant containing another PCR product (the constant product) (Figure 3.4A). To examine the complete quantitative range the converse titration was also established. This changing ratio series was electrophoresed through PAGE gels (Figure 3.4B) to determine optimal SYBR Green I staining times and scanned image background correction method as well as to confirm the ability to quantitate by fluorescence scanning.

Quantitated output ratios were fitted to a predicted line of direct proportionality between output and input ratios by deriving relative input ratios from the average result of the output ratio(s) closest to 1 (Figure 3.4C). The use of local average background correction originating from the perimeter of the rectangle defining the band gave output ratios with the best fit to this predicted line. The close fit of the data to this predicted line was indicative of directly proportional, linear quantitation over ratios to 1:100. Similar quantitation was achieved with post-PAGE SYBR Green staining times of 24 to 55 min (Figure 3.4C).

The two methods of quantitation are compared in Table 3.1. The disadvantage of scanning fluorescence is the cost of the instrumentation. However, more recent advances in image capture and analysis have resulted in CCD camera-based instruments becoming available which have quantitative and sensitivity properties rivalling that of laser-based systems, such as the FluorImager. Such machines are about a third of the cost of the FluorImager and not a lot more expensive than a high-quality densitometer. Densitometers are a common instrument in most established institutions, although they typically have basic and old-fashioned specifications. Chemiluminescence detection and indeed most X-ray film based methods are time consuming with a number of steps detracting from the ability to quantitate.

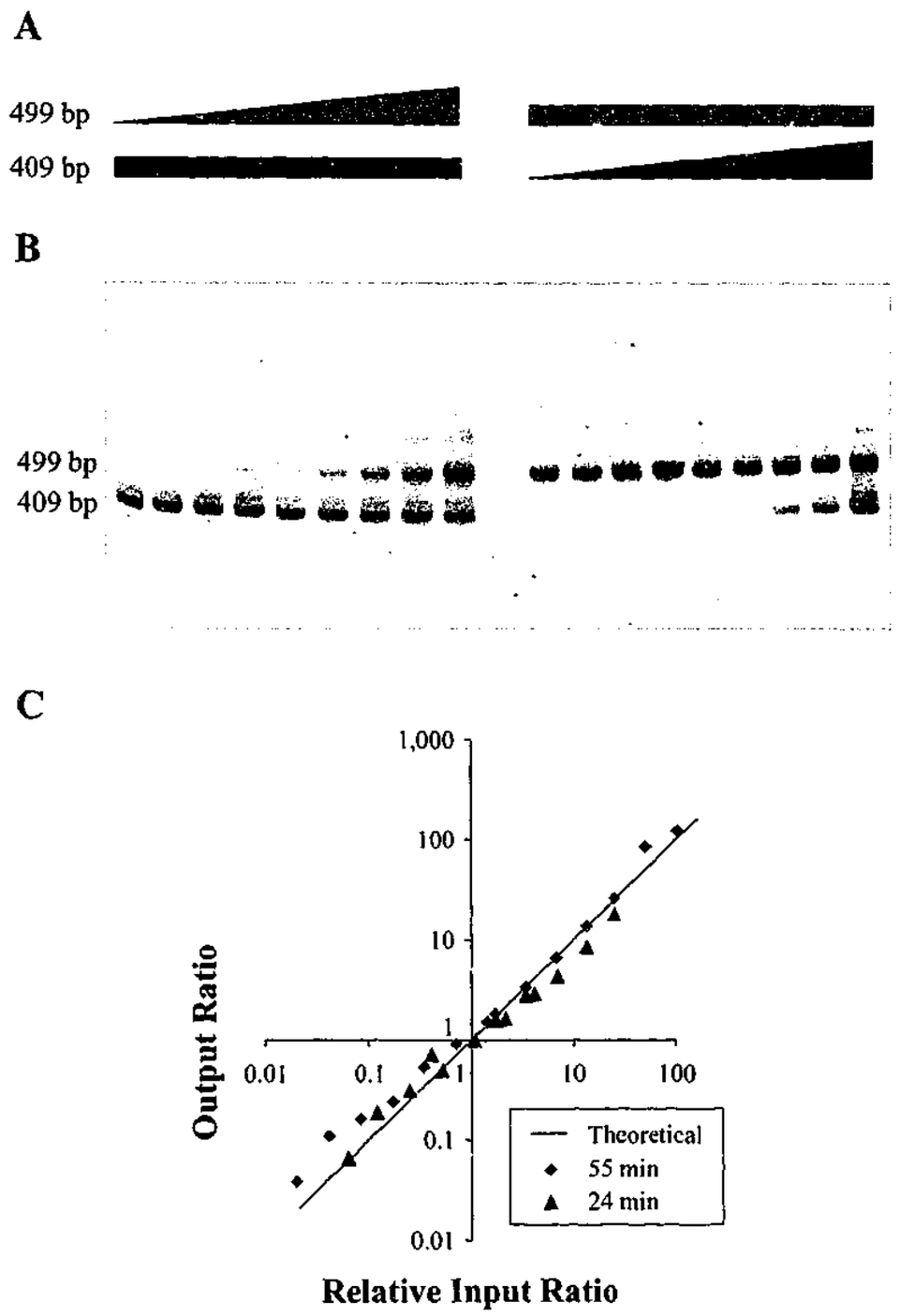

Figure 3.4 Effect of Input on Quantitation by PAGE and Scanning Fluorescence

A) Schematic diagram showing the mixing of IL-4 competitor (499 bp) and IL-5 competitor (409 bp) PCR products. B) PAGE gel of a typical changing input series loaded as in (A) and stained for 40 min. C) Plot of output ratios of changing input series stained for 24 and 55 min. The two series of data were derived from different experiments.

Table 3.1 Comparison of Chemiluminescence and Fluorescence Detection

	Chemiluminescence	Fluorescence
Mechanism of signal	Antibody-coupled, enzyme-induced chemiluminescence	Scanning laser induced fluorescence
Quantitative nature	May not be linear under some conditions Output rarely directly proportional to input	Linear, directly proportional to input[1]
Quantitative range	Variable	1:10,000 less background of gel[2]
Quantitation improved by	Pre-flashing X-ray film Calibration by standard curve	PAGE gels and SYBR Green I give lower background than agarose gels and/or ethidium bromide
Band saturation	Determined empirically Not clearly defined	Saturated image pixels are defined by colour
Time post-electrophoresis	> 9 hours[3]	< 1 hour
Potential problems	Uneven Southern transfer Lack of direct linearity reduces resolution	High gel background with agarose

[1] That is, input ratios of 1:2:4:8 give output ratios of 1:2:4:8. Note, for example, that output ratios of 1:1.5:2.5:4.5 (from output = 0.5 x input + 0.5), whilst linear, are not directly proportional to input.

[2] Ratios of 1:10 and 1:100 readily achievable with agarose and PAGE gels respectively.

[3] Time of some steps vary considerable between protocols, particularly Southern transfer, membrane blocking and autoradiography.

3.4 COMPARISON OF AGAROSE AND POLYACRYLAMIDE GEL ELECTROPHORESIS

Agarose gel electrophoresis has been the method of choice for the analysis of DNA due to the simplicity of equipment and gel formation and the rapidity of the technique (Table 3.2). The thick and cloudy nature of agarose gels results in high background, particularly at the high concentrations required to resolve small PCR products (Figure 3.6). Polyacrylamide gels are comparatively difficult to form by polymerisation and the equipment required for electrophoresis is considerable more complex (Figure 3.5). The equipment used in this study combined a number of non-essential features, such as the cooling system and the temperature controlled power supply, so that multiple gels could be electrophoresed at high speed. The lack of local laboratory experience with non-denaturing PAGE of nucleic acids further disadvantages the technique. The clarity of polyacrylamide affords a wide quantitative range, a necessary feature for assays of low expression mRNA conducted from limited biological tissue where only one reaction can be conducted. The native-competitor size difference of several of the assays, particularly TNF where the difference is only about 7.5 %, required the resolution characteristic of PAGE (Figure 3.6).

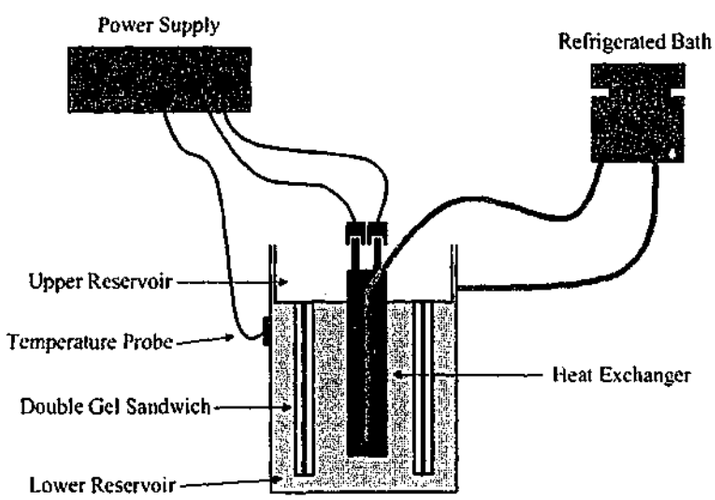

Figure 3.5 Apparatus for PAGE

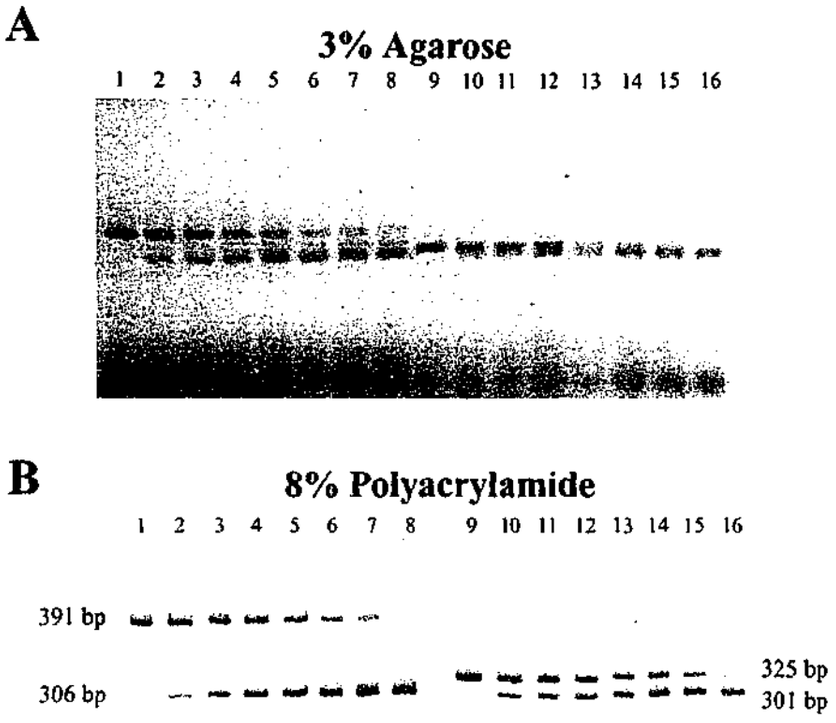

Figure 3.6 Comparison of the Resolution and Background of PAGE and Agarose Gels

IL-1-β (lanes 1 – 8) and TNF (lanes 9 – 16) competitive RT-PCR were electrophoresed through agarose (A) and polyacrylamide gels (B). The images are not of comparable scale, with the approximate size of the agarose image being 0.3 Mb in comparison to 1.3 Mb in (B). The grey-scale of the images has been individually adjusted across the background to maximum signal quantitative range of each scan. The quantitative range in (B) is about 20 times that of (A).

Table 3.2 Comparison of Gel Methods

	Agarose	Polyacrylamide
Type of matrix	Hydrocolloid of polysaccharide chains held together by hydrogen and hydrophobic bonds	Covalently cross-linked monomers of acrylamide and cross-linker (bisacrylamide)
Mechanism of formation	Melting of powder in buffer	Peroxide initiated, free-radical catalysed polymerisation
Reagents	Agarose, buffer	Acrylamide, bisacrylamide, TEMED (catalyst), ammonium persulfate (initiator), buffer
Method of electrophoresis	Horizontal submarine Single buffer reservoir	Vertical gel connects upper and lower buffer reservoirs
Resolution	> 10 % difference	To 1 base pair difference
Background fluorescence	High Concentration dependent	Very low Very little difference with concentration
Typical quantitative range	1:10	1:100
Methods of incorporation of fluorophore	Labelled PCR products Added to gel and buffer[1]	Labelled PCR products Post-staining
Set-up and electrophoresis time	Dependent on number of samples 1 hour for small gel	Dependent on resolution required[2] > 4 hours
Source of artefacts	Reagent impurities Unmelted agarose Excessive heat generation	Reagent impurities Uneven polymerisation Excessive electrophoresis Excessive heat generation Sample overloading

[1] Post staining is possible but is poorly quantitative in the high concentration gels required for PCR product separation.

[2] Multiple-gel equipment allows large numbers of samples to be ran together comfortably in one day, such as 4 gels of 20 tracks (Figure 3.5).

3.5 SELECTION OF FLUOROPHORE AND LABELLING METHOD

Laser-based fluorescence scanning of fluorescently labelled PCR products has been shown to be 1,000 times more sensitive than transilluminator visualisation of ethidium bromide-stained DNA [68]. In selection of fluorophore and labelling method for this quantitative study, it was important to balance sensitivity and quantitative ability as well as practical issues such as time (Table 3.3).

Fluorophores can be introduced into PCR products by two key strategies; chemical addition and intercalation of a stain [68]. A fluorophore can be chemically attached to a primer or to a nucleotide, usually dUTP. The latter method of chemical attachment, whilst being highly sensitive, may not be quantitative when there is a difference in the number of thymidine bases between native and competitor PCR products. Primers are most efficiently labelled during primer synthesis utilising the same phosphoramidite chemistry but can also be labelled after synthesis. The majority of the PCR assays in this study include one primer that had been synthesised with a fluorescein phosphoramidite addition at the 5' end. This label was used in some of the developmental work but was found to lack sensitivity for the assay of patient samples. Fluorescently labelled primers result in up to one fluorochrome per PCR product, whilst intercalating dyes are far more sensitive as they may bind many fluorophores per PCR product.

Intercalating fluorescent stains, such as ethidium bromide and SYBR Green I, can be added at several stages of the quantitative process but the choice of method was determined by the electrophoresis method, background fluorescence and cost. Ethidium bromide is old-fashioned and inexpensive but gives high background in comparison to SYBR Green (Table 3.3). SYBR Green exhibits a large fluorescence enhancement upon binding to DNA, resulting in very low background fluorescence [69, 70]. The high background of ethidium bromide is kept to a minimum if small amounts are added to both agarose gel and buffer during electrophoresis and this was found to give adequate sensitivity. Charged stains like ethidium bromide can not be added to PAGE buffer or gels during electrophoresis as the dye is mobilised at the expense of migration of DNA through the gel. Consequently, SYBR Green I post-staining was the method of choice for PAGE. When resolution and quantitative

range were not important, ethidium bromide staining during agarose gel electrophoresis was used.

Table 3.3 Comparison of Fluorophores and Their Labelling Methods

	Fluorescent Primer	Staining During Electrophoresis	Staining Post-Electrophoresis
Mechanism of labelling	Chemical During primer synthesis[1]	Intercalation Added to gel and buffer	Intercalation Overlay with stain
Used with gel type	Polyacrylamide Agarose	Agarose	Polyacrylamide[2]
Reagent used	Fluorescein phosphoramidite	Ethidium bromide[3]	SYBR green I[4]
Relative sensitivity	Low	Moderate	High
Background due to fluorochrome	None	High	Low
Stability of reagent	Anecdotal evidence of deterioration after 1 year	> 2 years	~ 2 years Moisture sensitive
Relative Cost	Economical	Cheap	Expensive

[1] Primers can also be labelled post-synthesis but this was not used in this study.

[2] Agarose gels also post-stain with high sensitivity but it was found that the quantitative range is compromised by the stain not being able to penetrate high concentration bands.

[3] SYBR Green I can also be used for this method but the cost is prohibitive. It is noted, however, that the cost in Australia has been 5 times that in the USA.

[4] Ethidium bromide can be used but the background is too high for signal quantitation.

3.6 OPTIMISATION OF BASIC PCR CONDITIONS

Traditionally PCR optimisation has focussed on the magnesium concentration and the annealing temperature to achieve specific PCR amplification [71-75]. This simple strategy alone, I found, does not deliver sensitivity for many primer pairs below 10^4 copies. The approach to insufficient sensitivity used by most researchers has been to supplement the PCR with additives, which include dimethylsulfoxide [76, 77], formamide [78], glycerol [76] and ammonium sulfate [79]. Most often, in my hands, these additives had little effect on sensitivity. As a result, I developed an optimisation strategy that focussed on the outcome of replicate reactions. Surprisingly, replicates are rarely used in the development of PCR methodology, even in reports of factors that influence PCR amplification [for example 80, 81, 82].

3.6.1 The Strategy

The strategy of investigating PCR sensitivity by replicate analysis was born of experiments trying to solve irregular negative reactions. One of these was the result of an unexpected contaminated water source and another was due to dust falling from the matrix of a batch of filter tips. On these occasions and similar situations where a factor has the effect of sub-optimising the reaction, the factor was only evident as the cause when direct replicate reactions were made. For example, to test various sources of water, large reactions differing by water source were made and then each was divided into 3 or 4 replicate reactions. A poor source of water may give 2 positive bands out of 4 compared to good quality water resulting in 4 bands of consistent intensity.

When a small amount of template is added to a PCR, below a certain threshold copy number, there is a large variation in amplification. The resulting non-reproducibility of positive reactions has been termed the Monte Carlo effect [83]. The outcome of such reactions is determined largely by the events of the early cycles of PCR and is reflective of the randomness of reaction components interacting together efficiently [83, 84]. The strategy of PCR optimisation by analysis of direct replicates allows selection of the factor(s) giving the lowest threshold of the Monte Carlo effect and

thereby increasing sensitivity and reproducibility. As evident in the optimisations discussed below (Section 3.6.2, Figure 3.7), choosing PCR factors from comparison of single reactions is not a reliable method of PCR optimisation unless it is intended to always use large amounts of template.

This method of replicate analysis was used to optimise all of the PCR assays in this study as well as to 'PCR-certify' new batches of water. The first optimisation experiment of a new PCR typically included a magnesium titration of 1.5, 2.0, and 2.5 mM $MgCl_2$, competitor template copy numbers of 10^2 and 10^4 and two annealing temperatures with three replicates of each combination (total of 36 reactions). The two annealing temperatures were chosen from the middle of the range suggested by Oligo Primer Analysis software (National Biosciences, Plymouth, MN, USA) [85, 86], usually differing by about 4 °C. It has been my experience that the lower and upper values of the suggested range represents closely the empirical most sensitive, least specific annealing temperature and the most specific, least sensitive annealing temperature, respectively. It was found that most primer pairs amplified 10^4 copies of template over 40 cycles with a combination of these magnesium concentrations and annealing temperatures. Frequently, 10^2 rather than 10^4 copies of template were more informative of the best combination of these parameters, being below the threshold of the Monte Carlo effect but yielding some positive reactions.

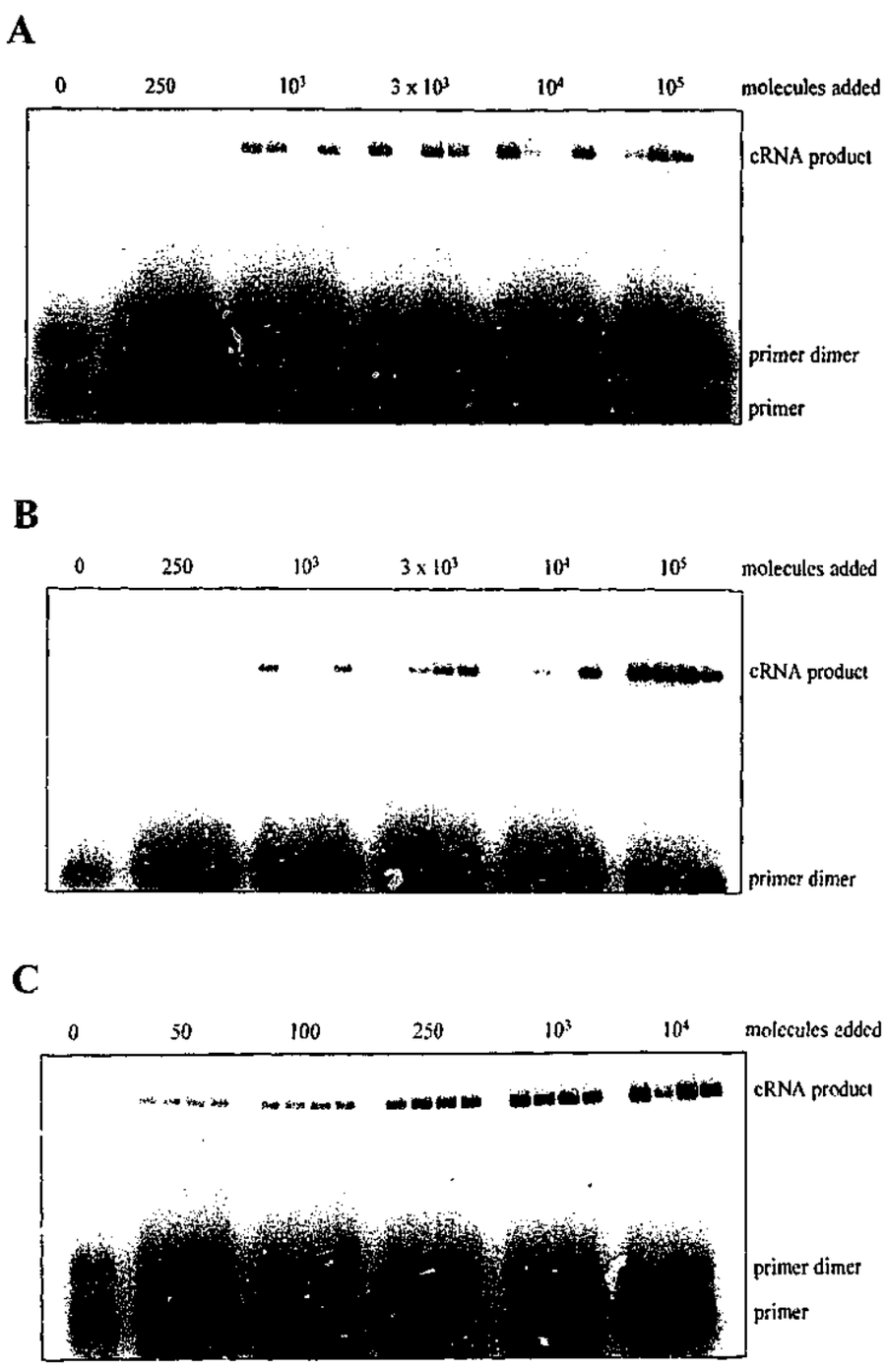

Figure 3.7 Optimisation of High-Sensitivity PCR by Analysis of Replicates

IL-1-β PCR sensitivity using Promega Taq polymerase (A) after basic optimisation of annealing temperature and magnesium concentration, and (B) with hot-start using neutralising anti-Taq polymerase. (C) PCR sensitivity with the same conditions as (B) but with primers at 0.2 μM instead of 0.5 μM and with Taq polymerase from Applied Biosystems.

3.6.2 Factors Improving Sensitivity

I established standard conditions for high-sensitivity PCR with IL-1-β PCR, using a large batch of cDNA synthesised from the RNA competitor molecule, pAW109 cRNA (Figure 4.1, Section 4.3.1) [8]. With the traditional optimisation of annealing temperature and magnesium concentration without replicate analysis (Section 3.6), I found that cDNA copies equivalent to 10^3 copies cRNA[3] were detectable but even 10^5 copies were not consistently amplified across all four replicates (Figure 3.7A).

During the set-up of PCR, the primers anneal to template but due to the low temperature, this binding is mostly non-specific. Whilst the optimal synthesis temperature of Taq polymerase is 72 °C, significant polymerisation occurs at lower temperatures [2]. When a set of reactions are placed in a PCR machine, as the temperature rises a portion of the non-specifically bound primers are quickly stabilised by synthesis. These non-specific products are now flanked by the incorporated primers that allow them to amplified efficiently for the course of the PCR, competing with the specific product for the sensitivity of the reaction [87]. The synthesis of non-specific products of this type is prevented by several hot-start strategies, including a wax barrier separating reaction components [87] and neutralising anti-Taq polymerase antibodies [88]. In my optimisation of IL-1-β PCR, the use of a neutralising monoclonal antibody was shown to increase sensitivity, with consistent detection of 10^5 copies (Figure 3.7B).

At the time that these experiments were being conducted, I noted that Applied Biosystems (known as Perkin Elmer at the time), a major manufacturer of Taq polymerases, had began to recommend 0.2 μM rather than 0.5 μM primer concentration as the starting parameter against an optimal range of 0.1 – 0.5 μM. For IL-1-β PCR, a 0.2 μM primer concentration was shown to be more sensitive than that used previously (Figure 3.7C). The concentration of primers is determined at synthesis by the manufacturer by theoretical projection from base coupling

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[3] Referred hereafter simply as copies although the actual copies of cDNA are likely to be at the very most marginally fewer than this but could even be less than 10 % of this.

efficiencies. Subsequent experience with PCR primer pairs lacking sensitivity has shown that sometimes these measurements can be inaccurate causing unbalanced strand synthesis. Sensitivity in these situations can be improved by titrating the primers against each other, determining the empirical balance of the primers.

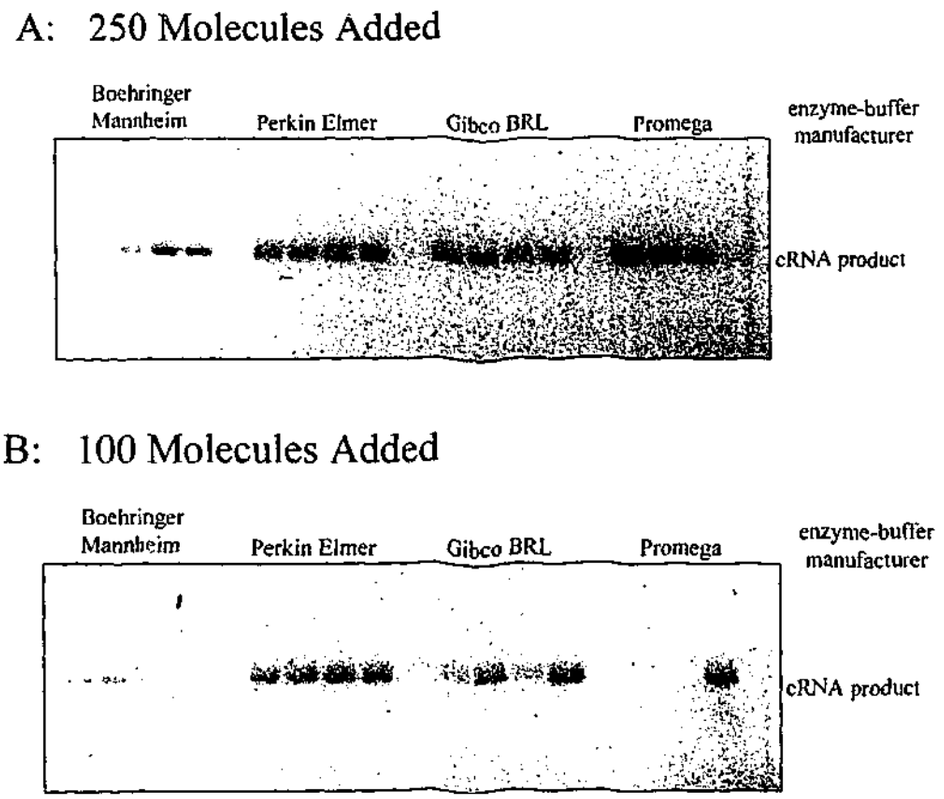

Figure 3.8 Comparison of IL-1-β PCR Sensitivity with Taq DNA Polymerases and Reaction Buffers from Different Manufacturers

Enzyme manufacturers differ in their mode of preparation of Taq DNA polymerase from both recombinant and natural sources, in their specifications of enzyme activity and in the composition of their storage and reaction buffers. Three enzymes from different manufacturers, Applied Biosystems, Gibco-BRL and Boehringer Mannheim were compared for sensitivity of IL-1-β PCR with the Taq DNA polymerase from Promega, which had been used for the previous studies (Figure 3.8). The enzymes were tested in their own buffer but otherwise the PCR conditions were the same. The enzyme from Promega produced some of the brightest bands but also several negative bands (3 of 4 replicates with 100 copies of template). The Taq

polymerase from Applied Biosystems was the only enzyme that consistently amplified all four replicates of 250 and 100 copies of pAW109 cDNA (Figure 3.8). The combination of optimised primer concentration and the use of Taq polymerase allowed the consistent amplification of less than 50 copies of template (Figure 3.7C).

These conditions for IL-1-β PCR were found to be generally transferable to other primer pairs and were used as the basis for subsequent optimisations. Late in the development of the PCR assays in this study, Taq Gold, a chemically modified polymerase became available (mid 1996). Taq Gold is activated by heat requiring more than 12 min at 94 °C to become fully active in contrast to antibody complexes which are rapidly dissociated above 70 °C [89, 90]. The enzyme has the advantage over antibody complexes of Taq polymerase in that it is considerably more stable at room temperature, allowing reactions to be set-up at room temperature without ice. The IL-4 and IL-5 competitive PCR assays in this study used 40 copies of competitor and Taq Gold. In these assays, the competitor was consistently amplified allowing mRNA negative reactions to be distinguished from the low number of failed reactions.

3.6.3 Avoiding False Positive PCR

The ability to amplify very small copy numbers of template confers a responsibility to prevent false positive PCR. In fact, when I first observed the Monte Carlo effect (Section 3.6.1), the experiments were repeated several times with fresh reagents to eliminate PCR contamination as the cause of the positive reactions. False positive reactions may arise from carryover from previous PCR or from the templates being used. Of particular concern with competitive PCR strategies is the use of concentrated stocks of competitor of up to 10^{11} copies/μL during the set-up of PCR. A small aerosol of even the more dilute stocks will contain sufficient template to render all reactions of a master mix positive. For example, a 0.1 μL droplet of a 10^4 copies/μL competitor stock diluted into a 10-reaction master mix of 500 μL will yield 100 copies in each 50 μL reaction.

The principles of Kwok and Higuchi [91] were followed to avoid PCR contamination but with some important additions. Pre and post-PCR procedures,

reagents and equipment were physically segregated in adjoining laboratories. All of the equipment (other than the biohazard cabinet) and consumables for pre-PCR work were used exclusively for these procedures. The majority of reagents and consumables were purchased pre-sterilised and ready to use. Set-up of cDNA synthesis and PCR was conducted in a class II biohazard cabinet and the work surface was covered with fresh laboratory bench paper. Aerosols were reduced by vortex mixing followed by centrifugation within the cabinet before opening any tube. Snap-capped tubes were used only for reactions and were opened with a cap lever to reduce aerosol-to-finger tip contamination. Standard pipettes were used with filter tips. After set-up, the biohazard cabinet and the equipment used was irradiated with UV for at least 30 min and often overnight for several hours.

3.7 CONCLUDING COMMENTS

I found that laser-based fluorescence scanning of agarose or PAGE gels could be established as a highly sensitive detection technique with excellent quantitative properties. This methodology, coupled with the ability to amplify 40 copies of target sequence consistently, laid the foundation for me to develop competitive PCR assays of cytokines in airway samples in asthma. As discussed in the introduction, these factors have previously constrained the ability to quantitate cytokines in asthma, reflecting poorly on the overall utility of molecular biology assays in asthma and similar diseases.

4 THE DEVELOPMENT OF COMPETITIVE PCR ASSAYS

4.1 INTRODUCTION

The proceeding chapters have described the acquisition of RNA from patients samples and the development of high-sensitivity PCR. This chapter follows the latter in the development of competitive PCR assays. Validation studies were used to determine the best method for measuring the PCR products as well as establishing the quantitative nature of each assay. All three stages of development are then drawn together to establish a series of competitive PCR assays of 8 mRNAs from a single cDNA synthesis.

4.2 MATERIALS AND METHODS USED

4.2.1 PHA-Stimulated PBMC

PHA-stimulated PBMC were prepared as described [62]. Aliquots of 3 x 10^6 cells were stimulated with 3 µg/mL PHA (Sigma-Aldrich, Castle Hill, NSW, Australia) for 24 hours, then collected by centrifugation, snap frozen in liquid nitrogen and stored at -80 °C. RNA was extracted using the spin column method (Section 2.2.4.2)

4.2.2 Competitor Templates and Primers

DNA competitor templates for IL-4, IFN-γ, TGF-β1, β-Actin and GAPDH PCR were obtained from Clontech (Palo Alto, CA, USA). IL-1-β and IL-2 PCR utilised a synthetic mRNA competitor, pAW109 cRNA (Perkin-Elmer, Foster City, CA, USA). Primers were synthesised by GeneWorks (Adelaide, SA, Australia) and were redesigned using Oligo 5.0 Primer Analysis Software (National Biosciences, Plymouth, MN, USA) to improve their specificity and annealing profiles (Table 4.2, page 65) [85, 86]. All competitors were diluted to working concentrations with 50 µg/mL nuclease-free glycogen (Roche Diagnostics Australia, Castle Hill, NSW, Australia) in 10 mM Tris-HCl, pH 7.5.

An IL-5 competitor was synthesised by PCR-mediated mutagenesis as previously described [92]. The 42 base composite primer was used with the lower IL-5 primer

(Table 4.2) in 30 cycles of PCR with 250 ng of human chorionic male DNA (Sigma Aldrich, Castle Hill, NSW, Australia) as the template. The PCR product was diluted 1:30 and 2 µl aliquots were amplified in five secondary reactions as for samples for 35 cycles. The resulting PCR product was purified by phenol-chloroform extraction and ethanol precipitation [62]. The competitor was resuspended in 10 mM Tris-HCl, pH 7.5 and quantitated by capillary spectrophotometry. The IL-5 competitor was sequenced bi-directionally by the Nucleotide Sequencing Facility, Monash University (Clayton, VIC, Australia) using Dye Terminator Cycle Sequencing Kit with AmpliTaq DNA Polymerase FS and an automated DNA sequencer (all from Applied Biosystems).

4.2.3 Validation of Competitive PCR

PCR of each cDNA was optimised using the principles described in Section 3.6 and included an analysis of sensitivity and specificity with both cDNA and competitor in each reaction. Reactions of 50 µL consisted of 2 U of AmpliTaq Gold, 1 x GeneAmp PCR buffer, 200 µM deoxyribonucleotide triphosphate (all from Perkin-Elmer), 0.2 µM upper and lower primers and $MgCl_2$ as listed in Table 4.1 (page 60). Thermal cycling was conducted in a MJ Research PTC-200, with the temperature profile commencing with an initial denaturation of 8 min at 94 °C, followed by cycles of 94 °C for 1 min, annealing temperature (Table 4.1) for 1 min and 72 °C for 1 min, concluding with 7 min at 72 °C and 1 min at 4 °C. IL-5 PCR was conducted differently in that a 2-step cycling protocol was used, omitting the usual extension step at 72 °C.

To establish that mRNA and competitor templates amplify equally, a "changing input-output ratio" series of reactions was set up for each gene. To test the validity of IL-2 competitive RT-PCR, a serial titration of pAW109 cRNA from 2 x 10^3 to 5.12 x 10^5 copies per reaction was established in 15 µl cDNA synthesis reactions (as in Section 3.2.3) containing 180 ng PHA-stimulated PBMC cell RNA. Aliquots of 2 µl cDNA were added to 100 µl PCRs, which were then split into duplicates and amplified for 35 and 40 cycles. For IL-5 competitive PCR, competitor was titrated in 2-fold steps from 5.12 x 10^4 copies to 200 copies against PHA-stimulated PBMC cDNA (synthesised as in Section 3.2.3) and duplicate reactions were amplified for

35 and 40 cycles. IFN-γ competitive PCR was tested similarly titrating from 8 x 10^4 copies to 3.13 x 10^2 copies competitor and amplifying for 30 and 35 cycles. For GAPDH and β-Actin, aliquots of BAL cell cDNA were combined in 100 µl PCR with a serial titration of competitor DNA from 8 x 10^5 to 4 x 10^2 copies. Reactions were split into duplicate 50 µl aliquots and amplified for 40 cycles. PCR products were electrophoresed through 4 % 19:1 polyacrylamide gels and stained with SYBR Green I before fluorometric quantitation (Section 3.2.2.2)

To test TGF-β competitive PCR, RNA was extracted from alveolar macrophages, which had been isolated from BAL by adherence to a microplate and then stimulated with lipopolysaccharide. The DNA competitor for TGF-β was titrated from 1.6 x 10^2 to 1.64 x 10^5 copies in 2-fold steps against a constant aliquot of cDNA synthesised from the alveolar macrophage RNA. The titration was prepared in triplicate 50 µL reactions and amplified for 37 cycles before electrophoresis in 3 % agarose gels. Ethidium bromide was not used as the signal from the fluoresceinated upper primer was found to be sufficient.

4.2.4 Reverse Transcription and Competitive PCR of Study Sample RNA

cDNA was synthesised from 0.27-3.86 µg RNA (mean 0.95 and 2.03 µg for biopsies and BAL cells, respectively) in a volume of 57 µl consisting of 1 x GeneAmp PCR buffer, 5 mM $MgCl_2$, 1 mM deoxyribonucleotide triphosphate, 0.8 U/µL RNase Inhibitor, 2 µM Oligo $d(T)_{16}$ and 2 U/µL MuLV Reverse Transcriptase (all from Perkin-Elmer). For cDNA synthesis from BAL cell RNA, 3 x 10^5 copies of pAW109 cRNA was added to each reaction, whilst for biopsy RNA, 4 x 10^3 copies of pAW109 RNA was used. Reactions were incubated for 1 min at 15 °C and then the temperature was increased to 42 °C over 9 min, followed by 1 h at 42 °C, 5 min at 85 °C and 1 min at 4 °C.

Table 4.1 Primer-Specific Conditions for PCR

cDNA	Annealing Temperature (°C)	Mg^{++} Concentration (mM)	PCR Product Size (bp)	
			Native	Competitor
IL-1-β	55	2	391	306
IL-2	56	1.5	229	305
IFN-γ	60	2	423	570
TGF-β1	60	2	161	270
IL-4 and IL-4δ2[1]	64	2.5	339 and 291	499
GAPDH	64	1.5	983	630
β-Actin	60	1.5	838	619
IL-5	60	1.5	493	409
IL-5 mutagenesis	55	1.5	-	409

[1] The development of IL-4 and IL-4δ2 competitive PCR is the subject of Section 5.

The assay specific parameters of annealing temperature and magnesium chloride concentration (Table 4.1), the amounts of cDNA reaction and competitor used (Table 4.3, page 85), and the primer sequences (Table 4.2, page 65) are detailed in the respective tables. For GAPDH and β-Actin competitive PCR, 2 µl of 1 in 80 diluted cDNA was added to a 50 µl reaction, consisting of 1 U of AmpliTaq Gold, 1 x GeneAmp PCR buffer, 1.5 mM $MgCl_2$, 200 µM deoxyribonucleotide triphosphates (all from Perkin-Elmer), 0.2 µM upper and lower primers and appropriate amounts of competitor. The temperature profile commencing with an initial denaturation of 8 min at 94 °C, followed by 40 cycles of 94 °C for 1 min, annealing temperature for 1 min and 72 °C for 1 min, concluding with 7 min at 72 °C and 1 min at 4 °C. IL-2 and

IFN-γ PCR was conducted in 50 µl reactions as above except that 5 µl and 3 µl of cDNA respectively and 45 cycles of amplification were used. For IL-4 (see Section 4.2.4) and IL-5 competitive PCR, 20 µl of cDNA was amplified for 50 cycles in a 100 µl reaction. The temperature profile for IL-5 competitive PCR differed in that a 2-step protocol omitting the usual extension at 72 °C was used.

All PCR products, except IL-4 and IL-4δ2, were resolved by electrophoresis through 1.5 mm, 4% 19:1 PAGE gels (acrylamide : bisacrylamide; Bio-Rad Laboratories) (Section 3.2.1.2). To quantitate IL-4 and IL-4δ2, 8 % 29:1 PAGE gels were used (Section cross ref). Gels were stained by overlay with 1:10,000 SYBR Green I (Molecular Probes, Eugene, OR, USA) in 2 x TAE for 45 min and then scanned directly in a laser-based fluorescence scanner (FluorImager 575, Molecular Dynamics). PCR product band volumes were quantitated with ImageQuaNT software (Molecular Dynamics). Native-competitor product ratios were calculated and the levels of gene expression reported as copies of mRNA per µg total RNA.

4.3 PCR COMPETITORS, PRIMERS AND THEIR DESIGN

4.3.1 Commercial PCR Competitors

The purchase of commercially available competitor templates is a cost effective means of obtaining such templates. At the time of the developmental phase of this work, two types of non-homologous competitors were available. A complementary RNA molecule, pAW109 cRNA, transcribed from a plasmid, was purchased. This synthetic mRNA-like molecule contains an array of upper primer sequences followed by the complementary sequences of the corresponding lower primers (Figure 4.1) [8]. The 3' end of the cRNA contains a polyadenylylated sequence allowing priming during reverse transcription of both the cRNA and sample mRNA with oligo(dT). DNA competitors, called PCR Mimics, were obtained from Clontech (Table 4.2, page 65). Each gene-specific competitor consists of primer sequences ligated to an irrelevant DNA sequence, V-erb B gene fragment [93]. The pAW109 cRNA was used in preference to PCR mimics for IL-1-β and IL-2, which were also available, predominantly because a cRNA allows for control of both the reverse transcription and PCR phases of the quantitation.

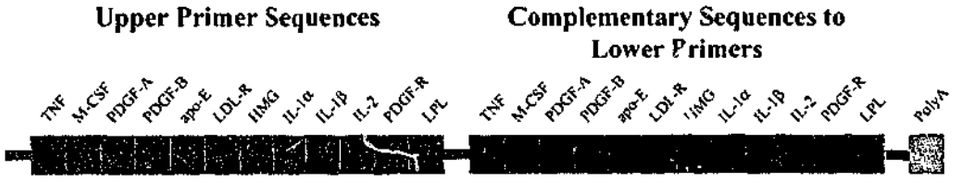

Figure 4.1 Schematic Representation of pAW109 cRNA

The diagram was deduced from the product specifications and the related cRNA described by Wang, Doyle and Mark [8].

The main problem with the use of commercial PCR competitors in this study was that the primer sequences were designed early in the development of PCR. Oligo Primer Analysis Software identified a number of unfavourable characteristics of the

primers, including runs of identical bases, 3' dimer formation, duplex formation and false priming [86, 94]. The software is also able to select primers likely to be highly specific by two mechanisms [85, 86]. Firstly, primers are chosen with internal stability (or strength of binding) that is high at the 5' end but low at the 3' end of the primer. With this stability profile, potential 3' mismatches of non-specifically bound primers are not sufficiently stable to be extended by Taq polymerase. Secondly, the software scans the gene sequence and user-selected related sequences for complementarity to potential primers. Because of these deficiencies, a number of the primers were re-designed with improved annealing profiles within the confines of the original primer sequences incorporated in the PCR competitors (Table 4.2, page 65).

4.3.2 Synthesis of IL-5 Competitor by PCR-Mediated Mutagenesis

Commercial competitor molecules for IL-5 were not available. A semi-homologous mutant was obtained by PCR-mediated deletion mutagenesis using human chromosomal DNA as the template. Primers were designed to amplify IL-5 cDNA with the upper primer spanning the exon 1–exon 2 junction and the lower primer sequence from the 3' untranslated region (Figure 4.2A). Oligo Primer Analysis software was used to find potential upper primers within the IL-5 genomic sequence that was compatible with the lower PCR primer. The last stage of the primer design involved using the software to analyse the efficacy of the lower primer and the cDNA-genomic composite upper primers as primer pairs in PCR. A primer sequence from intron C was chosen and when used as part of a composite primer, it has the effect of deleting exon 2 and 3 sequences and adding part of intron C in comparison to the native IL-5 PCR product (Figure 4.2B). This mutated PCR product is 17 % smaller than the native IL-5 amplicon.

To obtain the predicted mutated PCR product, PCR was carried out with chromosomal DNA, the composite upper primer and the lower primer using PCR conditions that had been determined by the usual optimisation process (Figure 4.2A). This product was then re-amplified with the cDNA-specific primer pair, to select for properly constructed PCR products. To help ensure that a non-specific product of a similar size was not amplified and to confirm that effective diluting out of the

composite primer was achieved, the optimisation of this step also included reactions with only individual primers. The mutated PCR product was sequenced and confirmed as the predicted sequence by BLAST (basic local alignment search tool) analysis [95]. This latter nucleic acid search tool accessible via the Internet searches Genbank and associated databases for sequences that have homologous alignments to a query sequence.

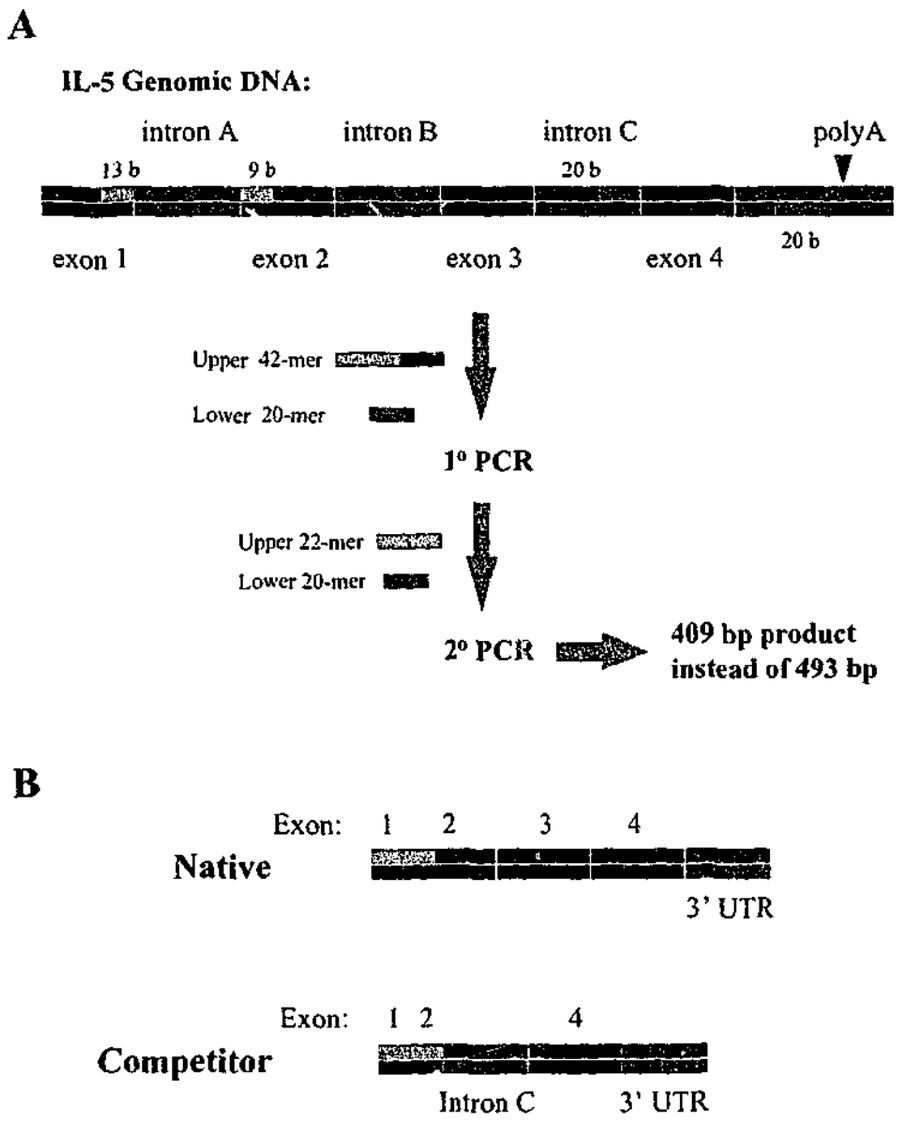

Figure 4.2 Construction of IL-5 Competitor by Deletion Mutagenesis

A) Schematic diagram of construction of IL-5 competitor. B) Schematic comparison of IL-5 native and competitor PCR products, depicting the origin of the sequences. 3' UTR, downstream untranslated region.

Table 4.2 Sequence of Primers Used

cDNA	Primer Sequence Used [1]	Source	Original Primer Sequence [2] where different from that used
IL-1-β	U: AAACAGATGAAGTGCTCCTTCCAG L: TGGAGAACACCACTTGTTGCTCCA	pAW109 cRNA	U: AAACAGATGAAGTGCTCCTTC-agg [3]
IL-2	U: GAATGGAATTAATAATTACAAGAATCC L: TGTTTCAGATCCCTTTAGTTCCAG	pAW109 cRNA	U: GAATGGAATTAATAATTACAAGAATCCc
IFN-γ	U: CGTTTTGGGTTCTCTTGGCTGTT L: CTCCTTTTTCGCTTCCCTGTTTT	Clontech Mimic	U: gcatCGTTTTGGGTTCTCTTGGCTGTTactgc L: CTCCTTTTTCGCTTCCCTGTTTTagctgctgg
TGF-β1	U: GCCCTGGACACCAACTATTG L: AGGCTCCAAATGTAGGG	Clontech Mimic	U: GCCCTGGACACCAACTATTGct L: AGGCTCCAAATGTAGGGgcagg
IL-4 and IL-4δ2 [5]	U: cCGGCAACTTTGTCCAC [4] L: TCTGGTTGGCTTCCTTCAC	Clontech Mimic	U: CGGCAACTTTGaCCACggacacaagtgcgata L: acgtacTCTGGTTGGCTTCCTTCACaggacag
GAPDH	U: TGAAGGTCGGAGTCAACGGATTTGGT L: CATGTGGGCCATGAGGTCCACCAC	Clontech Mimic	
β-Actin	U: GCACCACACCTTCTACAATG L: TGCTTGCTGATCCACATCTG	Clontech Mimic	U: atctgGCACCACACCTTCTACAATGagctgcg L: cgtcatactccTGCTTGCTGATCCACATCTGc
IL-5	U: GATAGCCAATGAGACTCTGAGG L: TTGCCTGGAGGAAAATACTT	In House	
IL-5 mutagenesis	U: GATAGCCAATGAGACTCTGAGGTGGCTGTGCCTATTTCTATG [6] L: TTGCCTGGAGGAAAATACTT	In House	

Notes

[1] Upper (U) and lower (L) primers are written 5' to 3'.

[2] Bases that differ from the primer sequence used is indicated by lowercase.

[3] Difference in 3' end of this primer is due to deletion of a C (indicated by a dash) in this sequence.

[4] An extra base (indicated by lowercase) not present in the mimic was added to the primer.

[5] The development of IL-4 and IL-4δ2 competitive PCR is the subject of Section 5.

[6] The sequence in the upper composite primer identical to the IL-5 upper primer is indicated by underlining.

4.4 VALIDATION OF COMPETITIVE PCR

The legitimacy of an assay is dependent on the documentation of validation studies of the assay. Given the complexity of competitive PCR and competitive RT-PCR, the exponential amplification nature of PCR and the range of detection and quantification strategies, it is surprising that a large number of publications do not refer to validation studies [for example 9, 96, 97, 98]. However, it is noted that, after the initial description of a method, many journals (and authors following their lead) prefer to concentrate on the biologically relevant data of a study and validation tests may often not be published. The complexity of competitive PCR assays is highlighted by a number of studies where the validation test actually indicates failure of quantitative competition but this has not been recognised or has been misinterpreted [99-101].

A large number of studies and reviews state that reactions should only be analysed in the exponential phase and not in the plateau phase of amplification [102-104]. However, an equally large number of validation studies of competitive PCR indicate that reactions in some assays can be conducted during the plateau phase [3, 105, 106]. There are three main reasons for this dissent: 1) The concept may be a carry-over from semi-quantitative PCR methods where all samples must be amplified to the same degree. 2) Non-specific products are most influential in latter stages of amplification and may begin to detract from the quantitative nature of some assays during the plateau phase. 3) Heteroduplex formation between native and homologous competitor PCR products have effects on quantitation similar to non-specific products [10, 107, 108]. During the exponential phase, most templates in the reaction are extended to form homoduplexes. During the plateau phase, most templates are not extended and can form heteroduplexes during reannealing [109]. Dependent on the detection system employed, heteroduplexes may or may not be distinguishable from competitor and native templates [110]. Validation studies should be designed to confirm that the competitive PCR assay as conducted allows quantitation of the initial native template.

4.4.1 The Mathematics of Competitive PCR

Competitive PCR is dependent on the starting ratio of native and competitor remaining unchanged throughout all subsequent steps. The accumulation of molecules of native (N_n) and competitor (C_n) templates after n cycles of amplification can be expressed mathematically by:

$$N_n = N_0(1+E_N)^n$$
$$C_n = C_0(1+E_C)^n$$

Equation 4.1

where N_0 and C_0 are the starting number of copies and E_N and E_C are the efficiencies of amplification expressed as a fraction between 0 and 1 for the native and competitor templates, respectively [111]. Validation experiments usually aim to test for equal rates of amplification of native and competitor templates with tne complete quantitative method producing equality between input and output ratios:

$$\frac{N_n}{C_n} = \frac{N_0}{C_0}$$

Equation 4.2

This validation allows the original copies of native template to be calculated:

$$N_0 = \frac{N_n}{C_n}C_0$$

Equation 4.3

Often absolute quantification is not required but the measurement of all samples must be directly relative to each other, such that:

$$\frac{N_n}{C_n} = k\frac{N_0}{C_0}$$

Equation 4.4

where k is a constant. In this instance, if Equation 4.3 is used to calculate N_0, the data will be transformed by a fraction of k from their absolute values [12]. The majority of validation methods of competitive PCR are incapable of distinguishing absolute and relative quantitation. Know amounts of input native template are required to detect the transformation indicated in Equation 4.4 [111, 112]. Equal amplification efficiencies are required for absolute quantitation but for relative quantitation the ratio:

$$\frac{(1+E_N)}{(1+E_C)}$$

Equation 4.5

must remain constant across the quantitative range of native inputs. The efficiencies of amplification of native and competitor need not be identical and may be different for different inputs [111]. A mathematical treatise of the effect of amplification efficiencies is given in Appendix 8.1. Changing amplification efficiencies are a reality of PCR and as reactions enter the plateau phase the efficiency changes dramatically (Section 1.1.1, Figure 1.2).

4.4.2 Methods of Validating Competitive PCR

A wide range of validation methods have been used, without any real consensus to date. Methods follow three general strategies with reactions comprising of: 1) a single starting ratio amplified for various numbers of cycles, 2) a changing input ratio series of reactions, or 3) a series of known amounts of native template. Often a combination of these methods is used but for simplicity, they are discussed here individually.

4.4.2.1 The cycle number validation method

The analysis of a reaction or replicates over various cycles of amplification is the simplest strategy. A variation is to keep the number of cycles constant, but to serially dilute a combination of native and competitor templates [113]. Whilst the effect of cycle number on the output ratio is analysed in some studies [114-117], the more common analysis is to qualitatively compare curves of native and competitor band intensities over various cycles [8, 118-120]. Parallel band curves are considered indicative of quantitative competition. However, if output ratios do change or band curves are not parallel with the number of cycles, then relative quantitation can still be achieved providing all ratios in the quantitative range change proportionally at a given assay cycle number as indicated by Equation 4.4 (Section 4.4.1) [4]. The major disadvantage of this strategy is that it only looks at one starting ratio and therefore does not identify relative quantitation and nor does it define the quantitative range.

If absolute quantitation is the aim of the assay, then not knowing the initial input copy number of native template means that it is not possible to determine if the output ratio is correct or to preclude deviation from the initial input ratio in the cycles before detection. When using the cycle number validation method, PCR products are often not detectable until the beginning of the plateau phase by which time the ratio may have stopped changing.

4.4.2.2 Validation with known inputs

Validation studies using known amounts of native templates have utilised plasmids [121-123], purified PCR products [124], cRNA molecules [125], genomic DNA [126-128] and even RNA [4, 129] with known copy numbers of target sequence. Whilst the PCR products of these native templates are indistinguishable from that obtained from test samples, the composition of these control samples often differs widely from test samples, particularly in the amount of background nucleic acids. Boivin et al, in an attempt to simulate a typical test sample, found a decrease in amplification efficiency when 1 µg negative cellular DNA was added to control reactions containing plasmid as the source of native template, presumably because of interference from cellular DNA [123].

Typically, a standard curve analysis is conducted with linearity being the favourable outcome rather than a comparison of expected and obtained values. A standard curve may be used as a once-only calibration of the assay [4, 127, 128] or it may be used in every PCR run [121-123, 129]. The latter approach is particularly important when a microplate capture-hybridisation detection method is employed [121, 122, 124]. Standard curves, whether conducted from PCR amplification or only within the detection system itself, are required to normalise each batch for variations between native and competitor PCR products in capture and probe hybridisation efficiencies. The calibration of competitive PCR assays by standard curves allows correction for incorrect slope of input versus output curves [4, 122], often unwittingly by the user [118, 121, 130-132]. The slope of input-output curves is important for the resolution of the method. A small compaction of the output range with respect to the input

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[4] This critique of the method was inspired by the principles discussed in Section 4.4.1 but are my own deductions.

range is amplified when copy numbers are calculated from the ratio. The use of standard curves adds to the complexity and cost of the assay and may increase the number of batches of reactions needed in a study.

4.4.2.3 The changing input ratio validation method

Validation of competitive PCR by analysis of a changing input ratio series of reaction was first used by the one of the founding groups that developed competitive PCR [133]. Usually the competitor molecule is titrated against a constant aliquot of a sample or cDNA known to be positive for the native template. Whilst the reverse titration is more reflective of the input of native template when the assay is in use, competitive PCR assays mostly use a relatively consistent amount of test sample and by inference, a consistent amount of background nucleic acids. In addition to indicating quantitative competition between native and competitor templates, the titration can define the quantitative range of the assay. Unless known inputs are used, this validation method can not distinguish between relative and absolute quantitation [111].

The concept of testing the outcome of a series of input ratios is simple but the analysis of the data has often been flawed, with a number of researchers failing to recognise an unsuccessful validation test [12]. Mostly, the mathematical basis of competitive PCR has been neglected and journals do not seem to have been critical enough. Commonly, the logarithms of the output ratios are plotted against the logarithm of the number of competitor molecules added to the reactions. Most researchers consider linearity to be the valid outcome, but, even for relative quantitation, output ratios should actually follow a line of slope -1 [111]. The output ratios in the TGF-β example (Figure 4.3) followed a least-squares regression well (r = 0.98) but the slope was -0.49 (compare to theoretical line)[5]. When either template is added in excess, it out competes the other during amplification. Whilst a linear relationship does exist between input and output ratios, if the copy number of the native template is calculated using Equation 4.3 (Section 4.4.1) the result is highly dependent on the amount of competitor used (Figure 4.3C). Given the simplicity of

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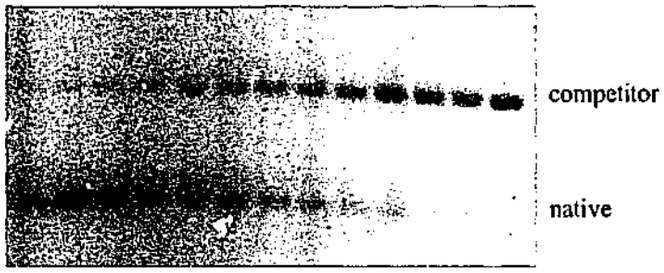
[5] The TGF-β assay was developed in conjunction with Dr Ling Zheng as part of a process of teaching her how to conduct competitive PCR (1998).

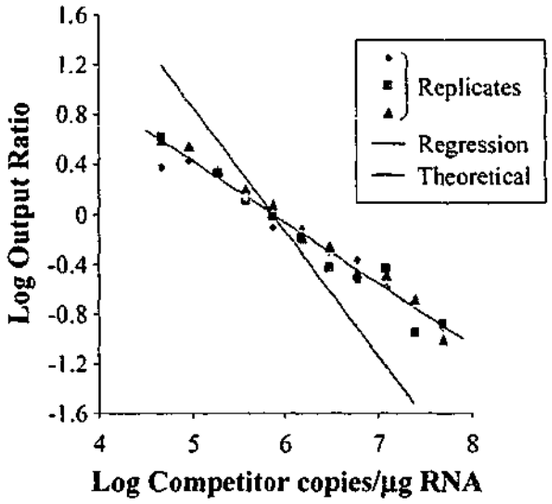
this latter plot, it is surprising that so many researchers have failed to recognise the erroneous use of assays that do not yield titrations that conform to a slope of -1 [for example 99]. Competitive PCR with the commercial TGF-β competitor has been described previously but the report did not refer to any validation studies [134].

Plots of output ratios against competitor used are difficult for visualising the validity of the competitive PCR assay, particularly if theoretical or predicted curves are not used. I have found that plots of output ratios against input ratios are far simpler to understand. It is intuitive that input ratios should remain unchanged by the quantitative process and titrations on such plots should map to a line with a slope of 1 as predicted by Equation 4.4. Some researchers have used known inputs (as in Section 4.4.2.2) to obtain input ratios [107, 128]. I developed a method of calculating relative input ratios by obtaining an estimation of the input native copies from the average result of the closest ratios to 1.0. Plots of output ratios against relative input ratios result in the data being centred around the 1:1 ratio of a theoretical line of the relative ratios plotted against themselves (Figure 4.3D).

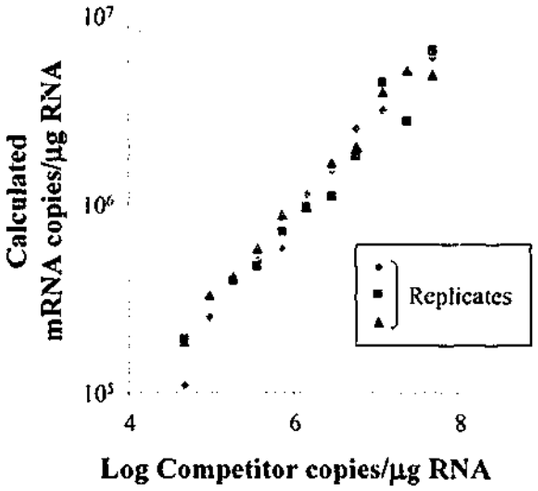
A


competitor
native

B


1.6
1.2
0.8
0.4
0
-0.4
-0.8
-1.2
-1.6
Log Output Ratio
4
5
6
7
8
Log Competitor copies/µg RNA
Replicates
Regression
Theoretical

C


10^7
10^6
10^5
Calculated mRNA copies/µg RNA
4
6
8
Log Competitor copies/µg RNA
Replicates

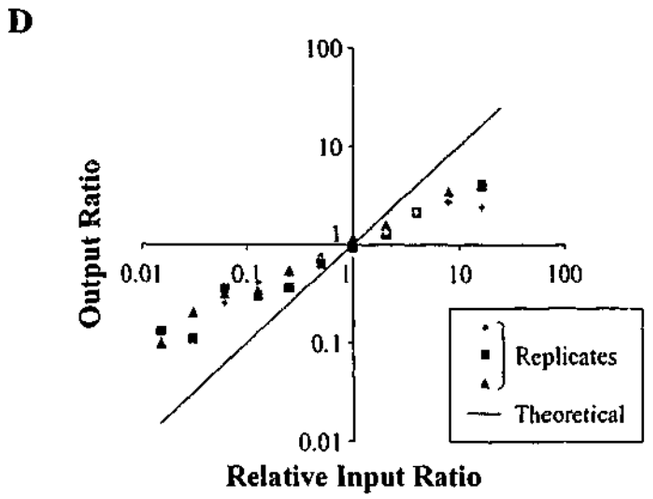


Figure 4.3 Validation Studies of TGF-β Competitive PCR Indicate Non-quantitative Competition.

TGF-β competitor was titrated in triplicate 2-fold steps against a constant aliquot of BAL cell cDNA and PCR was conducted for 37 cycles. A) Agarose gel of titration. B) Plot of native to competitor output ratios against the number of molecules of competitor added. The data fit the regression well but do not conform to theoretical line of slope –1 required for quantitative competition. C) Data from (B) showing the effect of non-quantitative competition on the calculated copy number of TGF-β cDNA in the single sample used in the titration. The copy number was calculated using Equation 4.3 (Section 4.4.1) and shows a dependence on the amount of competitor added. D) Plot of same titrations using relative input ratios. The average result of the three replicates closest to 1 was used to calculate the relative input ratios.

4.4.3 Validation Studies Supporting the Assays in this Study

Commercial competitors are not purchased with any guarantee that they will compete quantitatively with their native template during PCR amplification. It is important to test user-specific parameters of the assay such as cycling profile and method of band measurement. The detection system was also tested independently of the PCR (Section 3.3.2, Figure 3.4). The changing input ratio method was used in these studies, usually with replicate reactions being amplified for different numbers of cycles. The titrations were used to define the native and competitor PCR products within gel images, which at times involved the inclusion or avoidance of minor bands close to the expected specific band.

The expression of cytokines in BAL cells and biopsies was generally found to be less than 10^3 copies per μg RNA. This low expression level and the difficulty in obtaining relatively large amounts of RNA ruled out using study samples to validate the competitive PCR assays. The mRNA levels of IL-5, IL-2 and IFN-γ, were found to be approximately 10 times higher in PHA-stimulated PBMC. This expression level and the ease of obtaining plenty of RNA made PHA-stimulated PBMC a practical source of native template for validation tests. The expression of the housekeeping genes GAPDH and β-Actin in BAL cells was more than 10^4 times that of these cytokines enabling pooled BAL cDNA to be used in titrations.

The DNA competitors for IL-5, IFN-γ, β-Actin and GAPDH were titrated in 2-fold steps against a constant aliquot of cDNA using 3 dilutions of competitor (Figure 4.5). For each titration, the output ratios followed a theoretical line of slope 1 reasonably well. The numbers of cycles of PCR for each titration were observed to be in the plateau phase of amplification. A qualitative comparison of GAPDH and β-Actin titrations, where replicate reactions were amplified for 40 cycles, with that of IL-5 and IFN-γ indicates that the variability with number of cycles is similar to that obtained with replicates. PAGE gel artefacts, predominantly fluorescent contaminants and band smearing, were observed to be the main cause of variability. The effect of band smearing artefacts is seen in the low output ratios of the GAPDH and β-Actin titrations (Figure 4.5 C and D). Subsequent improvements to the PAGE

technique essentially eliminated this artefact. The validation of IL-2 competitive RT-PCR was conducted in a similar manner and with comparable results except that pAW109 cRNA was titrated in cDNA synthesis reactions before plateau phase PCR amplification (Figure 4.4).

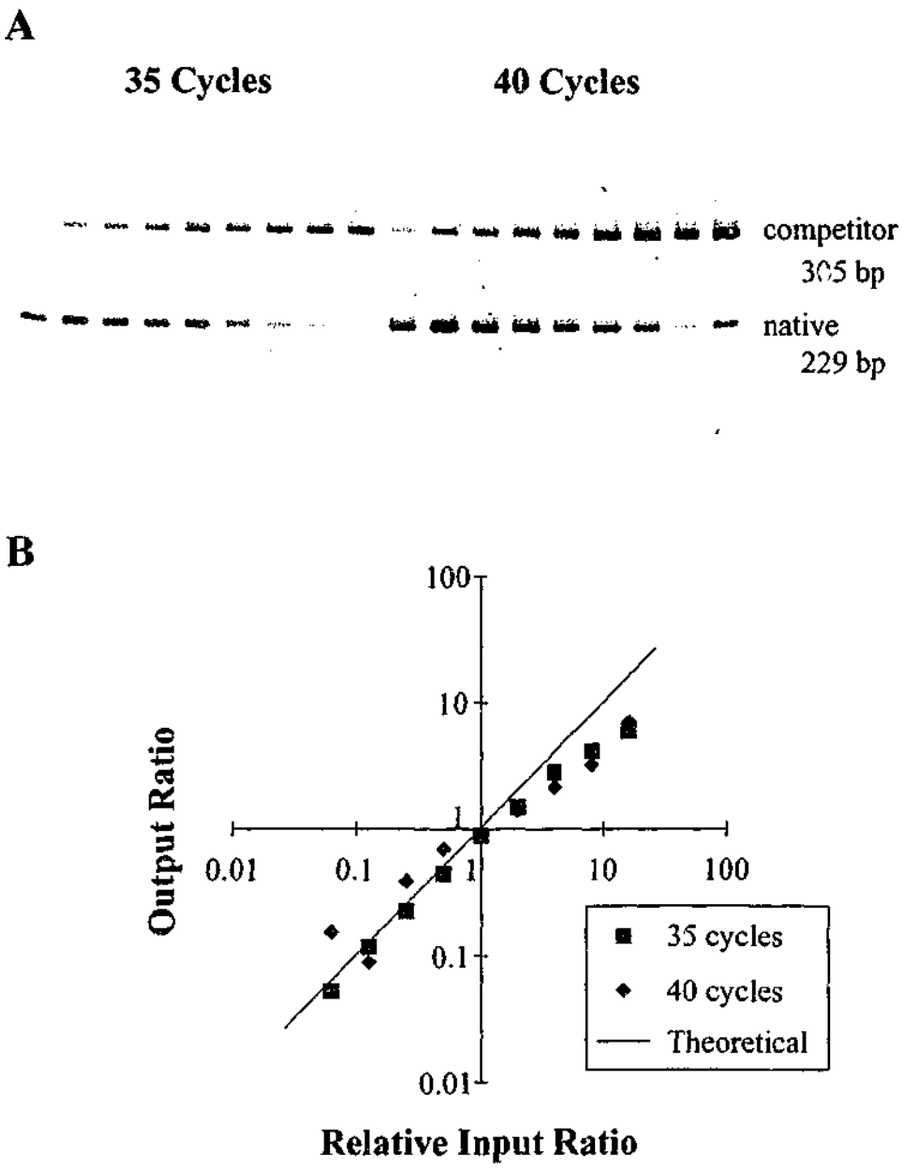


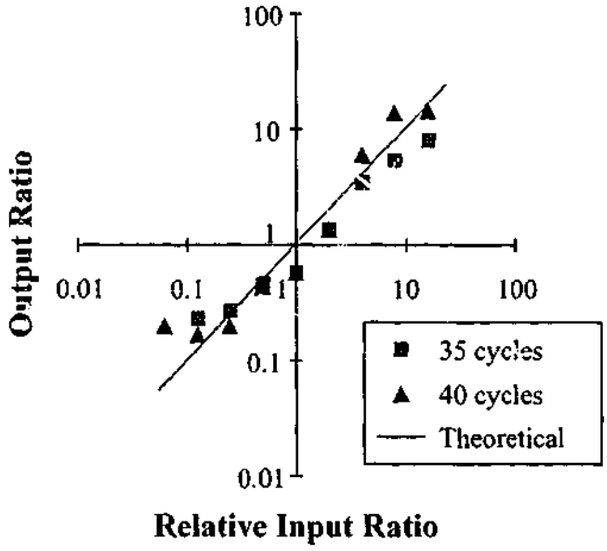
Figure 4.4 Validation of IL-2 RT-competitive PCR

A) The IL-2 competitor, pAW109 cRNA, was titrated in 2-fold steps against a constant aliquot of PHA-stimulated PBMC RNA in cDNA synthesis reactions. After subsequent PCR, aliquots were electrophoresed on a PAGE gel. B) Output ratios from (A) plotted against relative input ratios.

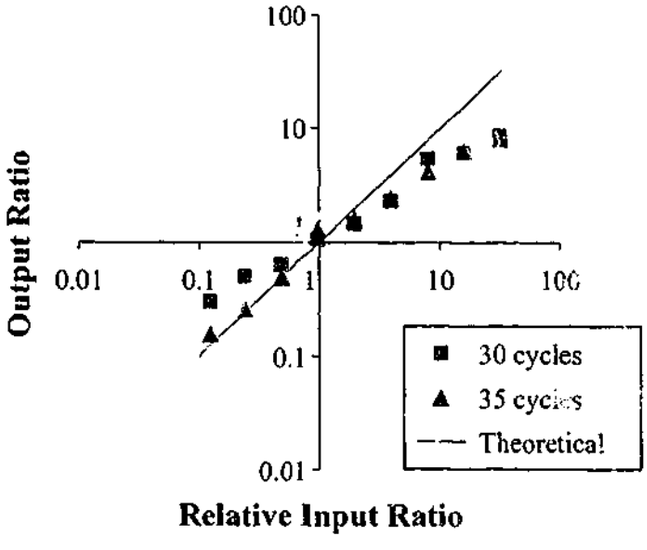
A


35 Cycles
native
competitor

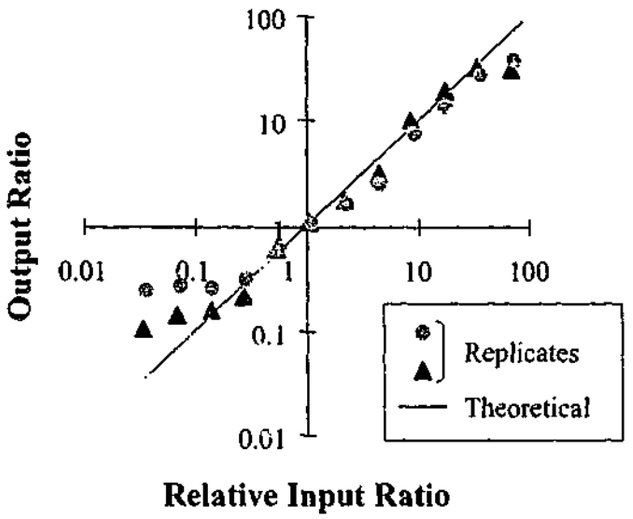
B


Output Ratio
100
10
1
0.1
0.01
0.01
0.1
1
10
100
35 cycles
40 cycles
Theoretical
Relative Input Ratio

C


Output Ratio
100
10
1
0.1
0.01
0.01
0.1
1
10
100
30 cycles
35 cycles
Theoretical
Relative Input Ratio

D



E

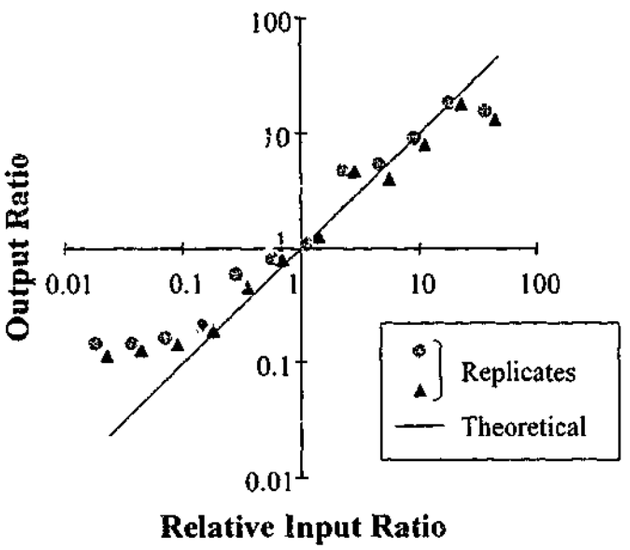


Figure 4.5 Validation of IL-5, IFN-γ, β-Actin and GAPDH Competitive PCR

A) PAGE gel of titrations of IL-5 competitor (increasing left to right in 2-fold steps) against a constant aliquot of PHA-stimulated PBMC cDNA. B) Output ratios from (A) plotted against relative input ratios. C) Changing input ratio series of reactions of IFN-γ competitive PCR were amplified for 30 and 35 cycles. Similar titrations were conducted to test β-Actin (D) and GAPDH (E) competitive PCR assays but replicate reactions were amplified for 40 cycles.

4.4.4 Methods of Calculating Native Input Copies

Most researchers (including me, in this study) calculate the input copies of native template from Equation 4.3 derived from the input ratios being equal to the measured output ratios (Section 4.4.1). In contrast, studies using competitor molecules that differ from the native by size in conjunction with detection systems that are size dependent (ethidium bromide staining for example) have adjusted the calculation by the ratio of the size of the templates:

$$N_0 = \frac{N_n}{C_n} C_0 \frac{L_C}{L_N} \qquad \text{Equation 4.6}$$

where L_C and L_N are the lengths of the competitor and native templates respectively [117, 135]. It is intuitive that large PCR products will stain more than smaller DNA fragments and that staining-based detection systems do require correction to achieve absolute quantitation. However, it is my observation that corrections, such as in Equation 4.6, have been applied incorrectly and in the case of relative quantitation, have been applied unnecessarily[6]. Equation 4.6 assumes that there is a direct linear relationship between the length of the PCR products and the detection signal. Intercalating dyes, such as SYBR Green I, stain DNA dependent on size but there is no published evidence of a direct linear relationship (of the form y = mx). However, my test of SYBR Green I staining of PAGE and subsequent fluorometric quantitation indicated that a linear relationship does exist (of the form y = mx + c) but this experiment is unable to determine if it is directly linear (Section 3.3.2). The length ratio has the effect of mathematically transforming the data such that absolute values change but relative values do not. Such correction factors, if not applied consistently, may simply add another potential source of error to relative quantitation.

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[6] I do not recall seeing this critique in the literature.

An alternative strategy of determining native template copies focuses on the output ratio of 1. A titration of competitor is conducted for every sample and a regression of the logarithms of the competitor copies and output ratios is used to calculate the competitor copies that would give an output ratio of 1 [105, 136, 137]. Most often it is assumed that an output ratio of 1 would originate from an input ratio of 1 and that the calculated competitor copies is equivalent to the input copies of native template. This assumption is rarely validated but some studies have conducted parallel titrations with different amounts of test sample [93, 138]. This validation test can confirm relative quantitation if there is the expected relationship between the different amounts of test sample, but can not confirm absolute quantitation.

The use of regression analysis to calculate native template copy numbers can be a solution to the problem of non-quantitative competition such as that I found for TGF-β (Section 4.4.2.3) [105, 135]. The distinct disadvantage of this method is that it adds considerably to the number of reactions, the amount of sample used and the complexity of data analysis. Most studies have used a 4-point titration to conducted regression analysis [93, 138, 139] but some studies have used up to 9 reactions per sample [140]. It has been shown that, when validation studies indicate quantitative competition, the use of titrations for quantitation does not afford any more accuracy than conducting replicate single-point assays [110].

4.4.4.1 Utilisation of the Linear Relationship Between TGF-β Input and Output Ratios

As discussed previously (Section 4.4.2.3), the validation studies of TGF-β competitive PCR clearly indicated that Equation 4.3 should not be used to calculate the native input copies. To avoid the complexity of standard curves or titrations for every sample, I developed a relative standard curve method that utilised the consistent linear relationship between the input and output ratios (Figure 4.3, page 74).

As the output ratios in the TGF-β assay are distorted by the amplification procedure, the aim of the standard curve construction process was to establish the relationship between the output ratios and input ratios (Figure 4.6). Firstly, the linear regression method discussed above was used to estimate the TGF-β copy number for the

BAL cDNA used in the triplicate titration. Using this input copy number relative input ratios were calculated. Linear regression was then conducted with the logarithms of the input and output rations:

$$Log(output\ ratio) = 0.49Log(input\ ratio) + 1.48\times10^{-6}$$

This equation allows a relative input ratio to be calculated from a single-point measurement of an output ratio. Using the input copies of competitor, relative inputs of native template can then be calculated (Figure 4.6B). Once the relative standard curve is established, this method allows quantification of TGF-β mRNA levels with only one calculation more than for traditional assays.

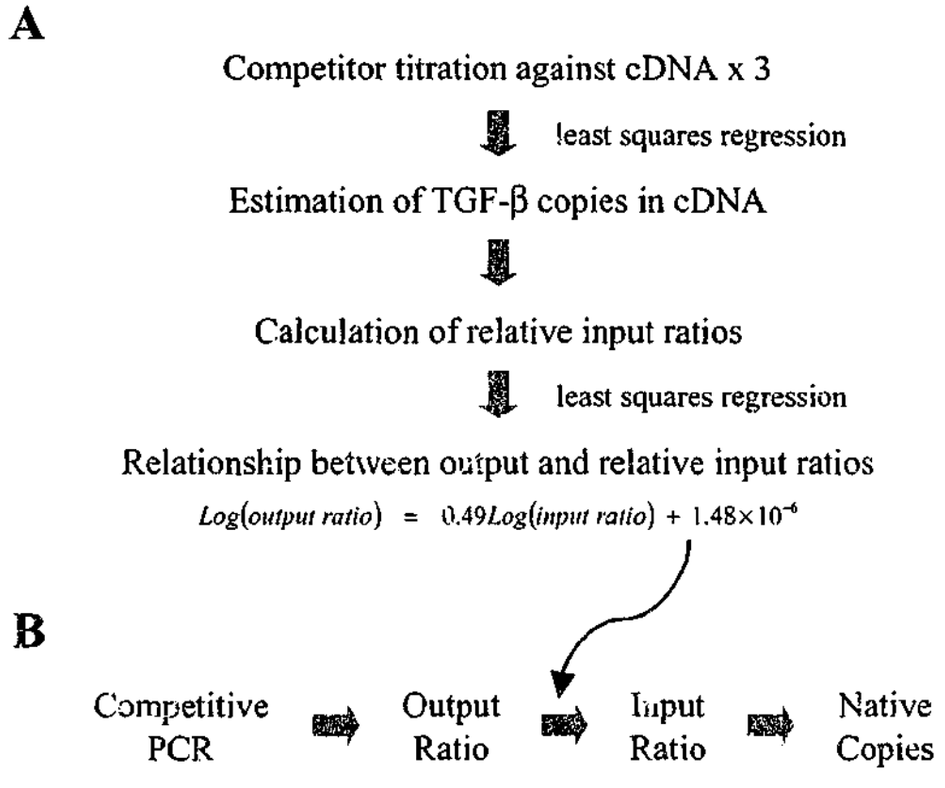

Figure 4.6 Construction of relative standard curve for TGF-β competitive PCR

A) Schematic diagram showing the process of constructing a relative standard curve for TGF-β competitive PCR. B) Schematic diagram showing the use of the relative standard curve in calculating TGF-β copy numbers in patient samples.

4.5 APPLICATION OF COMPETITIVE PCR TECHNOLOGY TO A CROSS-SECTIONAL STUDY

4.5.1 Pilot studies to determine optimal competitor amounts

Initial work with BAL cells and airway biopsies as part of the assay optimisation process indicated that cytokine expression in these samples was very low and assays for IL-4, IL-4δ2 and IL-5, in particular, may often not detect any mRNA. The amount of RNA extracted from the study samples was also very low with median yields of 1.1 µg and 3.1 µg for biopsies and BAL cells respectively (Section 2.5, Table 2.2). The competitive PCR assays were developed and validated with the aim of conducting only one PCR to determine mRNA expression levels. To achieve this aim, the low mRNA expression levels and total RNA input into the assays needs to be balanced by optimal amounts of competitors to obtain informative reactions.

To optimise the competitor amounts for each assay, the six BAL cell and biopsy samples from asthmatics with the highest yield of RNA were tested. An example of this process is given in Figure 4.7. For analysis of BAL cell samples, RNA was added at the recommended maximal rate of 1 µg per 20 µL cDNA synthesis reaction (Applied Biosystems product specifications). For cDNA synthesis from biopsy RNA, this rate was not possible with even the highest-yielding biopsy samples due to the low concentration of RNA in these samples. To test the six biopsy samples, the maximum possible volume of RNA was added, resulting in a median of 0.50 µg (range 0.35 – 0.69 µg) RNA being added per 20 µL of reaction. The amount of RNA competitor, pAW109 cRNA, added during reverse transcription was optimised so that both IL-2 and TNF (not discussed in this thesis[7]) assays could be conducted from the same cDNA. Subsequent optimised parameters included amount of competitor, amount of cDNA added to PCR, the volume of the PCR (50 or 100 µL) and the number of amplification cycles. In order to obtain reasonable sensitivity for IL-4 and IL-5 assays, 20 µL cDNA was added to each 100 µL PCR and

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[7] Whilst I developed and carried out the TNF assay, my colleague, Maja Divjak analysed the data as part of her PhD. Consequently this competitive RT-PCR assay is not elaborated on here.

amplification was conducted for 50 cycles (Table 4.3, page 85). For IL-2 and IFN-γ assays, 5 μL and 3 μL aliquots of cDNA respectively in 50 μL PCR was found to give adequate sensitivity. In contrast, GAPDH and β-Actin are expressed at levels about 10^4 times that of the cytokines and could be quantitated from a 1 in 80 dilution of the cDNA.

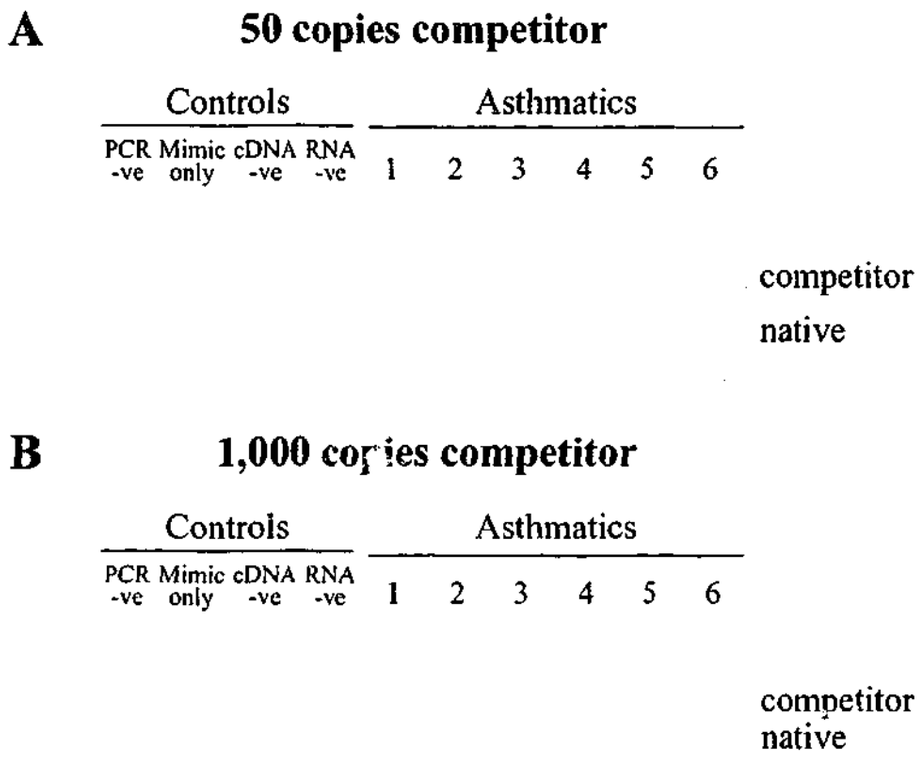


Figure 4.7 Optimisation of amount of IFN-γ competitor

Agarose gels of competitive PCR test of six asthmatic BAL cell samples with 50 copies (A) and 1,000 copies (B) of competitor per 5 μL of cDNA. With 50 copies of competitor, the native PCR product out-competes the amplification of the competitor in all samples except number 1. When 1,000 copies of competitor were used quantifiable reactions were obtained for all samples. The final IFN-γ assay used 600 copies of competitor and 3 μL of cDNA (Table 4.3). The negative (-ve) controls are indicated. Mimic only; competitor only control.

4.5.2 Multiple assays from a single cDNA synthesis

To make the most efficient use of each study sample RNA, a single 57 µL cDNA synthesis reaction was made from which all the competitive PCR assays were conducted (Table 4.3). These reactions allowed a maximum of 27 µL of sample RNA to be added. For all but 1 biopsy RNA sample and for 60 % of the BAL cell RNA samples, the maximum volume of RNA was added, as this amount of RNA did not exceed the recommended maximum input rate for reverse transcription.

To cope with variable inputs of RNA and to maintain competitor amounts relative to the quantity of RNA transcribed, the RNA samples were stratified according to RNA concentration and divided into batches. The bulk of the data obtained in this study (173 samples) was obtained from 6 batches of cDNA synthesis from BAL cell RNA and 5 batches of reverse transcription from biopsy RNA. The batching of reactions was maintained throughout PCR amplification and was adjusted to a minor extent before electrophoresis. For both BAL cell and biopsy samples, intermediate and low RNA yield batches were identified to which a half and a quarter, respectively, of the optimal amount of competitor was added. This approach may have been instrumental in achieving less than five non-informative reactions for the complete study. The strategy of conducting the assays in RNA concentration-stratified batches effectively randomised the samples with respect to the presence of asthma and atopy and the utilisation of treatment, which was an added bonus to the methodology.

Table 4.3 Division of cDNA synthesis and optimal competitor amounts

	cDNA Volume (µL)	RNA Volume (µL)	Copies of Competitor Added[1]	
			BAL Cells	Biopsies
Spectrophotometry	–	4	–	–
GAPDH and	–	1	2.5×10^3	500
β-Actin pseudogenes[2]			1×10^4	400
IL-5	20	9.5	40	160
IL-4 and IL-4δ2	20	9.5	40	40
IL-2	5	2.4	3×10^5	4×10^3
IFN-γ	3	1.4	600	200
TNF	2.5	1.2	3×10^5	4×10^3
GAPDH and	1	0.5	2.5×10^4	5×10^3
β-Actin[3]			1×10^5	4×10^3
Total volume required (µL)	51.5	29.3	–	–
Volume available (µL)	57	< 40[4]	–	–

[1] Optimal amounts of competitor. Amounts were reduced for lower RNA inputs.

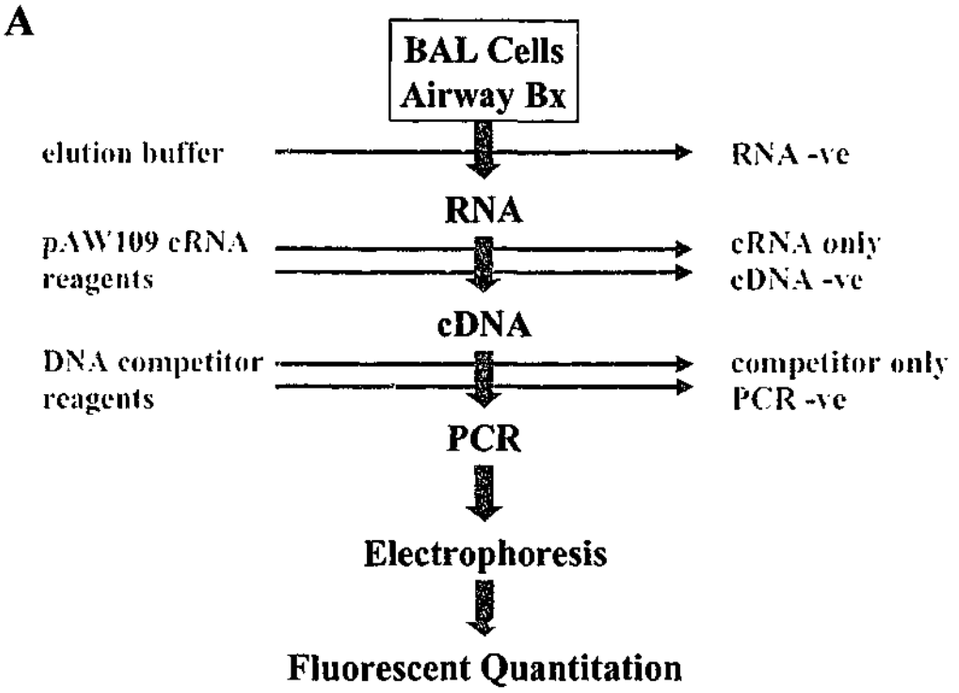
[2] Conducted from 2 µL aliquots of a 1 in 80 dilution of RNA, with out reverse transcription (Section 6.2.2)

[3] Conducted from 2 µL aliquots of a 1 in 80 dilution of cDNA.

[4] RNA was eluted from spin columns with 40 µL Tris buffer with variable recovered volume usually more than 30 µL (Section 2.2.4.2).

4.5.3 Controls for competitive PCR assays

The principle of a negative control at each stage [91] was followed for the competitive PCR assays in this study. Negative controls were usually made as the last step of each procedure to better reflect contamination events that may have occurred at that stage. Controls were treated identically to patient reactions in downstream steps of the assays. The aliquots of elution buffer reserved during each batch of RNA extraction served dual roles as the zero solution for spectrophotometry and as a control for contamination of the elution buffer (RNA negative control). During reverse transcription a 'pAW109 cRNA-only' control was added as well as a cDNA synthesis negative control. Likewise, during set-up of PCR for assays using DNA competitors, a 'competitor-only' control was incorporated in addition to the PCR negative control. A 'competitor-only' control functioned as a negative control for contamination of the competitor dilution with native template as well as a positive control of the set of reactions that is independent of the previous step(s). Assays of housekeeping mRNA levels required dilution of cDNA and a 'diluent-only' control was added to detect contamination of this step. The use of a comprehensive series of negative controls allowed contamination events to be readily traced. This was an important feature of the assays, as reactions could not be easily repeated due to the limited quantity of each sample.



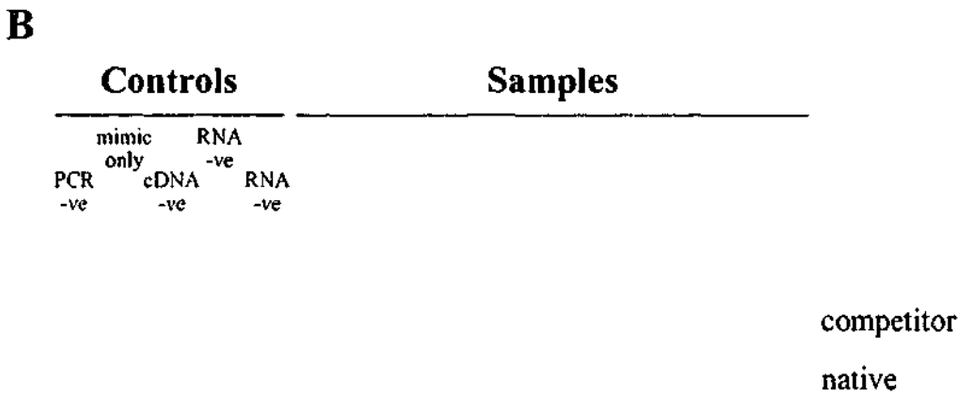


Figure 4.8 Controls of Competitive PCR Assays

A) Schematic diagram showing the steps at which controls are incorporated into the competitive PCR assays. Inputs into competitive PCR assays at each step are indicated on the left and the respective negative (-ve) controls are indicated to the right of the flow diagram of the assay. For assay of IL-2 mRNA by competitive RT-PCR, which utilises pAW109 cRNA, no DNA competitor is used and therefore there is no corresponding negative control in this case. B) Gel of IFN-γ competitive PCR assay showing typical control reactions.

4.6 CONCLUDING COMMENTS

I have performed validation studies for the competitive PCR assays, adhering to the mathematical principles of quantitative competition, which have established quantitative assays with defined quantitative ranges. A similar process was applied to the development of a novel method for quantitating IL-4 and its alternative splicing variant, IL-4δ2, as described in the following chapter (Section 5.3.3). The validation studies have allowed the assays to be applied with confidence to the cross-sectional series of RNA samples from asthmatics and normal controls.

5 DEVELOPMENT OF DUAL mRNA-SPECIES COMPETITIVE PCR FOR IL-4 SPLICING VARIANTS

5.1 INTRODUCTION

The human IL-4 gene contains 4 exons separated by 3 introns [141]. IL-4 mRNA contains all 4 exons spliced together whilst an alternatively spliced mRNA, IL-4δ2, contains the coding sequences of exons 1, 3 and 4 spliced together in an open reading frame with the sequence of exon 2 omitted (Figure 5.1) [142]. Alternative splicing of human IL-4 mRNA was first described in phytohemagglutinin (PHA)-stimulated peripheral blood mononuclear cells (PBMC) [142] and subsequently in phorbol myristate acetate stimulated leukemia and lymphoma cell lines [143] and even in some non-lymphoid leukemia/lymphoma cell lines [144]. Purified $CD3^+$ T cells [145] and B cells [144] have been shown to produce both IL-4 and IL-4δ2 mRNA when mitogenically stimulated. IL-4δ2 mRNA has been detected in human placental villi and in amniochorionic and decidual tissues [146].

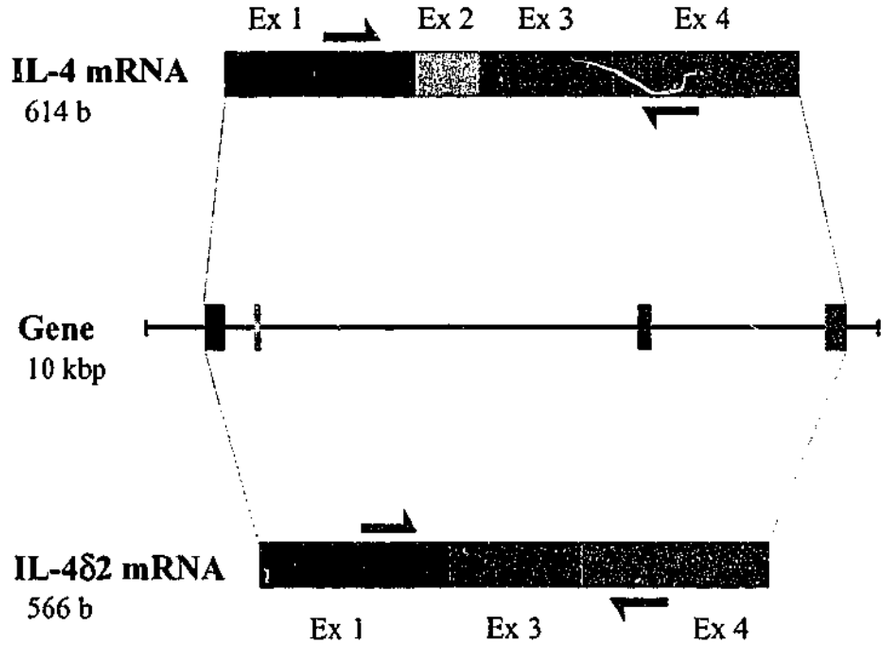


Figure 5.1 Schematic representation of IL-4 and IL-4δ2 mRNA

The exon arrangement of IL-4 and IL-4δ2 mRNA is indicated by colour coding. The approximate locations of the PCR primers are indicated by grey arrows and amplify PCR products of 339 bp and 291 bp from IL-4 and IL-4δ2 cDNA respectively.

The deletion of exon 2 does not result in a frameshift and IL-4δ2 mRNA encodes a truncated IL-4 protein. Studies with recombinant human IL-4δ2, indicate that IL-4δ2 is a naturally occurring receptor antagonist of IL-4. Unlike IL-4, IL-4δ2 did not act as a costimulator of activated T cells but rather was shown to inhibit the ability of IL-4 to stimulate anti-CD3 monoclonal antibody activated T cells [147]. In monocytes, IL-4δ2 blocked the inhibitory action of IL-4 on LPS-induced cyclooxygenase-2 expression and subsequent prostaglandin E_2 secretion [148]. IL-4δ2 was also an antagonist of IL-4-induced IgE synthesis and CD23 expression by B cells [148]. Receptor binding studies using lymphoma cell lines [147] or purified monocytes [148] showed that IL-4δ2 bound specifically in a dose-dependent manner and in competition with IL-4. The phenomenon of alternative splicing is shared by the interleukin-2 (IL-2) gene, which produces IL-2δ2 and IL-2δ3 mRNAs, missing exons 2 and 3 respectively, as well as IL-2 mRNA [149]. Similar studies with IL-2δ2 and IL-2δ3 indicate that they are also competitive inhibitors of IL-2 [149].

This chapter describes the development of a novel competitive PCR assay for the simultaneous quantitation of both IL-4 and IL-4δ2 mRNA. Previous studies of respiratory tissue have not attempted to differentiate these potentially competing IL-4 mRNA species and have only been able to report total IL-4 gene expression incorporating both mRNA forms.

5.2 MATERIALS AND METHODS USED

5.2.1 Competitor and Primers

A commercial non-homologous competitor was obtained from Clontech and used as previously described (Section 4.3.1). The primers were redesigned and synthesised as previously described (Section 4.2.2, Table 4.2, page 65).

5.2.2 Samples

PHA-stimulated PBMC were as described previously (Section 3.2.3). Nasal polyps were obtained from the operating rooms of the Alfred Hospital ENT Service and all

were resected from atopic individuals. Polyps were dissected and snap frozen in liquid nitrogen before storing at -80 °C.

5.2.3 Reverse Transcription and Competitive PCR

RNA was extracted from nasal polyps and PBMC using Qiagen spin columns (Section 2.2.4.2). cDNA was synthesised from up to 1 µg RNA in a volume of 20 µL consisting of 1x GeneAmp PCR buffer, 5 mM $MgCl_2$, 1 mM dNTP, 0.8 U RNase Inhibitor, 2 µM Oligo $d(T)_{16}$ and 2 U MuLV Reverse Transcriptase (all from Perkin-Elmer). Reactions were incubated for 1 min at 15 °C and then the temperature was increased to 42 °C over 9 min, followed by 1 hour at 42 °C, 5 min at 85 °C and 1 min at 4 °C.

For competitive PCR, 20 µL cDNA was made up to a 100 µL reaction consisting of 2 units AmpliTaq Gold, 1 x GeneAmp PCR buffer, 2.5 mM $MgCl_2$, 200 µM dNTP (all from Perkin-Elmer, Foster City, CA, USA), 0.2 µM upper and lower primer and 40 copies of IL-4 competitor. Thermal cycling was conducted in a MJ Research PTC-200 (Watertown, MA, USA) with an initial denaturation of 8 min at 94 °C, followed by 50 cycles of 94 °C for 1 min, 64 °C for 1 min and 72 °C for 1 min, concluding with 7 min at 72 °C and 1 min at 4 °C. The PCR products representing IL-4 and IL-4δ2 were quantitated by PAGE and fluorescence scanning. The PCR products were resolved by electrophoresis through 1.5 mm, 8% 29:1 polyacrylamide gels, stained by overlay with SYBR Green I and then scanned directly in the FluorImager (Section 3.2.2.2).

5.2.4 Analysis of Specificity of PCR

The IL-4 primers produce 339 and 291 bp PCR products as predicted by Oligo software when used to amplify cDNA from PHA-stimulated PBMC. To confirm the identity of the PCR products as being amplicons of IL-4 and IL-4δ2, the individual bands were isolated from a polyacrylamide gel using QIAquick PCR Purification Kit (Qiagen) and subjected to restriction enzyme analysis. Excised gel slices were crushed and the DNA eluted passively into 500 µL PB buffer incubating for 20 min at 94 °C, followed by 4 °C overnight. The gel fragments were collected by

centrifugation and washed with 500 µL PB buffer. The aliquots of PB buffer were combined and PCR products purified as directed by the manufacturer. Purified PCR products were re-amplified in 5 x 100 µL reactions as above and then purified directly by the QIAquick kit as above.

Aliquots of 400 ng of putative IL-4 and IL-4δ2 PCR products were restriction digested by 15 U Bpu AI (exon 1-2 splice junction), Hind II (exon 2), Pst I (exon 3) and Pvu II (exon 3; Boehringer Mannheim) according to the manufacturer's instructions. Digests were analysed by polyacrylamide gel electrophoresis (PAGE) and fluorescence scanning. The specificity of the PCR products was further tested by sequencing followed by BLAST analysis as described previously (Section 4.3.2).

5.2.5 Validation of Dual mRNA Species Competitive PCR

To establish that dual mRNA species competitive PCR is a valid quantitative method, a "changing input ratio" series of reactions was set up. Aliquots of PHA-stimulated PBMC cDNA, previously shown to be positive for both IL-4 and IL-4δ2, were combined in 100 µL PCR with serial titration of IL-4 competitor DNA from 8 x 10^5 to 3.9 x 10^2 copies. Reactions were split into duplicate 50 µL reactions and amplified for 35 and 40 cycles.

5.3 DEVELOPMENT OF THE METHOD

5.3.1 Outline of the Method

The commercial DNA competitor for IL-4 PCR was designed before the first report of IL-4δ2. The primer sites flanking the competitor are located in exon 1 and exon 4 (Figure 5.1, page 90). Consequently, the primers will amplify both IL-4 and IL-4δ2 resulting in PCR products that differ by 48 bp. The competitor and its primers were used to develop a PCR assay where both IL-4 and IL-4δ2 PCR products compete with the competitor template during amplification (Figure 5.2). I have called this assay dual-mRNA species competitive PCR.

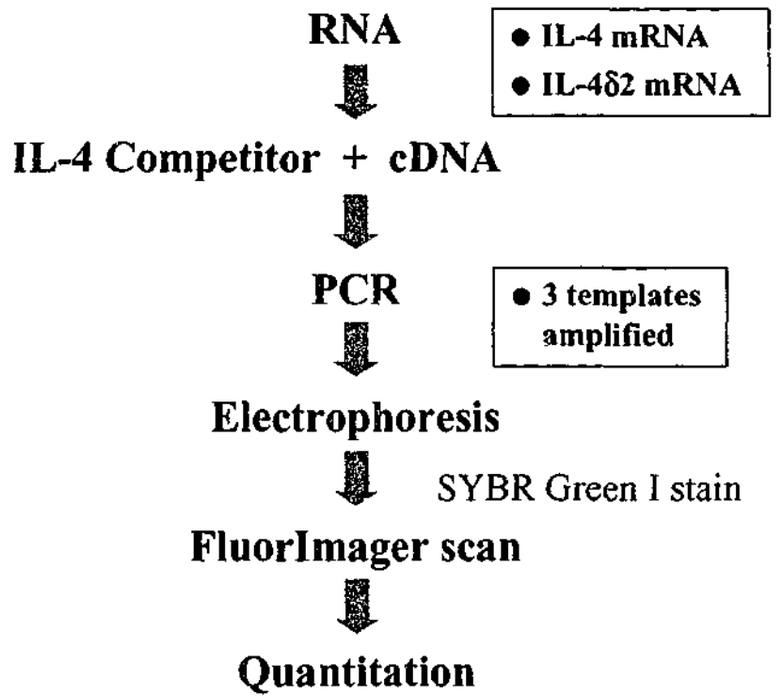


Figure 5.2 Flow diagram of dual-mRNA species competitive PCR assay of IL-4 and IL-4δ2

5.3.2 IL-4 PCR Specificity

RT-PCR of PHA-stimulated PBMC RNA yielded PCR products of 339 and 291 bp consistent with being derived from IL-4 and IL-4δ2 mRNA respectively. In optimisation of the PCR annealing temperature, it was noted that both products were present when 68 °C was used but neither were detected with 69 °C as the annealing temperature, indicating that both products were specific. Restriction enzyme analysis was conducted on PAGE purified and re-amplified PCR products (Figure 5.3). As predicted, putative IL-4δ2 PCR products were cleaved by Pst I (exon 3), Pvu II (exon 3) and Eco RI (exon 4) but not by Bpu AI (exon 1-2 splice junction) or Hind II (exon 2). IL-4 PCR products were cleaved by all five restriction enzymes giving fragments of the expected sizes. The identity of the PCR products was further confirmed by sequencing and BLAST analysis.

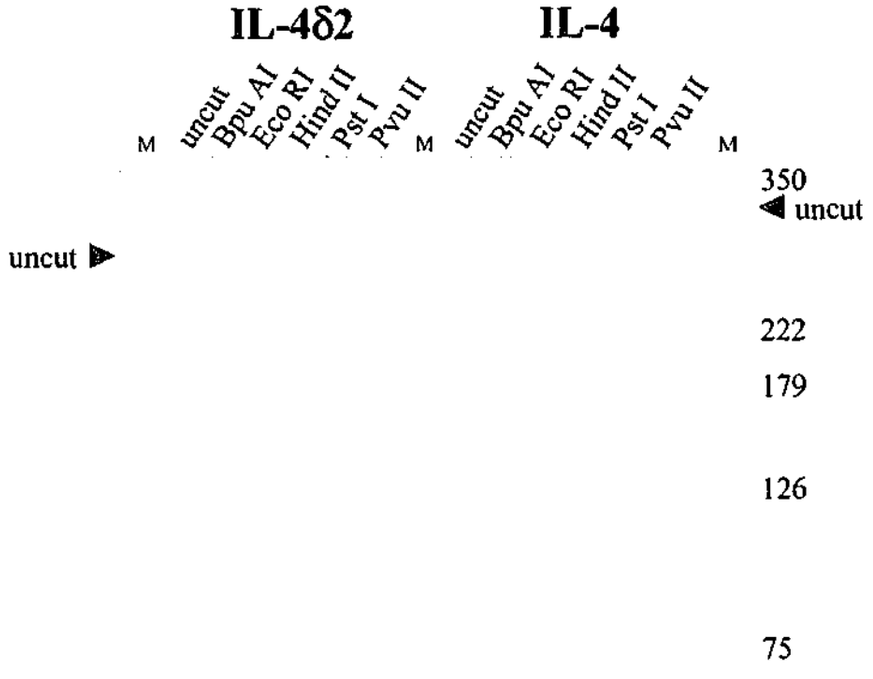


Figure 5.3 PCR Specificity; Identification of PCR products as amplicons of IL-4 and IL-4δ2 by restriction fragment analysis.

The sizes of the marker (M) bands are indicated to the right of the image.

When competitive PCR reactions were first established for IL-4 and IL-4δ2, a fourth band was observed co-migrating with the 499 bp competitor PCR product on 4 % PAGE gels, preventing its quantitation. When individually isolated PCR products were combined together in a subsequent PCR this band was produced but was not detectable if the PCR products were amplified separately. This test indicated that the band is a heteroduplex consisting of one strand each from IL-4 and IL-4δ2.

The presence of a heteroduplex band co-migrating with the competitor band prevented quantitation of the competitor. The migration of the heteroduplex is sensitive to the parameters of electrophoresis due to incomplete duplex formation of the larger IL-4 strand. It was found that similar resolution of the PCR products was obtained by electrophoresis in 8 % 29:1 acrylamide-bisacrylamide gels compared to the 4 % 19:1 PAGE gels used for the other assays in this study. The migration of the heteroduplex, however, was greatly impeded by the concentration and cross-linking combination of the 8 % 29:1 gel, allowing the competitor band to quantitated without interference from the heteroduplex.

5.3.3 Validation of Dual mRNA Species Competitive PCR

Dual mRNA species competitive PCR was validated using the changing input ratio method used for standard competitive PCR assays (Section 4.4.3). Titration of IL-4 competitor against a constant aliquot of PHA-stimulated PBMC cDNA resulted in output ratios that closely followed the predicted line (Figure 5.4), indicating that both IL-4 and IL-4δ2 products compete quantitatively with the IL-4 competitor in the PCR. Hence, the relative ratio of the 3 molecules remained unchanged by the amplification and detection process. Thus, dual mRNA species competitive PCR allows both IL-4 and IL-4δ2 cDNA molecules to be quantitated from one reaction. Despite the added complexity of dual mRNA species competitive PCR, the titrations were comparable to that obtained for standard competitive PCR assays (Section 4.4.3).

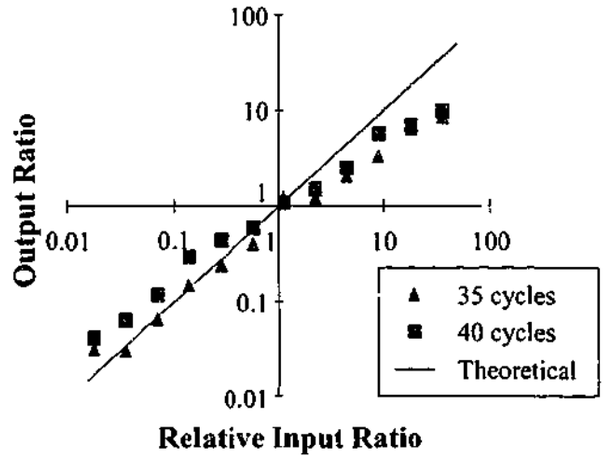
A

competitor

IL-4

IL-4δ2

B



C

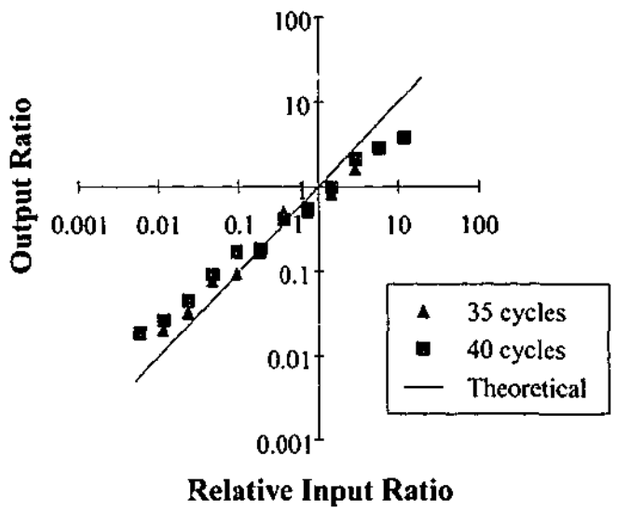


Figure 5.4 Titration test of dual mRNA species competitive PCR

A) PAGE gel of the titration of competitor, increasing from left to right, against a constant aliquot of PHA-stimulated PBMC cDNA. PCR was conducted for 35 cycles. Quantitation of the native:competitor ratios for B) IL-4 and C) IL-4δ2 for 35 cycles and 40 cycles.

The titrations did not allow relative and absolute quantitation to be distinguished and therefore it was not possible to determine if the ratios between IL-4δ2 and IL-4 are absolute or relative. Consequently, a quantitated 100 copies of IL-4δ2 may have not equated with a 100 copies of IL-4 in absolute terms. This anomaly of the method was unlikely to have biological significance, as a number of other factors will have influenced the biological potency of the splicing variants, such as mRNA degradation and translation rates.

5.3.4 IL-4 and IL-4δ2 in Nasal Polyps

IL-4 competitive RT-PCR was conducted on 4 nasal polyp samples, in preliminary experiments conducted to optimise the assay system (Figure 5.5). Two samples expressed only IL-4, but one expressed both IL-4 and IL-4δ2 while the other was negative for both mRNAs. This was the first demonstration of IL-4δ2 mRNA in human airway tissue.

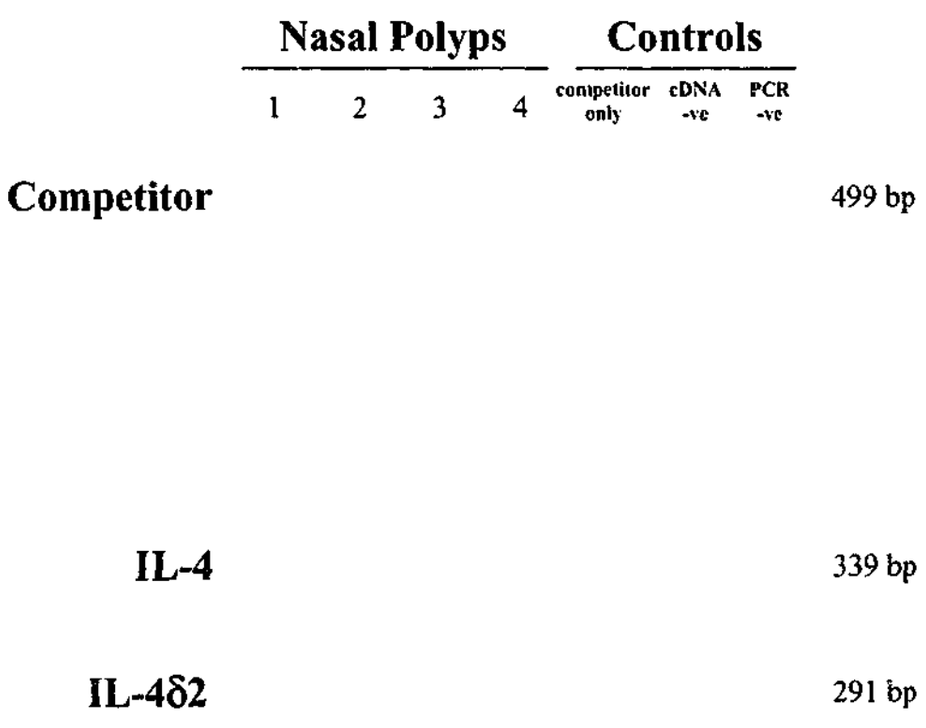


Figure 5.5 IL-4 and IL-4δ2 mRNAs are differentially expressed in nasal polyps.

5.4 DISCUSSION OF THE METHOD

5.4.1 The Limitations of Dual-mRNA Species Competitive PCR

The ability to quantitate by dual-mRNA species competitive PCR is dependent on the existence of a competitor band that has not been out-competed by the combined synthesis of IL-4 and IL-4δ2 PCR products. Therefore, dual species competitive PCR would not be suitable to quantitate samples where the two mRNAs differ widely in their expression so that the amplicon of the most expressed mRNA would out-compete the other and mask its expression. Such samples would need to be measured by conventional competitive PCR with distinguishing primers (ie, IL-4δ2 specific and IL-4 specific) and different levels of mRNA-specific competitor. In the nasal polyps, BAL cells and endobronchial biopsies (Section 7.3) tested in this study with 40 copies of competitor, we found that all reactions were quantifiable in that all reactions had a measurable competitor band. However, either or both IL-4 mRNAs were often not detectable and below the detection level of the assay, which was defined by the strength of the competitor band. The presence of a competitor band allowed negative samples to be distinguished from failed reactions. With the input of up to 1 μg of RNA into the assay along with 40 copies of competitor, very low levels of expression could be detected and hence the definition of negative samples is reasonable.

5.4.2 The Uniqueness of the Method

The dual mRNA species competitive PCR method is believed to be the first report of a directly quantitative assay with more than two simultaneously competing PCR products. A competitive RT-PCR method of simultaneous quantitation of four isoforms/splicing variants of the α subunit of the $GABA_A$ receptor in brain samples has been described [131]. In this assay the five PCR products did not compete quantitatively and a standard curve was used to semi-quantify the mRNAs. An alternative approach to quantitating splicing variants allows the corresponding PCR products to compete together without addition of a competitor molecule [110]. This strategy is restricted to quantitating the relative ratio of the splicing variants. The dual mRNA species method is analogous to that previously reported for HIV viral

load quantitation where multiple competitors, differing by size, are added in known amounts to patient samples allowing categorical reporting of HIV copy number [150].

Recently, IL-4 and IL-4δ2 mRNA have been quantified by separate competitive PCR assays using distinguishing primers located in exon 2 and the exon 1-exon 3 splice junction respectively [151]. In these assays, a nested PCR protocol was required to achieve adequate sensitivity for unstimulated PBMC samples.

5.4.3 Only mRNA-Based Methodology is Able to Distinguish IL-4 Splicing Variants

IL-4δ2 is missing 16 amino acids, encoded by exon 2, of the mature 129 amino acid IL-4 protein. Whilst molecular modelling of IL-4δ2 structure indicates some changes in folding compared to IL-4, including the formation of a new disulphide bond, 21 of 25 residues that contribute to the IL-4 hydrophobic core are retained in similar stereochemical positions [152]. Consequently, most monoclonal antibodies are likely to detect both IL-4 and IL-4δ2 [147] and distinguishing antibodies have yet to be described. An antibody specific for IL-4 alone would have to map to exon 2 epitopes and/or structural differences between IL-4 and IL-4δ2. Currently, expression of IL-4δ2 can only be detected independently of IL-4 by analysis of mRNA.

Two differentially staining anti-IL-4 monoclonal antibodies have been described [39]. One antibody stained intracellular granules thought to be stored IL-4 whilst the other gave a characteristic peripheral ring staining pattern perhaps detecting secreted IL-4 bound to the extra cellular matrix. The antibodies are thought to be specific for IL-4 since their binding can be ablated by prior incubation of the antibodies with recombinant IL-4. The two antibodies have been shown to bind to different epitopes and the different staining patterns may reflect different accessibility of the antibodies to their respective epitopes [39]. The difference in folding between IL-4 and IL-4δ2 described above is likely to produce a number of epitopes that are in different positions within the protein structures. It is possible that these two antibodies are distinguishing IL-4 and IL-4δ2 with sufficient cross-reactivity for the binding to be ablated by excess recombinant IL-4. This proposal needs confirmation by affinity

binding studies with recombinant IL-4 and IL-4δ2.

To detect IL-4 mRNA by in situ hybridisation independently of IL-4δ2, the probe needs to be limited to exon 2 sequences. For PCR-based detection of IL-4, either the PCR needs to be specific for IL-4, with one PCR primer in exon 2, or alternatively, the detection system must distinguish between IL-4 and IL-4δ2 PCR products. My assay has used the size difference of the PCR products to detect IL-4 and IL-4δ2 independently. An alternative strategy may have utilised hybridisation for detection and would once again require exon 2 specific probes to detect IL-4 PCR products and a probe from the exon 1-exon 3 boundary to detect IL-4δ2.

Increased expression of IL-4 and IL-5 in asthmatic airway biopsies and lavage has been reported [26, 153-157]. These studies have used varying combinations of immunochemistry, in situ hybridisation and semi-quantitative PCR methods, but all the methods were applied in the traditional manner, failing to differentiate between the two IL-4 mRNA species. For example, Humbert et al.[153] in a cross-sectional bronchial biopsy study of atopy and asthma, used the same primer sites as this study, with primer sequences differing by a few bases, but they used a dot blot detection method with the probe hybridising to exon 3. Exon 3 is common to both IL-4 and IL-4δ2. Likewise, their in situ hybridisations utilised cDNA riboprobes with sequences common to both IL-4 and IL-4δ2. My results indicate that both IL-4 and IL-4δ2 are commonly expressed in airway samples and therefore this study would have reported additive results. Indeed all studies that have measured total IL-4 gene expression need now to be repeated with differentiation of the two mRNA species.

5.5 CONCLUDING COMMENTS

Validation studies have shown dual-mRNA species competitive PCR to be a viable quantitative method. Both IL-4 and IL-4δ2 can be efficiently quantitated from a single assay. The simple example of IL-4 and IL-4δ2 quantitation in nasal polyps and the following application to airway samples indicated that IL-4δ2 is variably expressed. Given that IL-4δ2 is an IL-4 receptor antagonist [147, 148], these results indicate that it is crucial to be able to distinguish IL-4δ2 from IL-4 when assessing IL-4 gene expression.

6 HOUSEKEEPING GENE EXPRESSION, ASTHMA AND STEROID USE

6.1 INTRODUCTION

Attempts to develop quantitative assays of mRNA in samples have led to the common use of housekeeping gene expression to normalise the data. Assays based on Northern blots and semi-quantitative RT-PCR techniques are characterised by multiple steps, each introducing variation from sample to sample. In order to relate the results to each other and to the samples from which they originated, molecular biology assays need endogenous control(s), which are similarly influenced by the efficiency of each step [158].

Housekeeping genes are defined by specific gene promoter elements, which determine that they are expressed constitutively in every cell. However, that does not necessarily mean that their expression is not regulated or does not vary [reviewed 159]. Even so, the use of housekeeping genes in molecular biology assays relies on the assumption that their levels of expression remain the same from cell to cell, sample to sample, treatment to treatment and/or patient to patient. Whilst all methodologies need validation, it is difficult to determine whether housekeeping gene expression has biological variation in the absence of a validated alternative means of normalising the data. This is particularly so if housekeeping gene expression is the sole arbiter of the assay.

β-Actin, a cytoskeletal protein, and GAPDH, an enzyme of glycolysis, are the two most widely utilised housekeeping genes [158], on the basis of rather anecdotal evidence of their consistency of expression. The use of such housekeeping genes is complicated by the presence of pseudogenes in the human genome. These transcription-inactive cDNA-like sequences have high homology with their mRNA counterparts, from which they were probably derived by reverse transcription and integration into the genome [160, 161]. Genomic DNA contaminating RNA samples may produce PCR products indistinguishable from the cDNA-derived PCR product [162].

The rationale in measuring the expression of GAPDH and β-Actin in the cross-sectional study was to test the hypothesis that samples that differ in cellular

composition and/or activation status would also have different levels of expression of housekeeping genes. If this hypothesis proved to be incorrect, β-Actin or GAPDH could then be reasonably used in the assays of cytokine mRNA to control for sample-to-sample variability in the quality of the RNA and the efficiency of cDNA synthesis.

Table 6.1 Bronchoscopy Subject Profiles

	Asthmatics		Normal Controls	
	BAL Cells	**Biopsies**	**BAL Cells**	**Biopsies**
n	75	70	23	17
Gender (M:F)	46:29	40:30	15:8	9:8
Age median (range)	37 (21 – 69)	38 (21 – 69)	33 (20 – 61)	33 (20 – 61)
Atopy (+/-)	63/12	58/12	12/11	8/9
ICS use (+/-)	62/13	58/12	0/23	0/17
ICS dose/day (BDP)* **median (range)**	400 (100 – 900)	400 (100 – 900)	–	–
PD20 methacholine (mg) **median (range)**	0.027 (0.001 – 6.4)	0.026 (0.001 – 6.4)	>3.4	>3.4
FEV1 % predicted **median (range)**	90 (65 – 128)	88 (68 – 120)	111 (91 – 132)	107 (91 – 132)

*Dose in μg per day of beclomethasone diproprionate

6.2 MATERIALS AND METHODS USED

6.2.1 Subjects

Ninety-two asthmatic volunteers took part in the study. Airway endobronchial biopsies were obtained from 70 and BAL cells from 75 patients (Table 6.1). They all used albuterol for relief of symptoms as needed, with 79 patients using regular ICS. Salmeterol (50 μg twice daily) was used by 12 patients from whom BAL cells were obtained and in 5 from whom biopsies were taken.

6.2.2 Screen for Pseudogene Sequences in RNA

The primer sites used in this study are commercially available, widely used and known to produce PCR products from pseudogenes, whose size is within 2 or 3 bases of the cDNA-derived PCR products [162]. To determine if pseudogene amplification from contaminating DNA was contributing to the apparent quantitation of β-Actin mRNA, sample RNA was diluted 1 in 80 in water and 2 μL was added directly to β-Actin PCR containing 1 x 10^4 and 400 copies of competitor for BAL and biopsy, respectively. GAPDH pseudogene sequences were amplified similarly using 2.5 x10^3 copies competitor for BAL RNA and 500 copies competitor for biopsy. These reactions were quantitated on 2.8% agarose gels containing 0.1 μg/mL ethidium bromide.

6.2.3 Statistical Analysis

The Pearson correlation was used to test associations between GAPDH and β-Actin and between sample types. Generalised linear modelling was used for the cross-sectional analyses. The confounding variables used in the modelling as appropriate included age, sex, gender, atopic status, ICS usage, and use of long-acting β_2-agonist. The Mann-Whitney U test was used for intergroup comparisons for IL-2/β-Actin ratios. Statistical analyses were performed using SAS version 6.12 software (SAS institute, Cary, NC, USA) [163].

6.3 SCREEN FOR PSEUDOGENE CONTRIBUTION

The presence of β-Actin and GAPDH pseudogenes and the potential for cDNA-based primers to amplify pseudogene sequences is well known [160, 161, 164, 165]. Discriminating primer sites targeted at mismatched regions have been described [162] but commercially available competitors utilising these sequences are not available. Consequently, all RNA samples in this study were screened for contaminating DNA by direct competitive PCR of RNA without reverse transcription. The assay was designed to detect signals greater than approximately 1 % of that expected from cDNA.

Figure 6.1 Detection of pseudogene DNA sequences in biopsy RNA

Example agarose gel of GAPDH and β-Actin competitive PCR assay of biopsy RNA samples with out reverse transcription. The panels are flanked by DNA size markers. Arrowheads indicate the respective native PCR products.

Of 97 BAL cell RNA samples tested by PCR without cDNA synthesis, 5 were shown to have minor signals from RNA in the absence of reverse transcription. However, biopsy RNA samples were significantly contaminated with chromosomal DNA (Figure 6.1). Eighty three of 85 preparations had quantifiable levels of β-Actin pseudogene products, of which 16 % were contaminated by a level of β-Actin DNA molecules of 5 % or more of their cDNA derived signal (Figure 6.2). The GAPDH and β-Actin pseudogene products were expressed as DNA copies/μg RNA and used to correct the corresponding RT-competitive PCR quantitations of mRNA.

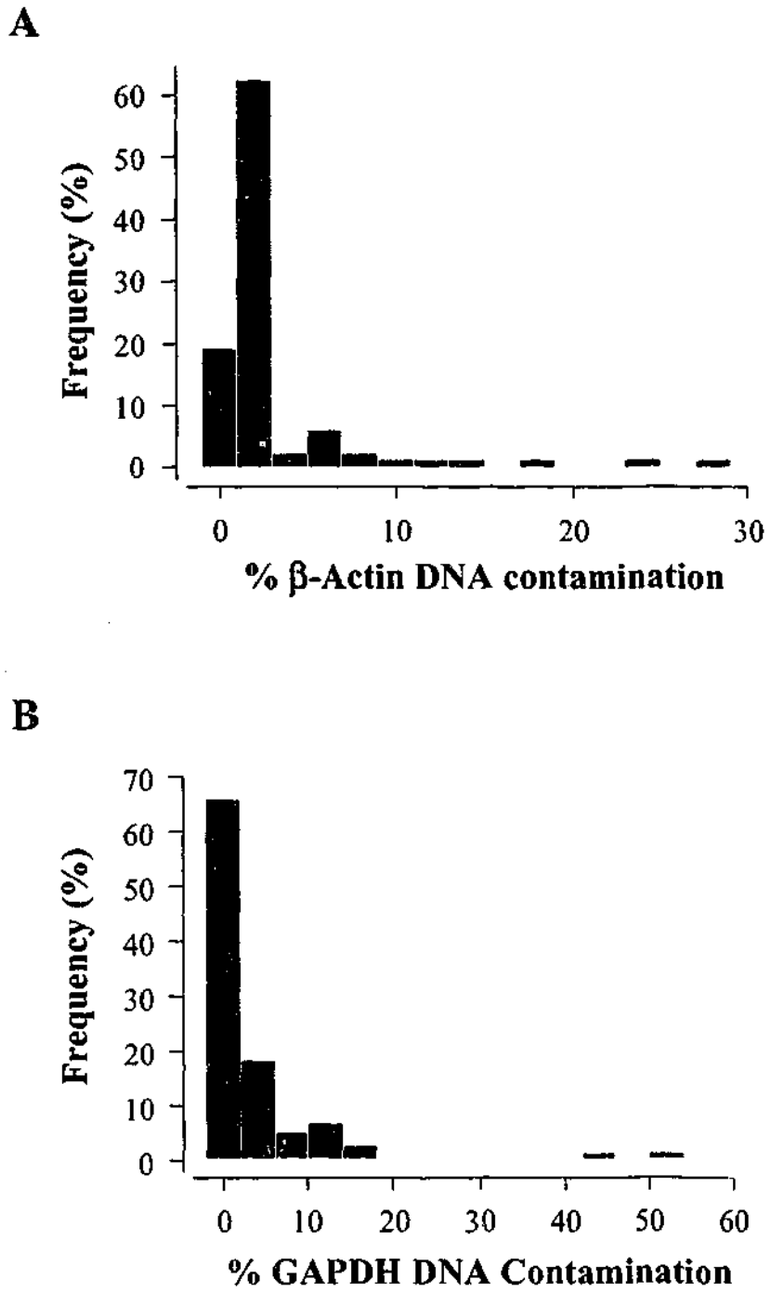


Figure 6.2 Frequency of DNA contamination of Biopsy RNA.

The frequency of biopsy samples contaminated with genomic DNA is plotted as a function of the percentage of the reverse transcription-competitive PCR of β-Actin (A) and GAPDH (B) attributable to pseudogene PCR products, as determined by β-Actin or GAPDH competitive PCR of sample RNA without reverse transcription.

The levels of GAPDH pseudogene DNA contamination of RNA samples were significantly different from the β-Actin pseudogene contamination levels. Analysis by Bland and Altman plots (Bland, 1986 #150) indicated that approximately two copies of β-Actin pseudogene were being detected for every copy of GAPDH pseudogene (Figure 6.3). The wide 95 % limits of agreement are a reflection of the expected poor quality of the contaminating DNA as templates in the PCR assays. Pseudogene homology searches on nucleotide sequence databases (Altschul, 1990 #146), followed by primer binding efficiency predictions using Oligo 5.0 Primer Analysis Software, indicated that there are at least 7 related pseudogenes, including a γ-Actin pseudogene and two β-Actin pseudogenes which might be amplified by the β-Actin primers to yield PCR products approximating the size of the mRNA-derived species.

Most researchers have been reliant on intron-spanning primers to avoid gene amplification contributing to that from cDNA. Whilst the genes for GAPDH and β-Actin are not readily amplified by such primers, the intron-less pseudogenes are frequently detected [166]. Whilst correction did not change the essence of the analyses in this study, it is possible that other studies have been biased by pseudogene contamination. Recently, an optional DNase-digestion step has been introduced into the RNA extraction procedure (personal communication, Qiagen) and based on my experience in this study, I believe it should be a routine step in airway RNA preparations.

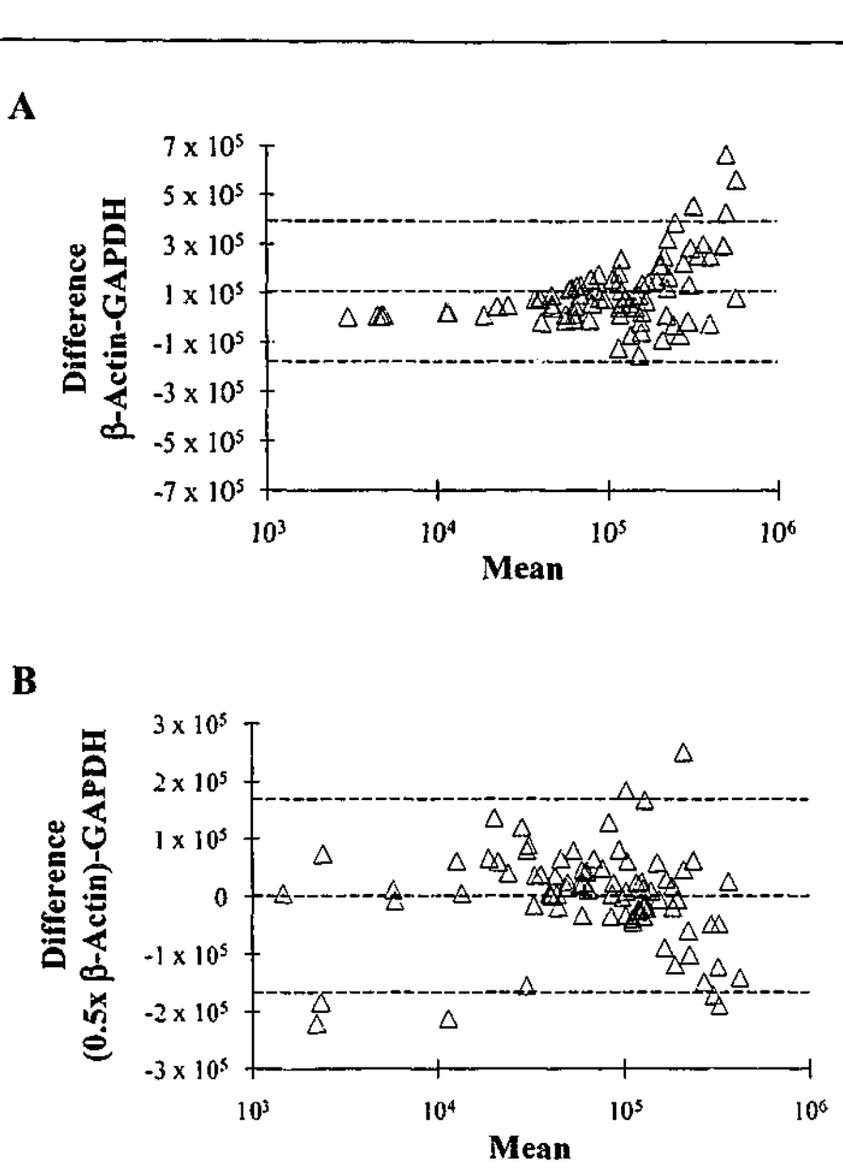
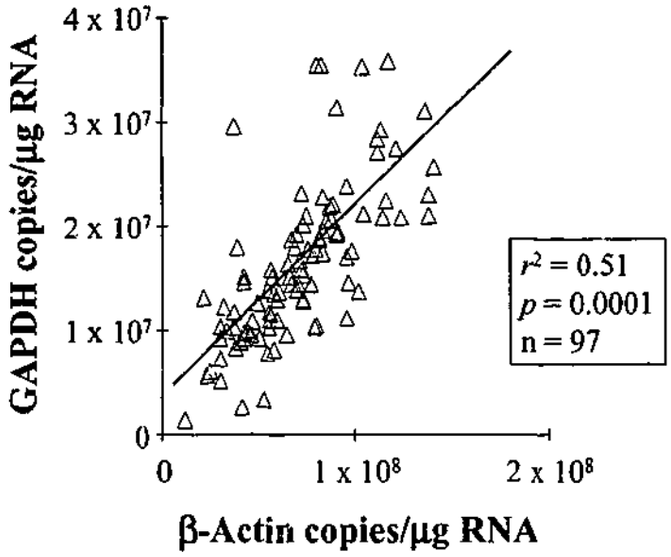


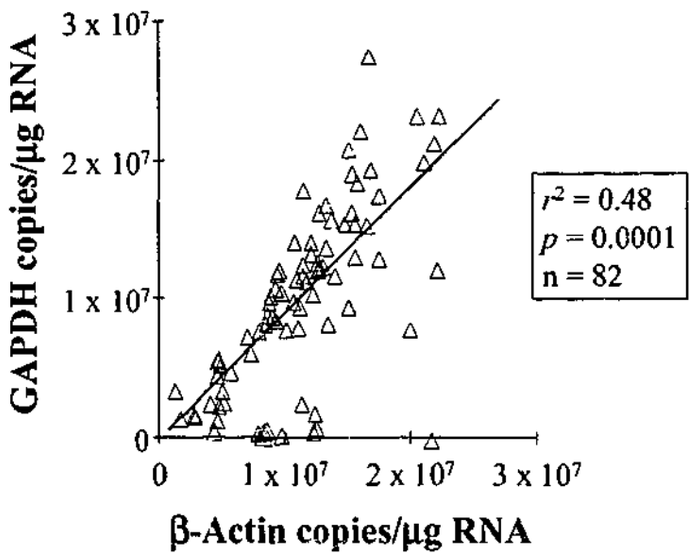
Figure 6.3 Two copies of β-Actin pseudogene are detected for every copy of GAPDH pseudogene

Bland and Altman plot of the β-Actin-GAPDH difference against the mean (A) indicates that the number of copies of β-Actin pseudogene per μg RNA is far greater than the copies of GAPDH pseudogene detected. The dotted lines indicate the mean difference (1.1×10^5 copies/μg RNA) and the mean difference ± 2 x standard deviations respectively. B) Bland and Altman plot as in (A) but with (0.5 x β-Actin)-GAPDH difference plotted against the corresponding mean. The mean difference ± 2 x standard deviations is $1.4 \pm 169.8 \times 10^3$ copies/μg RNA indicating that β-Actin pseudogene sequences are detected at approximately twice the rate of GAPDH pseudogenes in biopsy RNA.

A


4 x 10^7
3 x 10^7
2 x 10^7
1 x 10^7
0
GAPDH copies/µg RNA
0
1 x 10^8
2 x 10^8
β-Actin copies/µg RNA
r^2 = 0.51
p = 0.0001
n = 97

B


3 x 10^7
2 x 10^7
1 x 10^7
0
GAPDH copies/µg RNA
0
1 x 10^7
2 x 10^7
3 x 10^7
β-Actin copies/µg RNA
r^2 = 0.48
p = 0.0001
n = 82

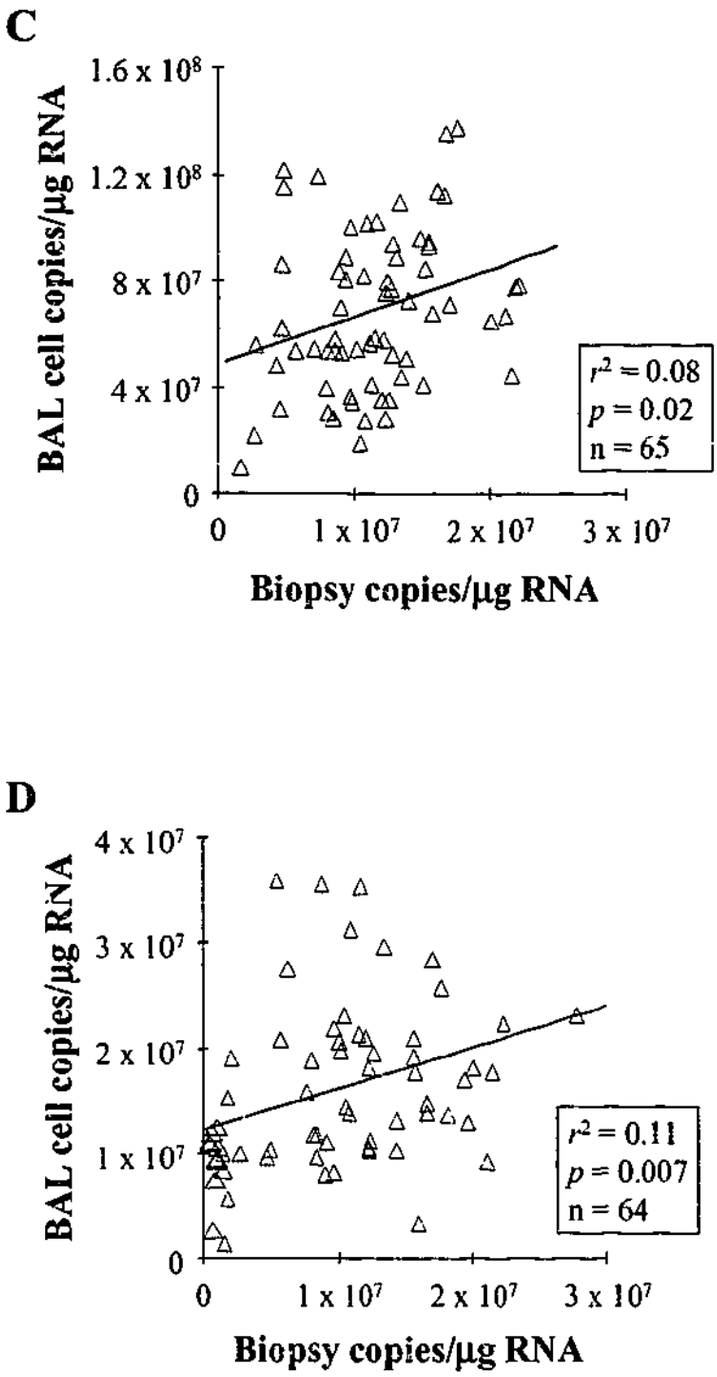


Figure 6.4 GAPDH and β-Actin gene expression are not the same.

Pearson correlations between GAPDH and β-Actin gene expression in RNA from BAL cells (A) and in RNA from biopsies (B) and correlations between BAL cells and biopsies from the same subject for β-Actin (C) and GAPDH (D).

6.4 GAPDH AND β-ACTIN GENE EXPRESSION ARE NOT DIRECTLY COMPARABLE

GAPDH and β-Actin mRNA levels significantly correlated in both biopsy ($r^2 = 0.48$, $n = 85$, $P = 0.0001$) and BAL cells ($r^2 = 0.51$, $n = 97$, $P = 0.0001$) (Figure 6.4A and B). The correlation coefficients indicate that only half of the variability of one is explained by the other, suggesting that at the resolution of this methodology GAPDH and β-Actin mRNA levels are not equivalent. Furthermore, GAPDH gene expression was more variable in biopsy samples and was approximately 5 times lower in BAL cells compared to β-Actin expression.

Comparison of GAPDH and β-Actin between BAL cells and biopsies from the same patient, indicated that the 2 samples types are substantially different in the expression of these housekeeping genes (GAPDH: $r^2 = 0.12$, $n = 67$, $P = 0.004$; β-Actin: $r^2 = 0.08$, $n = 67$, $P = 0.017$) (Figure 6.4C and D). In particular, β-Actin mRNA levels per μg total RNA extracted from BAL cells were approximately 5 times higher than those in biopsy specimens.

6.5 LACK OF CONCORDANCE BETWEEN REPEAT SAMPLING

Analysis of repeat measurement of housekeeping gene expression over 3 months was available for 24 BAL paired samples and 14 paired biopsy samples from asthmatic patients. For the paired BAL samples 6 had added fluticasone, 8 had added salmeterol and 10 had added placebo over the 3 months period. For both sample types, the change in housekeeping gene expression over 3 months was independent of treatment (Figure 6.5). The 95 % limits of agreement on Bland and Altman plots are of the same order of magnitude as the levels of mRNA indicating a lack of concordance of housekeeping gene expression over 3 months. Instead, substantial changes in GAPDH and β-Actin mRNA levels were observed, with changes in GAPDH mRNA ranging from –114 to 106 % (mean change 1.6 %) in BAL cells and –63 to 156 % (mean change 23 %) in biopsy. Bland and Altman plots of the percent change in β-Actin compared to GAPDH indicate that β-Actin and GAPDH changed independently of each other (Figure 6.6). Similar changes in β-Actin mRNA levels

in BAL cells from 3 patients with repeat sampling after 2 weeks have been reported previously [167].

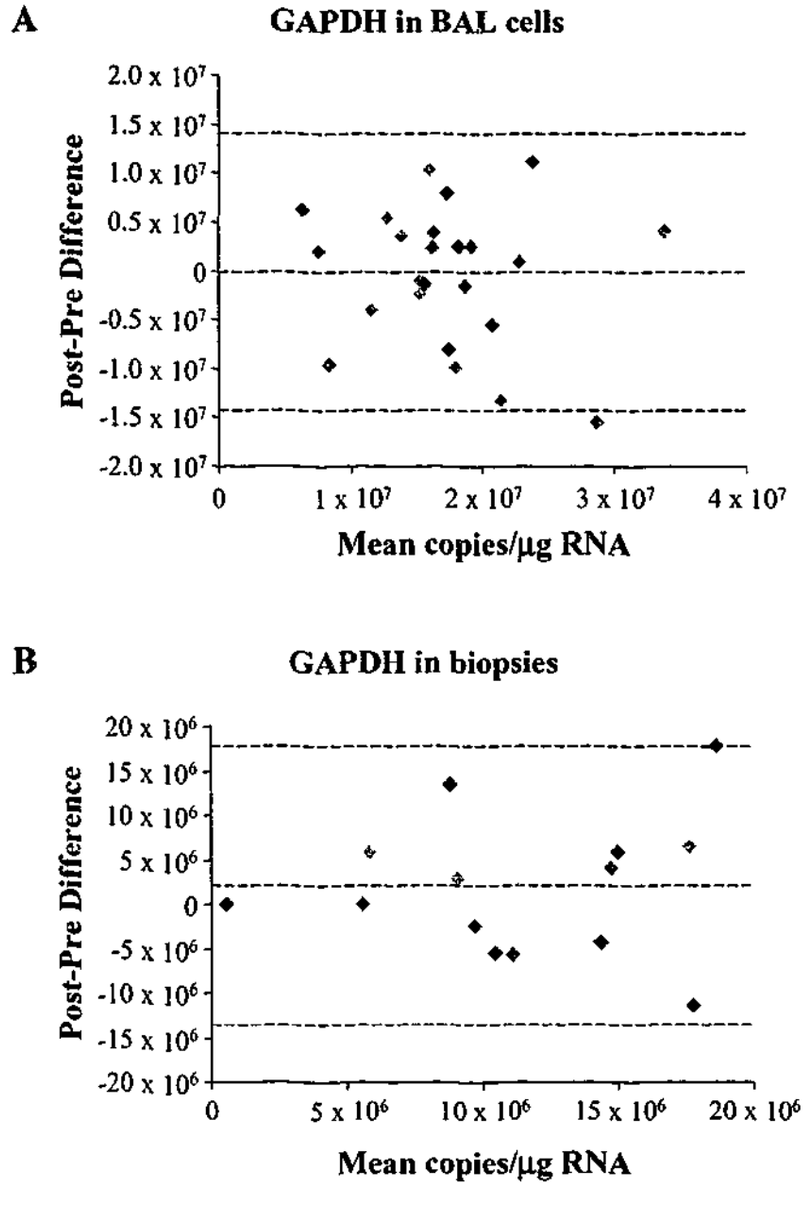


Figure 6.5 Lack of concordance in housekeeping gene expression in paired samples taken 3 months apart.

Bland and Altman plots of the difference of 3 months post –pre-intervention plotted against the mean of the two measurements. All patients were initially on low dose ICS up to 500 µg/day, except for two patients that were not using ICS and received placebo over the 3-month period. Paired biopsy samples were obtained from only one of theses latter two patients. Purple, placebo; Orange, salmeterol; Red, fluticasone as in Figure 6.6

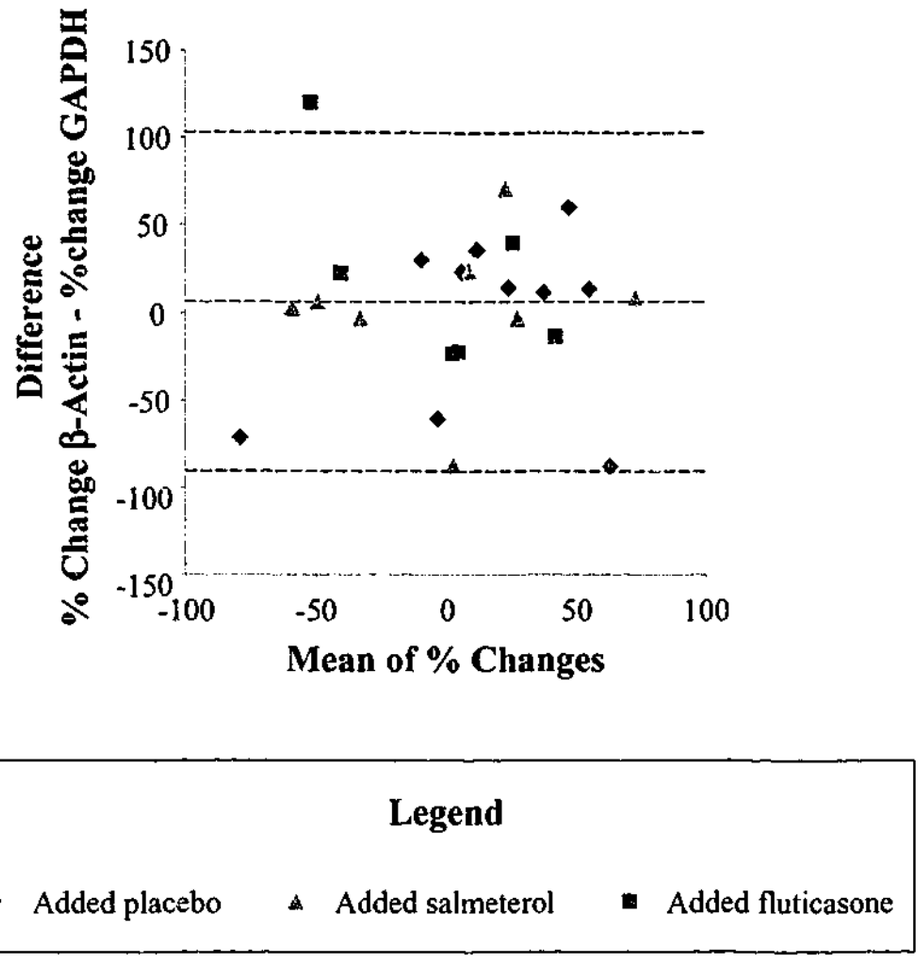


Figure 6.6 β-Actin and GAPDH expression changed independently of each other over 3 months

Bland and Altman plot of the difference in the percent change in β-Actin minus the percent change in GAPDH mRNA levels in BAL cells over 3 months plotted against the mean of the percent changes. A similar graph was obtained for mRNA levels in biopsies but there were only 12 matches of paired samples between β-Actin and GAPDH.

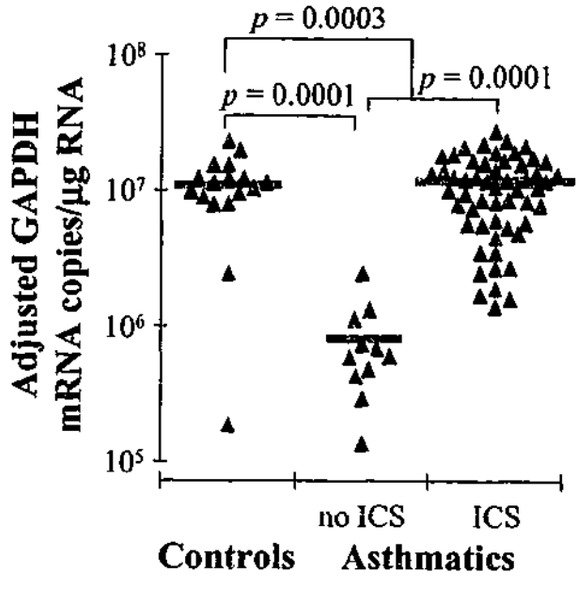
6.6 CROSS-SECTIONAL COMPARISONS OF GENE EXPRESSION

The gene expression of β-Actin and GAPDH were compared with respect to atopic status, asthmatic status, and ICS use (Figure 6.7A). GAPDH gene expression in biopsies from asthmatics not using ICS (least squares mean ± SEM, 8.1 ± 1.3 x 10^5 mRNA copies/μg RNA, n = 11) was significantly lower than in biopsies from normal controls (1.1 ± 0.1 x 10^7 mRNA copies/μg RNA, n = 17, $P = 0.0001$) and from asthmatics on ICS (1.2 ± 0.2 x 10^7 mRNA copies/μg RNA, n = 56, $P = 0.0001$). Asthmatic subjects as a group, whether taking ICS or not, were significantly different from controls (7.1 ± 1.9 x 10^6 mRNA copies/μg RNA, n = 67, $P = 0.0003$). The BAL cell levels of β-Actin and GAPDH mRNA showed similar differences between groups as for biopsy GAPDH mRNA levels, with corresponding *P*-values (Figure 6.7B and C).

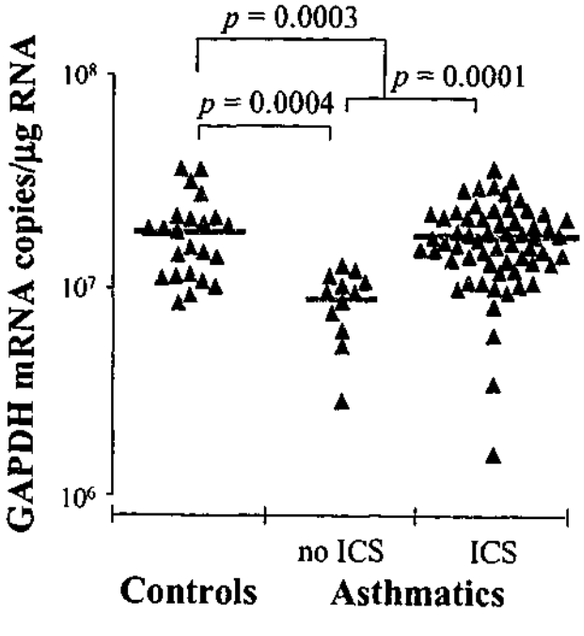
β-Actin mRNA expression in biopsies differed, in that asthmatics taking ICS were similar to those not on ICS ($P = 0.26$) (Figure 6.7C). However, in common with the other comparisons, normal controls (1.5 ± 0.1 x 10^7 mRNA copies/μg RNA, n = 17) had higher β-Actin mRNA levels than asthmatics not taking ICS (8.1 ± 1.4 x 10^6 mRNA copies/μg RNA, n = 10, $P = 0.001$) and higher levels than all asthmatics (9.3 ± 1.5 x 10^6 mRNA copies/μg RNA, n = 66, $P = 0.013$).

The large cross-sectional differences in housekeeping gene expression are in contrast to minimal differences in cytokine mRNA levels (Section 7.3) and the lack of significant differences in BAL cell profiles (Figure 6.8).

A


p = 0.0003
p = 0.0001
p = 0.0001
10^8
10^7
10^6
10^5
Adjusted GAPDH mRNA copies/µg RNA
no ICS
ICS
Controls
Asthmatics

B


p = 0.0003
p = 0.0004
p = 0.0001
10^8
10^7
10^6
GAPDH mRNA copies/µg RNA
no ICS
ICS
Controls
Asthmatics

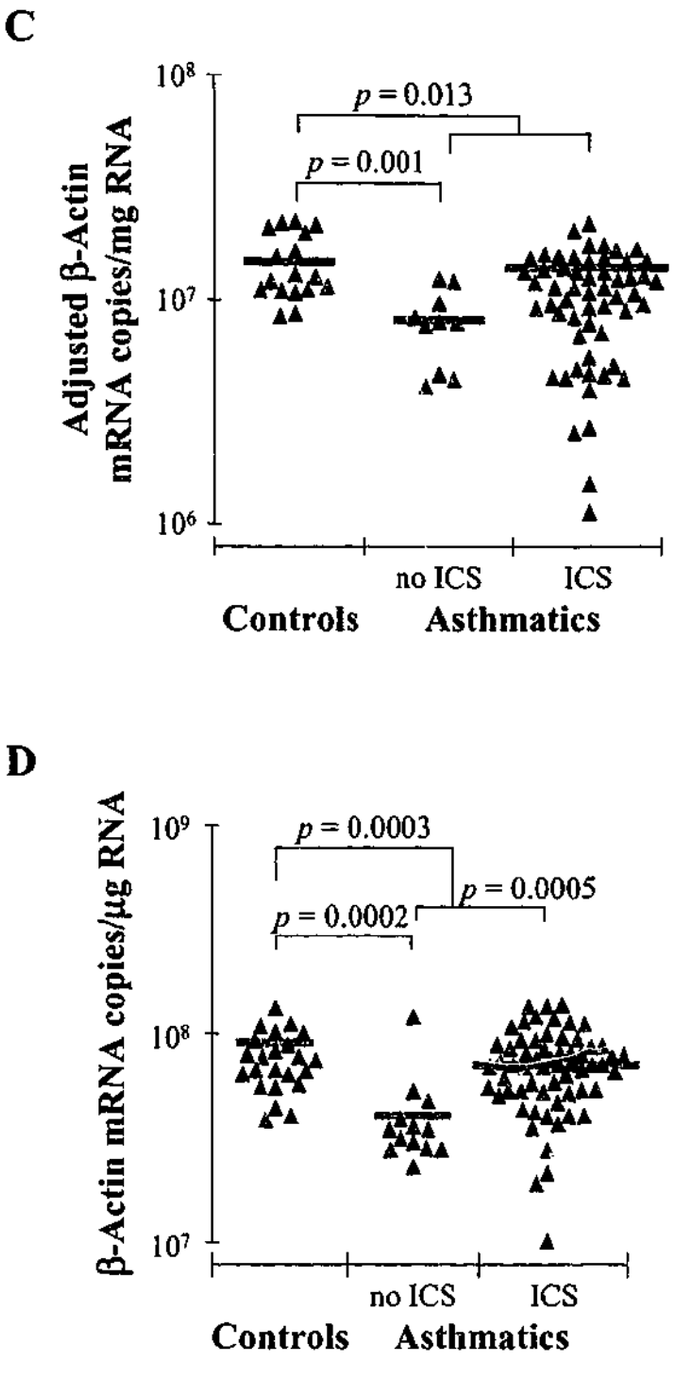


Figure 6.7 Cross-sectional comparisons of GAPDH and β-Actin gene expression.

GAPDH gene expression in biopsies (A) and BAL cells (B). β-Actin mRNA levels in biopsies (C) and BAL cells (D). The biopsy mRNA levels have been corrected for contamination with pseudogene sequences.

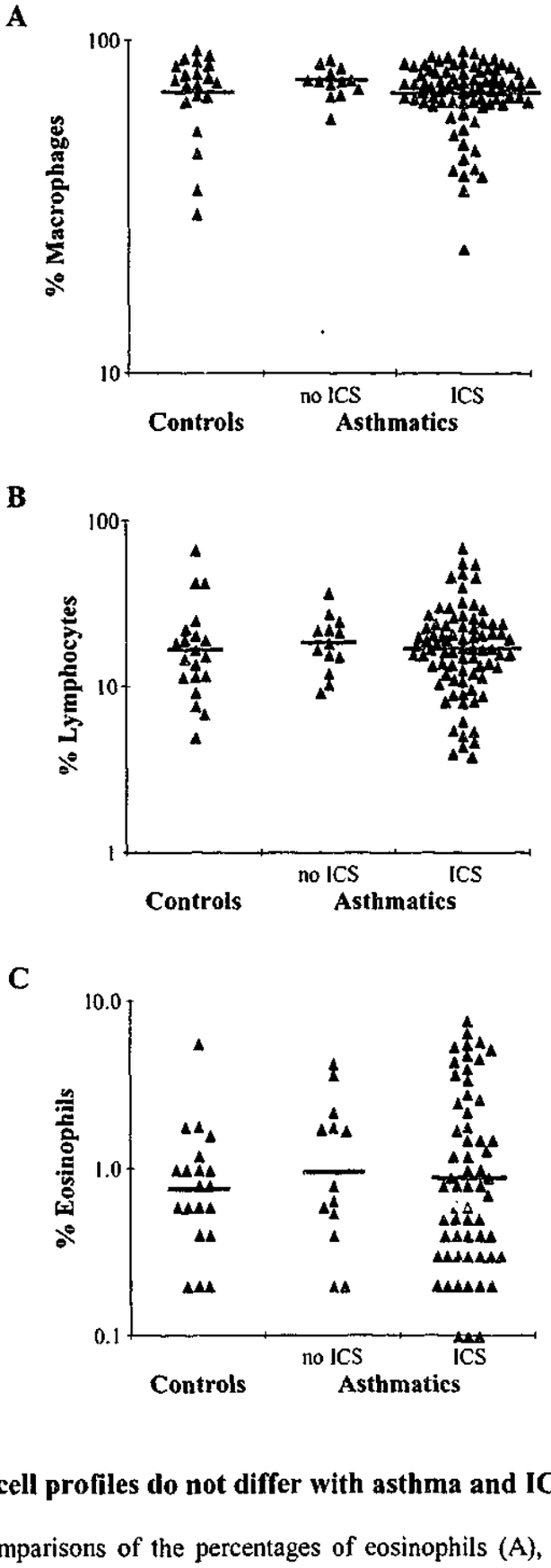


Figure 6.8 BAL cell profiles do not differ with asthma and ICS use

Cross-sectional comparisons of the percentages of eosinophils (A), macrophages (B) and lymphocytes (C) in BAL. Geometric means are indicated by horizontal bars.

6.7 EFFECT OF USING HOUSEKEEPING GENES AS DENOMINATORS

To demonstrate the effect of using β-Actin and GAPDH as denominators of mRNA expression for genes of interest, IL-2 and IL-4 mRNA levels in BAL cells (see Section 7.3) were expressed as ratios with β-Actin. IL-2 mRNA levels in BAL cells did not differ between normal controls ($4.6 \pm 0.9 \times 10^4$ copies/μg RNA), asthmatics not using ICS ($4.8 \pm 1.2 \times 10^4$ copies/μg RNA) and asthmatics taking ICS ($3.0 \pm 0.7 \times 10^4$ copies/μg RNA) (Figure 6.9). However, when IL-2 mRNA levels were expressed as a ratio with β-Actin, significant differences were seen, with asthmatics not using ICS having a higher ratio ($1.2 \pm 0.4 \times 10^{-3}$) than normal controls ($6.2 \pm 1.8 \times 10^{-4}$, $P = 0.03$) and asthmatics using ICS ($4.5 \pm 0.9 \times 10^{-4}$, $P = 0.003$). Similarly, IL-4 gene expression assessed directly in BAL cells did not differ between the same groups. When IL-4 mRNA levels were expressed as a ratio with β-Actin, significant differences were also seen, with asthmatics not using ICS having a higher ratio ($17.6 \pm 3.3 \times 10^{-6}$) than normal controls ($8.0 \pm 1.2 \times 10^{-6}$, $P = 0.0007$) and asthmatics using ICS ($8.4 \pm 2.7 \times 10^{-6}$, $P = 0.0003$) (Figure 6.9). Thus, apparent differences in the IL-2/β-Actin ratios and the IL-4/β-Actin ratios are confounded by the differences in β-Actin mRNA levels between the groups.

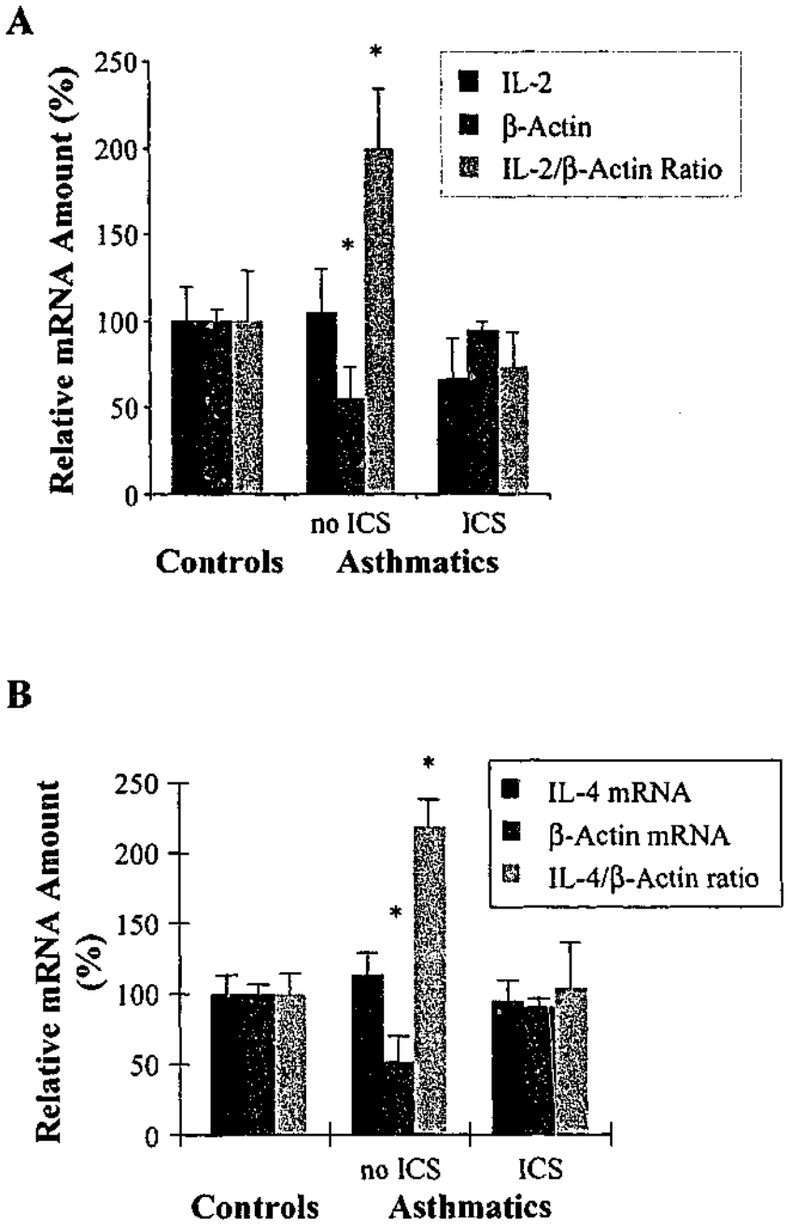


Figure 6.9 Effect of using β-Actin as a denominator of IL-2 and IL-4 BAL cell mRNA levels.

In order to compare IL-2 (A), IL-4 (B) and β-Actin mRNA levels and their ratios, the subject groups least squares means are presented as percentages of the respective normal control least squares mean. Error bars are given as percent SEM. Significant differences were observed (*) for β-Actin (as Figure 6.7D), IL-2/β-Actin ratios ($P < 0.05$) and IL-4/β-Actin ratios ($P < 0.001$) in that asthmatics not using ICS are significantly different with respect to normal controls and asthmatics using ICS.

6.8 DISCUSSION OF RESULTS

This study is the first systematic analysis of the validity of using housekeeping gene expression to normalise mRNA quantitation in lung samples. I have utilised reverse transcription followed by competitive PCR to quantitate β-Actin and GAPDH gene expression in airway biopsies and BAL cells from normal controls and asthmatic patients. GAPDH and β-Actin gene expression were found not to be directly comparable neither within samples nor between BAL cells and biopsies. Asthmatics as a group, and in particular asthmatics not using ICS, were found to have decreased expression of both β-Actin and GAPDH. Quantitation of mRNA in asthma will be confounded by these differences if GAPDH and β-Actin are used to normalise data, and this was demonstrated for IL-2 and IL-4 mRNA levels expressed as ratios with β-Actin.

6.8.1 The Methodology Used

To develop a technique of gene expression measurement that would be independent of housekeeping gene expression I sought to maximise the quantitative nature of each step. Capillary spectrophotometry with background correction was used to analyse the sample RNA accurately, so that a consistent quantity and quality was added to the assay. The competitive nature of the PCR and the subsequent direct quantitation by scanning fluorescence controls for the complete latter part of the assay. The only step without endogenous control is the cDNA synthesis.

Nevertheless, the assay is still highly controlled and I have assumed that there is similar reverse transcription efficiency between subject groups. To prevent systematic bias in efficiency effecting comparisons, the RNA samples were stratified according to RNA concentration before cDNA synthesis. Consequently, I believe that it is very unlikely that the differences in GAPDH and β-Actin gene expression ith asthma were due to the RNA from ICS-untreated asthmatic samples having been transcribed with 10-fold less efficiency.

Analyses of correlations between GAPDH and β-Actin and between biopsies and BAL cells suggest that the two housekeeping genes vary independently of each other. A single cDNA synthesis reaction was conducted for each sample so that β-Actin and GAPDH could be compared without confounding by differences in reverse transcription efficiency. In addition, a limited number of samples taken 3 months apart from clinically stable asthmatics showed a complete lack of concordance in mRNA levels. Normalisation of a gene of interest by housekeeping gene expression will incorporate the biological variability of the housekeeping gene and so add to assay variability and decrease the resolution of the method.

6.8.2 Alternative Interpretations of the Data

In a rat model of particulate-induced lung injury, RNA degradation has been observed concomitant with a time-dependent influx of eosinophils in inflamed pulmonary tissue [168]. The degraded RNA samples produced significantly reduced β-Actin levels in a RT-PCR assay. Asthmatics not using ICS will not have eosinophilic inflammation as severe as that generated by such an animal model, but increased eosinophil number and/or activation correlating with disease severity is a well-documented feature of asthma [169]. The asthmatics in this study were clinically stable and the percentage of eosinophils in the BAL samples in this study were not significantly different between the subject groups (Figure 6.8) making this potential source of confounding unlikely.

It is possible that my data could be interpreted as the mRNA levels of β-Actin and GAPDH per cell not being different between normal controls and asthmatics but rather the total RNA pool being increased with asthma. In the particulate-induced lung injury model discussed above, the authors noted a 2-fold increase in RNA yield with intense inflammation [168]. There were no differences in yield of RNA extraction from the subject groups, and it is unlikely that such an artefact could be responsible for the actual differences in housekeeping gene expression found.

Ribosomal RNA comprises more than 90 % of the total cellular RNA measured by spectrophotometry and varies with cell type, functional state of the cell and stage of the cell cycle [170]. With normalisation to total RNA, differences in the proportion

of mRNA in total RNA between patient groups could confound the quantitation of gene expression. However, in the BAL cell samples, there were no significant differences between the groups in cell types nor were there any significant correlations between housekeeping gene mRNA levels and the percentage of the major cell populations of macrophages, lymphocytes or eosinophils. Conversely, there was a substantial difference in cellular composition between biopsy and BAL cell samples but the same pattern of housekeeping gene expression with asthma and ICS use was found. Conceivably, the 5-fold difference in housekeeping gene mRNA levels between BAL cells and biopsies could be due to differing mRNA content of total RNA. However, there seems little chance that the 10-fold difference in housekeeping gene mRNA levels between asthmatics with and without ICS treatment could be due to effects on total RNA composition. It is more likely that there was a difference in transcription and/or stability of GAPDH and β-Actin mRNA between the groups.

The cross-sectional differences in housekeeping gene expression that I have found are in the context of no differences in BAL cell profiles and minimal differences in cytokine expression in both biopsies and BAL cells (Section 1.1). If β-Actin and GAPDH expression is sensitive to the relatively mild inflammation characteristic of such clinically stable asthmatics in samples that differ as widely as BAL cells and airway biopsies, differences in housekeeping gene expression are unlikely to be restricted to studies of asthma or indeed to the lung. However, housekeeping gene expression has been so widely used in medical research that it may be difficult for researchers to accept it.

6.9 PROBLEMS ASSOCIATED WITH THE USE OF HOUSEKEEPING GENE EXPRESSION

Quantitation of IL-2 and IL-4 mRNA levels was used to demonstrate the potential for confounding of gene expression measurements by housekeeping genes when they are used as denominators. IL-2, produced exclusively by T lymphocytes, is the principal differentiation and growth factor for T cells, in addition to stimulating other lymphocytes, such as B cells and NK cells [171]. In T cell-mediated inflammatory

diseases such as asthma, IL-2 and its specific receptor chain, IL-2Rα, can be viewed as markers of cellular activation. Given that the patients in this study were clinically stable asthmatics, we were not surprised that there were no differences in BAL cell IL-2 gene expression with asthma with or without ICS use. Increased expression of IL-4 with asthma and atopy was expected but in this study no significant differences were detected (Section 7.3). However, when both IL-4 and IL-2 data were normalised by either GAPDH or β-Actin, marked differences were seen due entirely to the underlying differences in housekeeping gene expression.

There are three key problems with the previous use of housekeeping gene expression in RT-PCR quantitation in asthma studies [37, 51-53, 172]. Firstly, proper validation studies have not been conducted. Secondly, my results indicate that such studies are likely to be confounded by differences in housekeeping gene expression as demonstrated here for IL-2 and IL-4. Finally, several studies on airway biopsy RNA have used the same primer sites as used in this study for β-Actin and such data could potentially be confounded by pseudogene detection [37, 52, 53, 172].

Two fundamentally flawed validation studies of β-Actin expression in asthma have been published by the group of Kay from the National Heart and Lung Institute (London, UK), which is responsible for the majority of PCR-based studies in asthma [53, 172]. The group has used a semi-quantitative RT-PCR method, which utilises external cDNA standard curves for β-Actin and genes of interest in bronchial biopsies to control for variability in both PCR amplification efficiency and dot blot detection [37, 52, 53, 172]. In the two validation studies, there were no significant differences in the number of β-Actin cDNA copies between asthmatics not using ICS, atopic controls and normal controls [53, 172]. They claimed that the data confirmed that there were "statistically equivalent efficiencies of the RNA extraction and cDNA synthesis in biopsy specimens from subjects in all groups" and by inference that β-Actin gene expression does not vary between controls and asthmatics. The real denominator of this data is per aliquot of cDNA and given its arbitrary nature does not allow any one sample to be compared to another. Consequently, it is incorrect to conclude anything at all regarding RNA extraction and reverse transcription efficiencies nor concerning the biological variability of β-

Actin gene expression in their biopsies. The analyses merely inform them that there was no difference between groups in the number of copies of β-Actin cDNA added to the subsequent steps of the assay.

If β-Actin mRNA levels were consistently expressed in Kay's samples, presenting the gene of interest as a ratio would allow β-Actin to link the samples together allowing them to be compared to each other. Given that the asthmatics in four of their studies had not been using ICS at the time of bronchoscopy [37, 52, 53, 172], it is likely that the expression of β-Actin was significantly lower in these asthmatics than in the atopic and nonatopic control groups. Whilst they concluded in accordance with their hypotheses, that RANTES, MCP-3 [172], IL-4, IL-5 [37] and IL-13 [53] are upregulated with asthma, this is what one would expect with 10-fold decreased β-Actin as the denominator. Indeed the pattern of the ratios is very similar to the inverse of that obtained with β-Actin in biopsies in this study. Upregulation of RANTES/β-Actin ratio was also detected in asthmatics not using ICS by another group using similar technology [51]. Conversely, there are no PCR-based studies indicating unchanged or decreased gene expression with asthma, except for our paper describing the competitive PCR assay of IL-4 and IL-4δ2, which was not reliant on housekeeping gene expression (Section 5) [173]. Without any quantitative comparator between these semi-quantitative methods and our β-Actin copy numbers, it is not possible to determine if there is any real increase in the mRNA levels of these genes with asthma.

6.10 DIFFERENTIAL EXPRESSION OF β-ACTIN AND GAPDH IS WELL DOCUMENTED

Whilst molecular biologists have tended to regard GAPDH and β-Actin as simple housekeeping genes, evidence has been growing over the past decade which indicates that, whilst constitutively expressed, their rate of transcription is influenced by a number of factors. Both β-Actin and GAPDH mRNA levels vary with cellular proliferation [174-180] and their expression is upregulated with the dedifferentiation-regeneration process in the pancreas [181-183] and liver [184]. The

transcription of GAPDH and β-Actin are upregulated rapidly in response to mitogenic stimuli including Epidermal Growth Factor, Transforming Growth Factor-β (TGF-β) and Platelet Derived Growth Factor [185-188]. Sequences in the 5' promoter of the β-Actin gene have been identified as serum responsive enhancers conferring both positive regulation by growth factors and negative regulation by one or more labile proteins [189, 190]. In view of this, it is ironic that both β-Actin and GAPDH have been used to normalise the quantitation of the mRNA of growth factors such as TGF-β, including in some studies in asthma [191, 192].

The observed variability of expression of β-Actin and GAPDH genes emphasises their important physiological roles. The β-Actin cytoskeleton functions in cellular shape and anchorage where transmembrane glycoproteins link fibronectin in the extra cellular matrix with actin microfilaments on the cytoplasmic side of the membrane [193]. It is not surprising, therefore, that cellular proliferation, activation and/or differentiation induces upregulation of β-Actin gene expression and subsequent cytoskeletal remodelling [193, 194]. Similarly, GAPDH expression would be expected to vary as it has a diverse range of functions unrelated to its glycolytic activity. GAPDH has been shown to be involved in membrane transport and membrane fusion, microtubule assembly, nuclear RNA export, protein phosphotransferase/kinase reactions, the translational control of gene expression, DNA replication and DNA repair [reviewed 195]. In addition, over expression of GAPDH is involved in apoptosis, age-related neuronal disorders, and a number of cancers including lung cancer [177, 196, 197]. Indeed these functions of β-Actin and GAPDH determine the need not only for their expression in every cell but also confer the requirement for tightly regulated and responsive gene expression. In retrospect, the rationale for accepting such genes as unchanging, constant denominators was always likely to be flawed, and it is difficult to understand how the concept became so widely accepted.

6.11 CONCLUDING COMMENTS

In spite of the growing body of evidence of differential regulation of GAPDH and β-Actin, these housekeeping genes have been widely used in mRNA quantitation for three main reasons. Firstly, there has been a substantial lack of validation studies to discredit the methodological concept. Secondly, control of RNA quality and cDNA synthesis efficiency without housekeeping gene expression is otherwise difficult. Alternatives include incorporation of radioactive labels during cDNA synthesis [198] and co-RT-PCR of synthetic mRNA exogenous standards [199]. Lastly, the use of housekeeping gene expression to try to control for the PCR amplification and detection phases has allowed relatively inexpensive and simple technology to be used. Such semi-quantitative methods do not allow housekeeping gene expression to be examined independently to validate the assumption that expression is consistent across the study samples. Often the justification for using semi-quantitative methods over quantitative assays has been the large differences expected to be found, therefore only requiring a low-resolution technique. However, the differences detected here in housekeeping gene expression, considerably question this justification.

The advent of real-time quantitation of PCR has circumvented the problems associated with controlling PCR amplification and detection [200, 201]. However, normalising samples for RNA quality and reverse transcription efficiency remain as potential sources of confounding of data. Obviously, mRNA assays need to be designed to allow validation of the internal standards being used and each new application of a given method should include an analysis of consistency of expression.

7 Th CYTOKINE GENE EXPRESSION IN ASTHMA

7.1 INTRODUCTION

As already discussed (Section 1.2), asthma is characterised pathologically by an infiltrate of activated T cells and eosinophils into the airways [156, 169]. Helper T (Th) cells can be broadly divided into two functional subsets based on their profile of cytokine production. Type 1 T cells (Th1) predominantly produce IFN-γ and promote delayed-type hypersensitivity. Type 2 T cells (Th2) secrete IL-5 and IL-4 and regulate B cell and eosinophil-mediated responses. IL-2 is recognised as the principle T cell growth factor and is produced by both classes of Th cells in humans [reviewed 202].

A number of studies of endobronchial biopsies and bronchoalveolar lavage (BAL) have shown a predominance of IL-4 and IL-5 gene expression in asthma and atopy [37, 154-157]. However, the quantitation of IL-4 gene expression in these studies is likely to have been confounded by the unrealised co-detection of the IL-4 alternative splice variant, IL-4δ2 (Section 5.4.3) [173].

As already highlighted, (Section 1.2.2), the use of PCR technology in asthma has usually been characterised by essentially qualitative assessments [33, 46, 47] or at best semi-quantitative assays dependent on "normalisation" by comparison with housekeeping gene expression [37, 49]. My cross-sectional analysis of GAPDH and β-Actin mRNA levels has indicated that such studies are likely to be confounded by differences in housekeeping gene expression (Section 6.8). It is likely that uncritical use of such housekeeping genes for normalising data has lead to false confidence in previous semi-quantitative data.

In this chapter, my cross-sectional analysis of the mRNA levels of IL-5, IL-4, IL-4δ2, IL-2 and IFN-γ in BAL cells and airway biopsies are described. This is the first documentation of the in vivo mRNA levels of these cytokines in asthma that is independent of housekeeping gene expression.

Table 7.1 Bronchoscopy Subject Profiles

	Asthmatics		Normal Controls	
	BAL Cells	**Biopsies**	**BAL Cells**	**Biopsies**
n	89	81	30	24
Gender (M:F)	46:29	40:30	21:9	15:9
Age median (range)	42 (21 – 70)	41 (21 – 69)	30 (20 – 61)	29 (20 – 61)
Atopy (+/-)	72/17	64/17	13/17	13/11
ICS use (+/-)	72/17	66/15	0/30	0/24
ICS dose/day (BDP)[1] median (range)	400 (100 – 1500)	400 (100 – 1000)	–	–
PD20 methacholine (mg) median (range)	0.028 (0.001 – 6.4)	0.026 (0.001 – 6.4)	>3.4	>3.4
FEV1 % predicted Median (range)	90 (60 – 128)	88 (58 – 120)	112 (80 – 132)	108 (91 – 132)

[1] Dose in μg per day of beclomethasone diproprionate

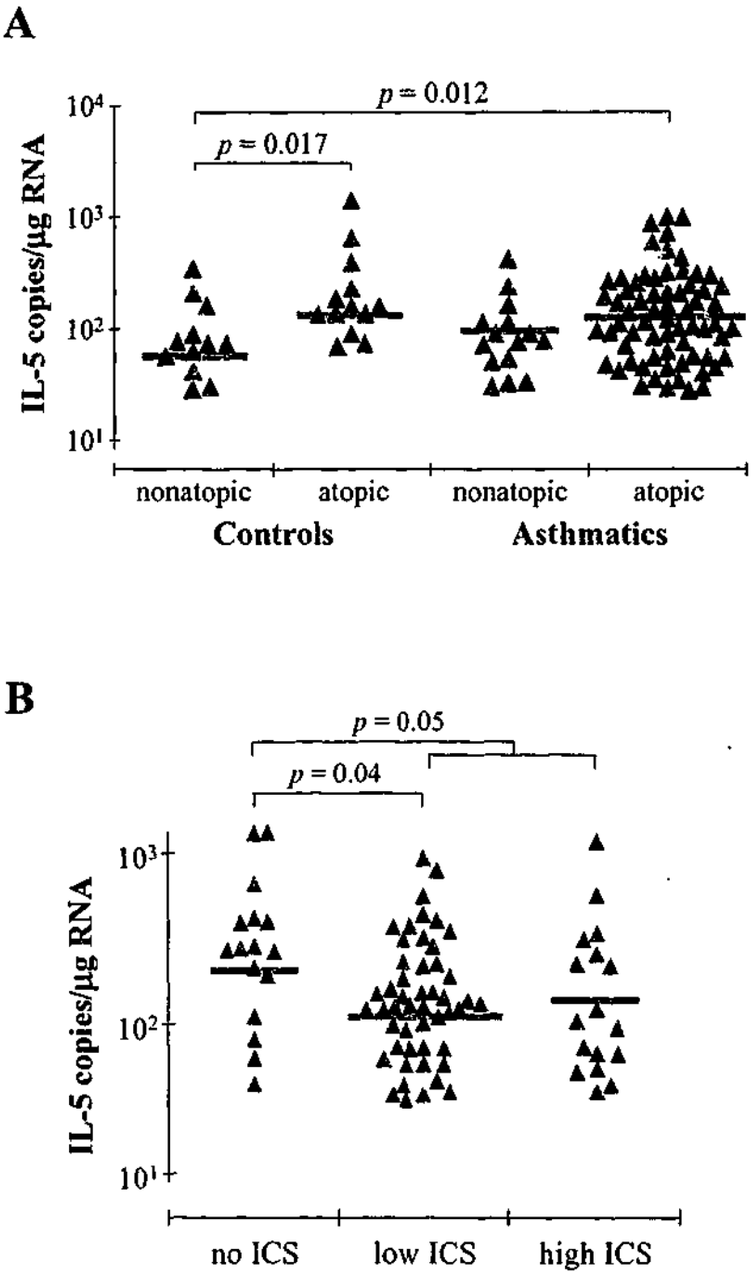


Figure 7.1 IL-5 gene expression related to atopy, asthma and ICS use

IL-5 mRNA levels were increased with atopy (A, left) and decreased with ICS use (B) in BAL cells but not in biopsy samples (C, right). Asthmatics as a group were not different from controls.

7.2 MATERIALS AND METHODS USED

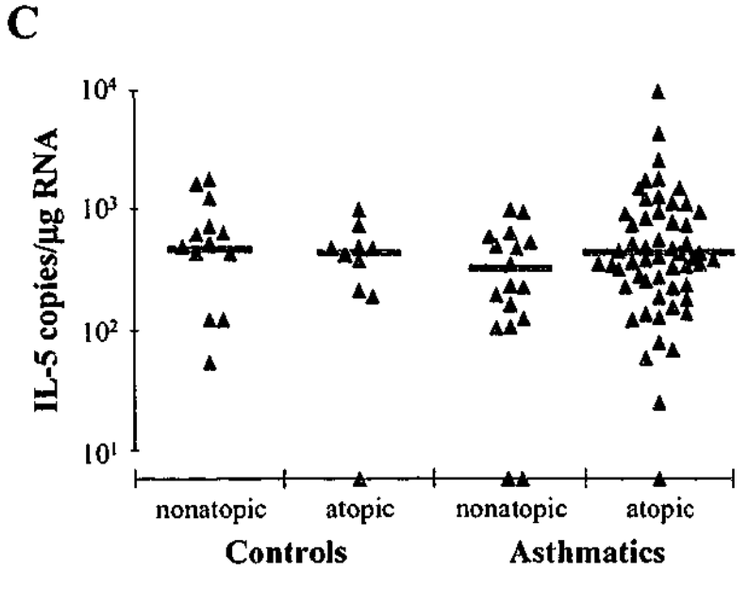
7.2.1 Subjects

111 asthmatic volunteers took part in the study. Airway endobronchial biopsies were obtained from 70 and BAL cells from 75 patients (Table 7.1). They all used albuterol for relief of symptoms as needed, with 93 patients using regular ICS. Salmeterol (50 μg twice daily) was used by 12 patients from whom BAL cells were obtained and in 5 from whom biopsies were taken.

7.2.2 Statistical Analysis

Generalised linear modelling was used for the cross-sectional analyses. The confounding variables used in the modelling included as appropriate: age, sex, gender, atopic status, ICS usage, use of long-acting β2-agonist and RT-PCR batch factors. The Pearson correlation was used to test associations between IL-4 and IL-4δ2. Statistical analyses were performed using SAS version 6.12 software (SAS institute, Cary, NC, USA) [163].

Figure 7.1, continued



7.3 ASTHMA, ATOPY AND Th CYTOKINE EXPRESSION IN BAL CELLS AND BIOPSIES

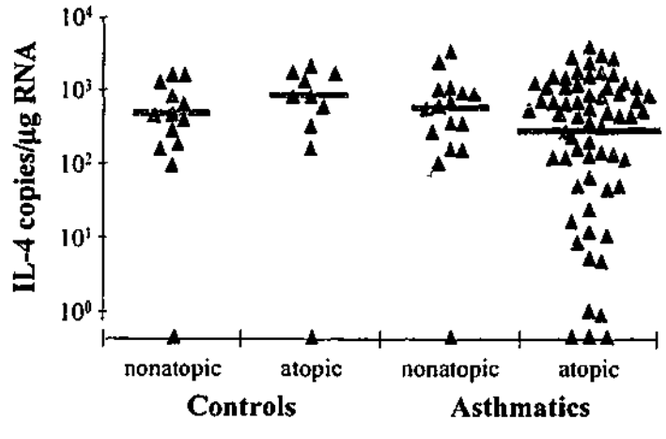
In biopsy samples, no cross-sectional differences in IL-5 mRNA levels were observed (Figure 7.1C, above). In BAL cells, the only signal for IL-5 was an increase in atopics, with nonatopic asthmatics being intermediate, but not significantly different from any other group (Figure 7.1A). Thus, in normal control BAL cell RNA samples, IL-5 mRNA levels were higher in atopics (least squares mean ± SEM; $1.5 \pm 1.0 \times 10^2$ copies/μg RNA) than in nonatopics ($6.0 \pm 2.6 \times 10^1$ copies/μg RNA, $P = 0.017$). Whilst atopic asthmatic levels of IL-5 ($1.4 \pm 0.3 \times 10^2$ copies/μg RNA, $P = 0.012$) were significantly higher than nonatopic controls, they were not different from atopic controls or nonatopic asthmatics ($1.1 \pm 0.3 \times 10^2$ copies/μg RNA).

BAL cells from asthmatics not using ICS expressed more IL-5 mRNA ($1.9 \pm 0.7 \times 10^2$ copies/μg RNA) than those using ICS ($1.0 \pm 0.2 \times 10^2$ copies/μg RNA, $P = 0.05$) but there was no obvious dose response effect (Figure 7.1B).

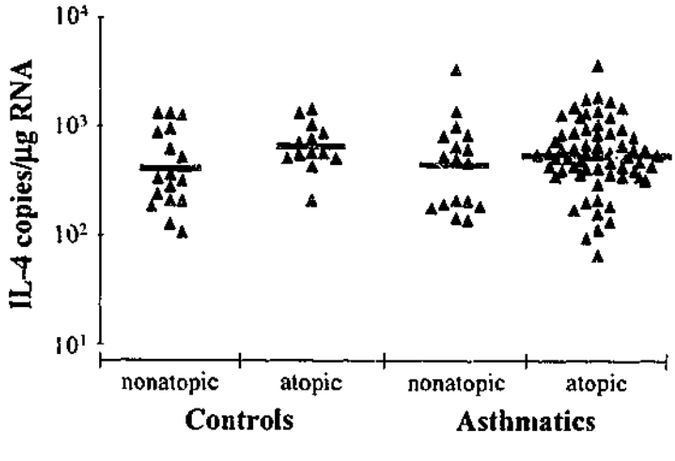
Neither in BAL cells nor biopsies did IL-4, IL-4δ2 and IFN-γ mRNA levels differ with atopic status or asthma (Figure 7.2). Furthermore, asthmatics using ICS were not different from those not taking ICS treatment.

IL-2 gene expression in biopsies did not differ significantly between controls ($1.3 \pm 0.6 \times 10^4$ copies/μg RNA) and asthmatics not using ICS ($2.7 \pm 2.9 \times 10^4$ copies/μg RNA) (Figure 7.3A). However, biopsies from asthmatics using up to 500 μg per day of ICS ($3.5 \pm 0.4 \times 10^3$ copies/μg RNA, $P = 0.042$) and those using 500–1,500 μg per day ($2.0 \pm 0.3 \times 10^3$ copies/μg RNA, $P = 0.014$) expressed less IL-2 mRNA than ICS-untreated asthmatics. In common with IFN-γ and IL-4 gene expression, IL-2 mRNA levels did not vary with atopy. No differences were detected in IL-2 mRNA expression in BAL cells (Figure 7.3B).

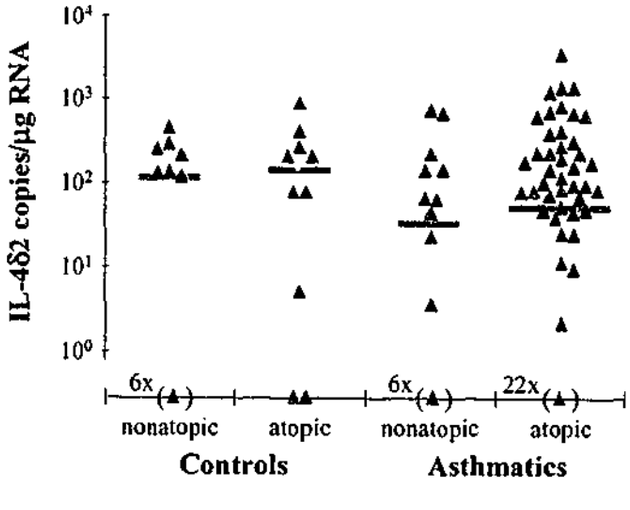
A


IL-4 copies/µg RNA
10⁴
10³
10²
10¹
10⁰
nonatopic
atopic
nonatopic
atopic
Controls
Asthmatics

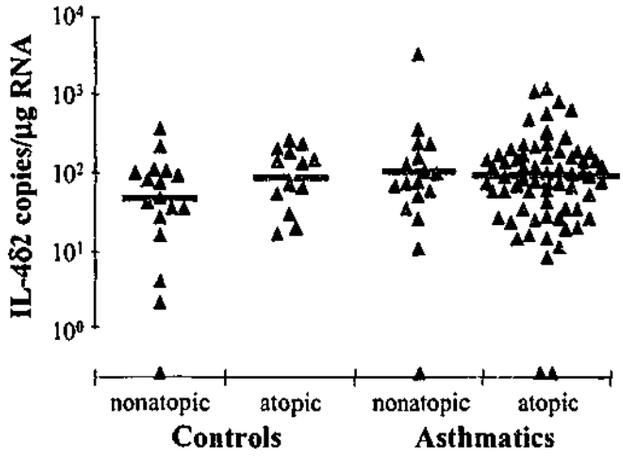
B


IL-4 copies/µg RNA
10⁴
10³
10²
10¹
nonatopic
atopic
nonatopic
atopic
Controls
Asthmatics

C


IL-4δ2 copies/µg RNA
10⁴
10³
10²
10¹
10⁰
6x
6x
22x
nonatopic
atopic
nonatopic
atopic
Controls
Asthmatics

D



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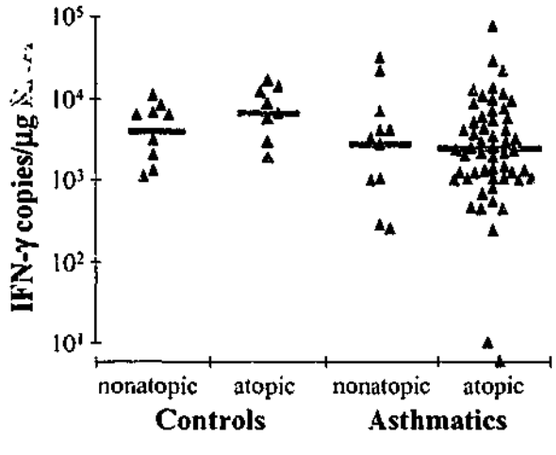

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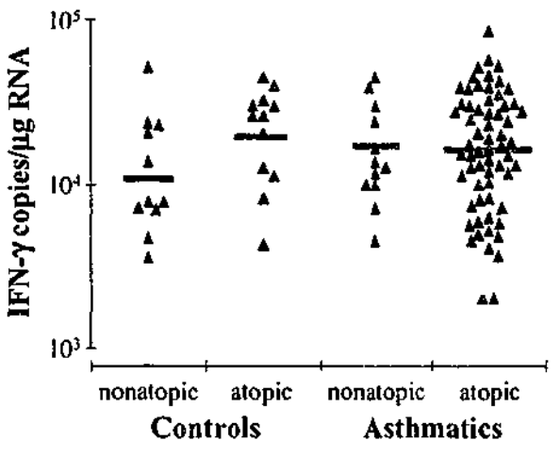

Figure 7.2 Cross-sectional analyses of IL-4, IL-4δ2 and IFN-γ mRNA levels

IL-4 (A and B), IL-4δ2 (C and D), and IFN-γ (E and F) in biopsies and BAL cells respectively.

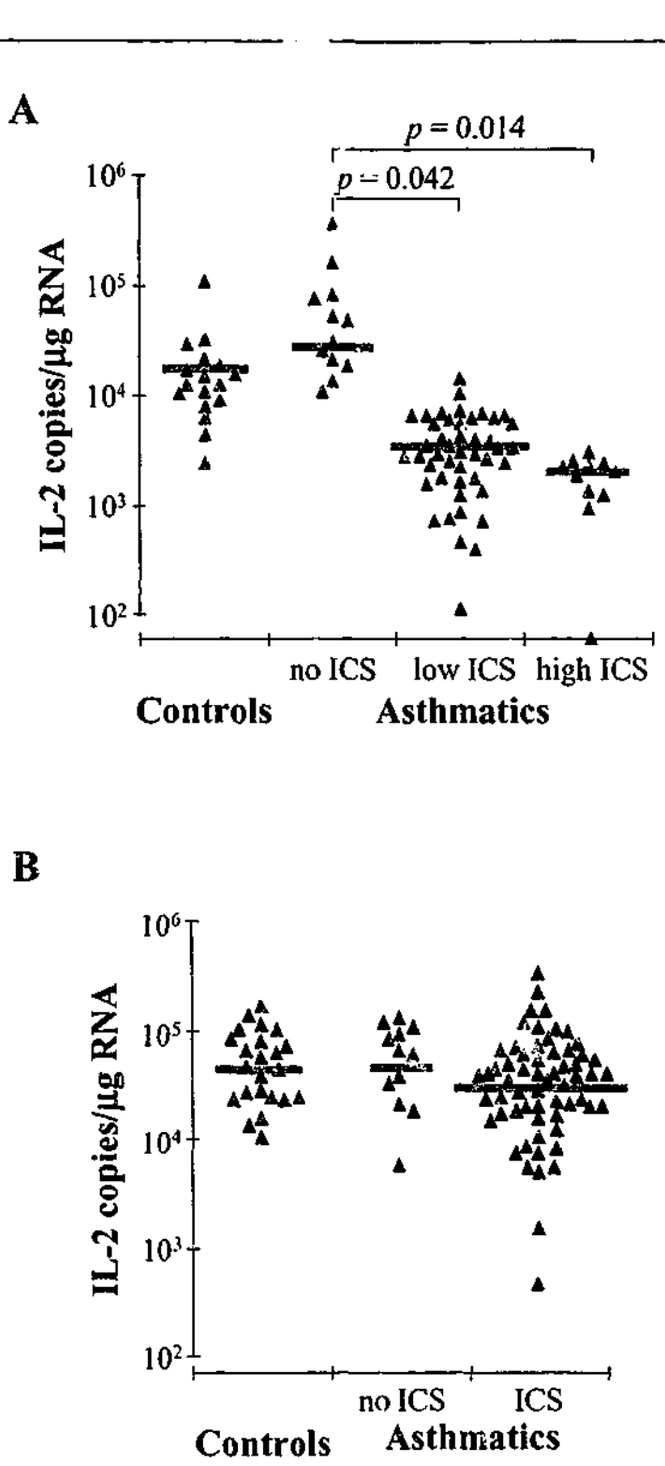

Figure 7.3 IL-2 gene expression, asthma and ICS treatment

IL-2 mRNA levels were decreased by ICS use in biopsies (A) but not in BAL cells (B).

7.4 EVIDENCE OF COORDINATED EXPRESSION OF IL-4 AND IL-4δ2

Pearson regression analysis indicated that IL-4 and IL-4δ2 mRNA levels positively correlated in both BAL cells ($r^2 = 0.53$, $P < 0.0001$, $n = 111$) and biopsies ($r^2 = 0.31$, $P < 0.0001$, $n = 65$) (Figure 7.4). In the majority of samples, the calculated IL-4 mRNA levels were higher than IL-4δ2 and the slope of the regressions of IL-4δ2 against IL-4 were 3.1 and 13.4 for biopsies and BAL cells respectively. Whilst dual-mRNA species competitive PCR has been established as a relative rather than an absolute quantitative method (Section 5.3.3), the positive slopes of the regressions, particularly of the levels in BAL cells, indicate that with up regulated IL-4 gene expression a greater proportion of the pre-mRNA is spliced to IL-4δ2.

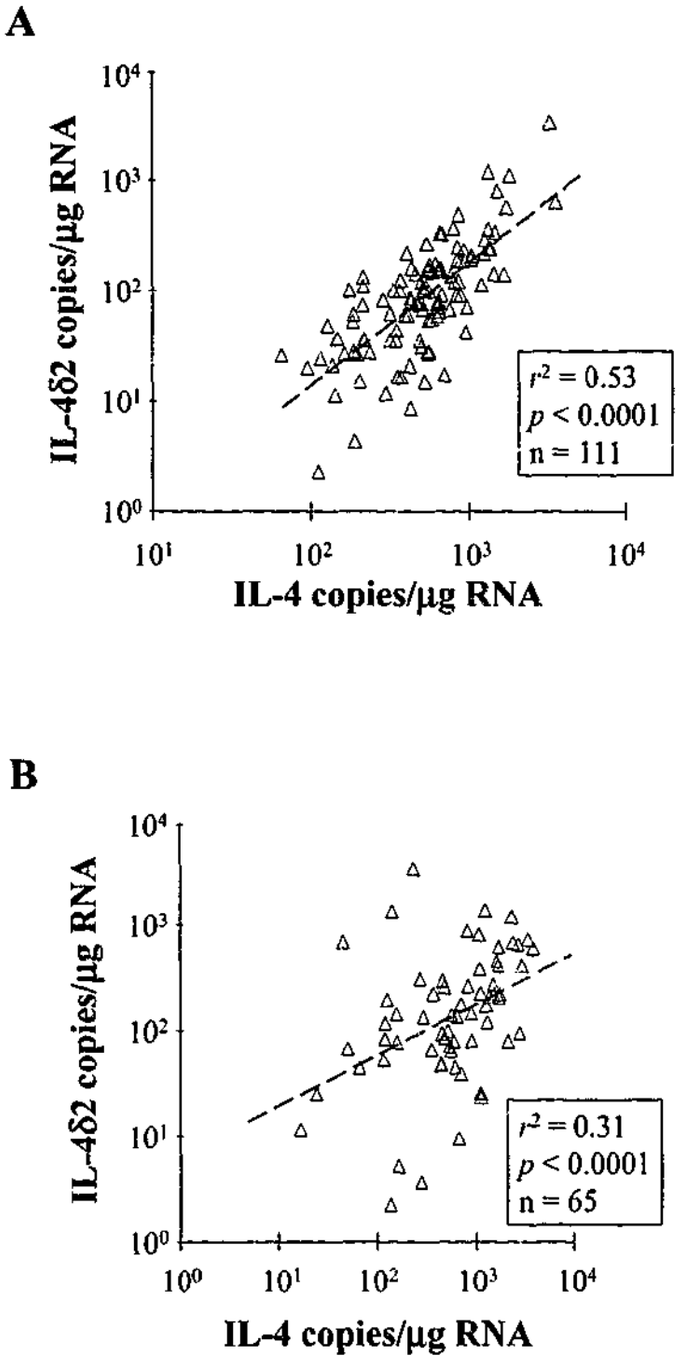

Figure 7.4 Coordinated expression of IL-4 and IL-4δ2

Pearson correlations between IL-4 and IL-4δ2 in BAL cells (A) and biopsies (B) indicated a degree of coordinated expression of IL-4 mRNA and its splicing variant, IL-4δ2.

7.5 DISCUSSION OF RESULTS

This study is the first to use quantitative, competitive PCR methodology to measure Th cell cytokines in a cross-sectional analysis of airway samples from asthmatics and normal controls. I found that in BAL cells atopic controls and atopic asthmatics expressed more IL-5 than non-atopic controls, with nonatopic asthmatics having intermediate levels. Within the asthmatics there was an inhibitory effect of ICS on IL-5 mRNA levels in BAL cells. However, in biopsy specimens no cross-sectional differences in IL-5 were detectable. Similarly, BAL cell and biopsy levels of IL-4, IL-4δ2 and IFN-γ did not differ significantly with atopy, asthma or ICS use. In contrast, IL-2 gene expression in biopsies but not BAL cells was decreased with ICS use. Strong correlations between IL-4 and IL-4δ2 in both BAL cells and biopsies suggest a degree of coordinated expression, above a threshold of IL-4 production.

Multivariant linear modelling statistics was used to control for confounding effects whilst testing the significance of other factors. This included correction for differences between groups attributable to atopy whilst testing the difference due to ICS use, for example. Whilst RNA samples were stratified by RNA concentration to facilitate cDNA synthesis and avoid systematic bias, I found that batch parameters were significant confounders, although differing batches were random between assays and not associated with the amount of input sample RNA. Likely causes include pipetting inaccuracies leading to differences in competitor amounts and differences in gel resolution, quality and staining influencing the measurement of native and competitor PCR products. Whilst multivariant statistics optimise power of such studies and allow a degree of correction for the methodological weakness, it is obviously important to avoid systematic biases where possible and in particular to minimise the number and differences between batches.

Despite the quantitative nature of the competitive PCR assays, I was only able to detect minor differences in cytokine gene expression with atopy, asthma, and ICS use. The asthmatics in this study were clinically stable but had a wide range of bronchial hyperreactivity and ICS-dose requirement, although current activity of the disease in terms of symptom levels was comparable between subjects (Table 6.1). Importantly, there were no differences in BAL cell profiles between the subject

groups and in particular, the percentage of eosinophils in samples from untreated asthmatics were not elevated compared to normal controls. In the context of minimal cellular differences, Th2-type cytokine expression would also not be expected to differ with asthma. However, the results are cross-sectional only and the absence of longitudinal PCR-based studies in the literature of asthma is testament to the difficulties associated with such studies.

PCR assays commence with cell homogenisation and as a strategy are good for detecting large numbers of cells expressing small amounts of mRNA but are relatively poor at detecting small numbers of upregulated cells producing larger amounts of message which become diluted with lysis. In situ hybridisation studies indicate that the latter pattern of expression is likely in asthma [37, 154, 155]. A major problem with the application of quantitative PCR to asthma is the low levels of mRNA assayed with some mean levels in this study being below 100 copies per μg total RNA. The pathological variability of the stable asthmatics in this study is reflected in their mRNA levels and contributed to the lack of contrast between stable asthmatics and normal controls.

My study of IL-4 and its receptor antagonist-encoding splicing variant, IL-4δ2, in nasal polyps, BAL cells and biopsies indicate that both IL-4 mRNA variants are commonly expressed in respiratory tract samples. The primers used in this study to amplify IL-2 mRNA do not amplify the splicing variants IL-2δ2 or IL-2δ3, which also encode receptor antagonists [149]. Given the difficulty of distinguishing cytokine splicing variants and in particular the absence of antibodies of confirmed specificity, previous studies are likely to be further confounded by co-detection [38, 154, 155].

7.6 ARE IL-4, IL-4δ2, IL-5, IL-2 AND IFN-γ IMPORTANT IN ASTHMA?

The strongest evidence for increased IL-4 and IL-5 expression being associated with asthma comes from airway allergen challenge in atopic patients. IL-5 [47, 203-206] and IL-4 [203, 204] gene transcription was reported to be upregulated 18 to 24 hours after challenge compared with that found immediately after challenge or compared with vehicle-only-challenge sites. These studies are further reinforced by nasal challenge in rhinitics [207, 208] and skin challenge in atopics [209], which also found increased IL-5 and total IL-4 expression.

Interestingly, even with acute allergen challenge, not all studies have found significant increases in IL-4 gene expression [205, 206]. As the methodology used in theses studies did not distinguish the IL-4 splicing variants, a shift in the relative splicing of IL-4 and IL-4δ2 can not be ruled out. IL-4 and IL-5 have been correlated with asthma symptoms [210] and may be more important in acute asthma than stable asthma. The inconsistency of antigen challenge studies suggests that repeat antigen stimulation (both natural and introduced) may be required to obtain uniform upregulation of IL-4 and IL-5 in airway tissue. The expression of IL-2 and IFN-γ was not changed by allergen challenge in the lung [203-206]. In the group of stable non-acutely challenged asthmatics in this study, I did not find significant mRNA increases compared to normal controls in IL-4, IL-4δ2, IL-2 or IFN-γ mRNA levels. Atopy rather than asthma differences in IL-5 gene expression were detected in BAL cells but not in biopsies.

7.7 THE POTENTIAL BIOLOGICAL ROLE OF IL-4δ2

In both BAL cells and biopsies, there was a strong positive correlation between IL-4 and IL-4δ2 but with IL-4 expression tending to predominate over IL-4δ2. This is strongly suggestive of coordinated expression of IL-4 and its receptor antagonist-encoding splicing variant. It is conceivable that splicing of IL-4δ2 may commence after a threshold of IL-4 expression is reached, allowing IL-4δ2 to act as a brake on IL-4 mediated responses (Figure 7.5). This latter concept is supported by the

observation that samples with higher levels of IL-4 expression also had proportionally higher levels of IL-4δ2 than those with less IL-4 mRNA.

In the absence of validated protein detection methods to discriminate between IL-4 variants, it is not possible to determine whether the transcribed mRNAs are actually translated to protein. IL-4 immunoreactivity has been found in storage granules of eosinophils and mast cells [211, 212]. If IL-4δ2 is indeed an in vivo physiological antagonist of IL-4 activity, then conceivably, IL-4δ2 could be co-expressed with IL-4 and stored to rapidly moderate an IL-4-mediated response. It is possible that the defect in some allergic responses is with the expression of IL-4δ2 rather than IL-4, so that events become explosive and uncontrolled leading to end-organ disease.

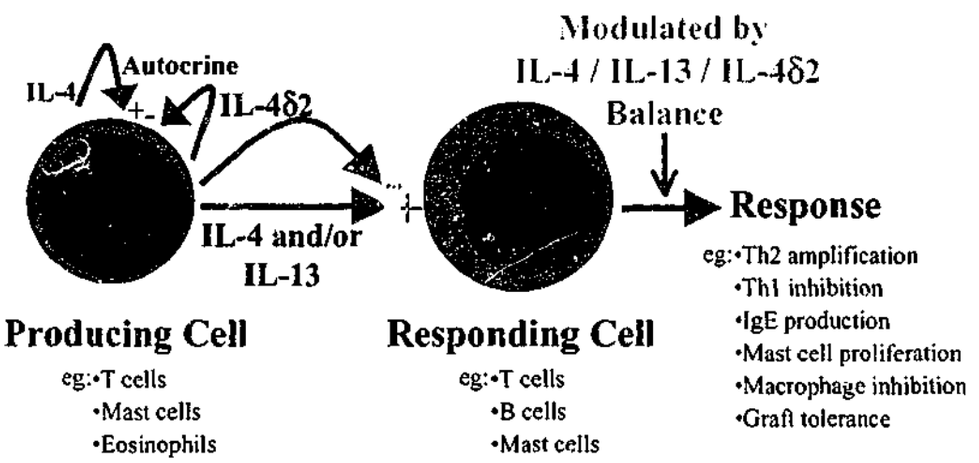

Figure 7.5 Potential biological role of IL-4δ2 in modulating IL-4 and IL-13 responses

There is preliminary evidence that IL-4δ2 has differing affinities compared to IL-4 for at least two receptors [147, 148]. Thus, IL-4δ2 may be expressed as a differential receptor antagonist to channel IL-4 from binding to the high affinity IL-4 receptor to the low affinity receptor. The IL-2 receptor antagonists, IL-2δ2 and IL-2δ3, are likely to function in the same manner and have been shown to specifically bind to the IL-2 high affinity receptor [213]. There is considerable overlap in the function of IL-4 and IL-13, mediated in part by shared receptors and receptor chains [214-217].

IL-4δ2 expression might also function to direct IL-13 from one receptor to another. The close linkage of IL-4 and IL-13 genes on chromosome 5 and common promoter sequences mediate co-regulation [218]. Concomitant expression of IL-4 and IL-13 has been observed in T cells [218]. However, IL-13 mRNA levels were not measured in this study. The relative amounts of IL-13, IL-4 and IL-4δ2 and their receptors may differ to mediate differential IL-4/IL-13 responses (Figure 7.5). IL-4δ2 expression may be a mechanism for critical control and rapid resolution of such responses.

Polarised Th2-cells are not as readily found in humans in comparison to mice and this might be due to rapid depolarisation of Th2 cells facilitated in part by IL-4δ2. IL-4δ2-mediated inhibition of Th2 responses may be an alternative to Th1 cytokine expression, such as IFN-γ, without the concomitant pro-inflammatory effects of these cytokines. Consistent with this concept is the finding that, unlike in mice, in humans reduced IFN-γ expression with respect to controls is not often observed with active Th2-type responses (Table 1.1, Section 1.2.1). Likewise, IL-2δ2 and/or IL-2δ3 may provide a similar inhibition of Th1 responses with out the pro-inflammatory effects of Th2-type cytokines.

The antagonistic actions of IL-4δ2 on IL-4 and IL-13 responses have been mimicked by recombinant mutants of IL-4 [219, 220]. Whilst providing important insights into the biology of IL-4, IL-13 and their receptors [221, 222], the ultimate goal of these endeavours is to find IL-4 and/or IL-13 antagonists that have clinical utility in modulating IgE responses [223]. IL-4δ2, as a naturally occurring receptor antagonist, could also have therapeutic potential for the treatment of allergies [148].

7.8 THE UTILITY OF COMPETITIVE PCR ASSAYS IN ASTHMA STUDIES; CONCLUDING COMMENTS

7.8.1 Possible future improvements to the methodology

The competitive PCR assays that I have established contain two aspects that were not as tightly controlled as I would have wished; the quality of mRNA and the efficiency of its reverse transcription during cDNA synthesis. Spectrophotometry partially controls for RNA quality but is unable to distinguish full-length mRNA from degraded RNA and some combinations of salts and solvents may give absorbance ratios identical to quality RNA.

Housekeeping gene expression has traditionally been used to control for RNA quality and reverse transcription efficiency. However, the concept of housekeeping gene expression remaining consistent across experimental groups has always been debated although rarely in the literature. In my cross-sectional study of asthmatics and normal controls, I found 10-fold differences in housekeeping gene expression in the presence of only minimal differences in the expression of T cell cytokines and in the absence of differences in BAL cell profiles. Housekeeping genes by definition are responsible for basic cellular function and therefore their expression is quite likely to vary between cell types and within cell populations at different activation levels. This suggests that the use of housekeeping gene expression as endogenous controllers of RNA quality and its reverse transcription are unlikely to be valid, especially in studies of inflammatory diseases such as asthma.

A number of studies that have found variable GAPDH and β-Actin have reported ribosomal RNA (rRNA) sequences to be consistently expressed [224-226]. The use of rRNA to normalise mRNA quantitation has been promoted by the co-development of semi-competitive rRNA assays and real-time quantitative PCR technology (personal communication Applied Biosystems). Despite the rapid utilisation of real-time quantitation, no validation studies with rRNA and this new technology have been published to date. rRNA sequences are likely to vary with the cell cycle, cellular activation and with cell type reflecting differential requirements for protein

synthesis. Indeed the variation of 16S rRNA with the cell cycle has been used to measure the generation time of pathogens such as Chlamydia trachomatis [227].

An alternative strategy is to add a cRNA competitor prior to RNA extraction allowing control of all the steps from extraction of RNA to competitive PCR quantitation [228]. The variation in size and amount of biological samples coupled with the range of expression of mRNA in a given study makes it difficult to optimise the amount of cRNA to add. A variation of this strategy is to add a cRNA specific for a sequence not expected to be expressed in the biological samples [229]. The cDNA derived from this cRNA molecule can then be quantitated by competitive PCR with a DNA competitor allowing the efficiency of RNA extraction and cDNA synthesis to be calculated for each sample. Whilst these strategies attempt to control for RNA extraction efficiency, RNA degradation is thought to occur within cells as they begin to lyse. During lysis, the internal cellular compartments are disrupted, exposing RNA to cellular RNases that have not been inactivated by the lysis solution. A cRNA added to samples will be protected by the denaturants in the cell lysis solution and will not be exposed to these RNases to the same degree as cellular RNA.

The pAW109 cRNA added to my assays at cDNA synthesis in this study could be utilised in a similar fashion to the latter strategies to determine the efficiency of reverse transcription. This would require the use of a mismatched upper and lower primer present on the cRNA (upper IL-2 primer and lower IL-1-β primer, for example; Figure 4.1, page 62) so that the cRNA could be amplified in PCR without co-amplification of a native mRNA sequence. The construction of a mutated pAW109 cRNA-like DNA competitor based around these primers would also be required to quantitate the amount of cRNA transcribed to cDNA. Unfortunately, time and expense did not permit development of this complex strategy.

7.8.2 How well did competitive PCR perform?

The major purpose of this study was to perform as definitive an evaluation of the utility of using quantitative competitive PCR in asthma research as current technology at the time allowed, mainly because previous PCR-based studies have been relatively methodologically inadequate. My initial response was that the lack of distinction within the asthmatics and between asthmatics and normal controls prevented a thorough evaluation of the usefulness of the competitive PCR assays. Whilst the sets of samples in this study have not allowed the method to be show-cased, it does allow an insight into the ability of competitive PCR to detect small differences.

From the studies reviewed in Section 1.2.1, differences in IL-5 expression might have been expected. Whilst four of theses studies did not look at the number of eosinophils, those that did quantitate eosinophils found significant differences between asthmatics and control groups [28, 30, 35-39]. Furthermore, IL-5 expression has been positively correlated with the number of eosinophils [28, 30, 230, 231] and asthma symptoms [29, 34, 37, 52, 232] and negatively with spirometry [28, 29, 34, 37, 52, 232]. Of ten studies that quantitated IL-2 expression, only four found significant cross-sectional differences (Table 1.1, page 11). In my study, despite the mildness of the asthmatics and their lack of BAL eosinophilia, some small differences in IL-5 and IL-2 gene expression were even then observed, suggesting that the technique is sensitive in measuring small differences in cytokine expression.

One major concern with RT-PCR technology is the amount of biological material required. Approximately 2 x 10^6 BAL cells and at least 2 airway biopsies are required for efficient RNA extraction and its quantitation by spectrophotometry, representing a substantial commitment to the technology at the commencement of a bronchoscopic study. However, from this pool of RNA a total of nine assays were able to be conducted for each sample; competitive RT-PCR assays for IL-4/IL-4δ2, IL-5, IL-2, IFN-γ, TNF, β-Actin and GAPDH and competitive PCR for β-Actin and GAPDH directly on the RNA with out reverse transcription to screen for genomic DNA contamination. The expression of IL-4, IL-4δ2 and IL-5 was quite low and the respective assays used a total of 65% of the RNA used. In contrast, the high level

of expression of housekeeping genes were quantified from a 1:80 dilution of cDNA using only 0.08 % of the RNA.

Clearly, the usefulness of PCR technology is dependent on the level of expression of the genes being investigated. Growth Factors have been detected in asthmatic and control airway biopsies by immunohistochemistry at much higher levels and by larger numbers of cells than T cell cytokines (preliminary results of my group). The percentage area of a biopsy section is difficult to quantitate when a majority of the cells are stained, even with sophisticated image analysis technoiogy. Perhaps competitive PCR will be more efficient at detecting differences in growth factor expression in asthma studies.

8 APPENDICES

8.1 MATHEMATICAL TREATISE ON AMPLIFICATION EFFICIENCIES

The aim of this appendix is to clarify the mathematical discussion of amplification efficiencies and competitive PCR by the use of simple mathematical modelling (Section 4.4.1). The modelling centres on the use of the previously discussed equations (Equation 4.3, page 67) to calculate yields of PCR products:

$$N_n = N_0(1+E_N)^n$$

$$C_n = C_0(1+E_C)^n$$

Simple data resembling a titration of competitor against a constant aliquot of native was entered in to the equations to observe the effect of changing the amplification efficiencies of native and competitor templates on the yield of the templates after 20 cycles of amplification (N_{20} and C_{20}, respectively). The data for absolute quantification where E_N and E_C are equal are given in Table 8.1

Table 8.1 Simulated absolute quantitation by competitive PCR

N_0	C_0	E_N	E_C	N_{20}	C_{20}	Output Ratio
100	10	1	1	104,857,600	104,857,600	10
100	20	1	1	104,857,600	20,971,520	5
100	40	1	1	104,857,600	41,943,040	2.5
100	80	1	1	104,857,600	83,886,080	1.25
100	160	1	1	104,857,600	167,772,160	0.625
100	320	1	1	104,857,600	335,544,320	0.3125
100	640	1	1	104,857,600	671,088,640	0.15625
100	1280	1	1	104,857,600	1,342,177,280	0.078125

When the amplification efficiency of the competitor is reduced (E_C = 0.8; E_N = 1), the titration is transposed in a similar fashion to the curve where more native template was added (Figure 8.1). Note that when E_N and E_C are not equal but are constant across the quantitative range, a curve of slope –1 is obtained. The output ratios retain their relative relationship to each other indicating that relative quantitation is possible even if amplification efficiencies are not identical. It can also be seen that known amounts of input are needed to distinguish the two titration curves and to distinguish absolute and relative quantitation.

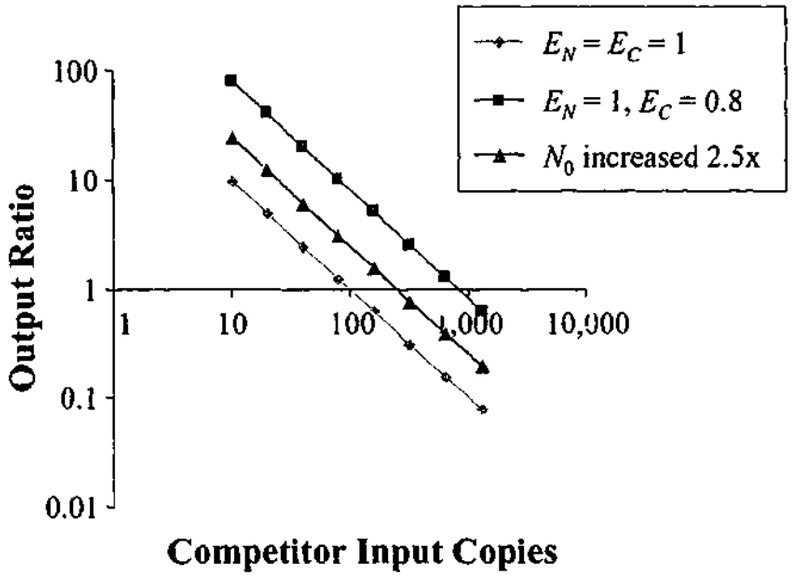

Figure 8.1 Effect of different amplification efficiencies on simulated titrations

The mathematical modelling of Raeymaekers [111] indicated that the amplification efficiencies could differ across the quantitative range providing the ratio of the amplification rates was constant (Equation 4.5, page 68). In the above example where E_N and E_C are not equal, this ratio was 0.9. If this ratio is kept constant but E_N and E_C are reduced to 0.6 and 0.44 respectively for the latter half of the titration, an identical titration curve is obtained (Figure 8.2). Despite the differences in amplification efficiencies, these four output ratios retain their relative relationship with the other output ratios in the titration, confirming the ability to conducted relative quantitation under these conditions.

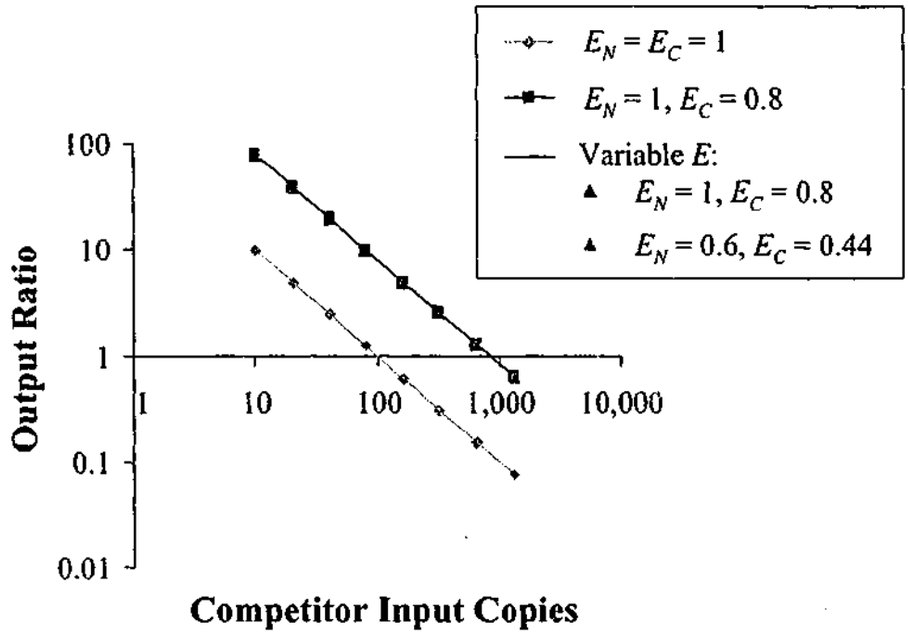

Figure 8.2 Effect of changing efficiencies of amplification but constant ratio of amplification rates on simulated titrations

8.2 ABBREVIATIONS

A_{260}	spectrophotometric absorbance measured at 260 nm
AP	alkaline phosphatase
BAL	bronchoalveolar lavage
bisacrylamide	N,N'-methylenebisacrylamide
BLAST	basic local alignment search tool
C_0 and C_n	amount of competitor template before and after n cycles of PCR, respectively
cRNA	complementary RNA
DIG	digoxigenin
DNases	deoxyribonucleases
dNTPs	deoxyribonucleotide triphosphates
ELISA	enzyme-linked immunosorbent assay
E_n and E_c	amplification efficiency of native and competitor templates, respectively
FEV_1	forced expiratory volume in 1 second
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
ICS	inhaled corticosteroid
IFN-γ	Interferon-γ
IL	Interleukin

N_0 and N_n	amount of native template before and after n cycles of PCR, respectively
PAGE	polyacrylamide gel electrophoresis
PBMC	peripheral blood mononuclear cells
PD_{20}	cumulative dose provoking a 20 % decrease in FEV_1 from baseline
PHA	phytohemagglutinin
RANTES	regulated on activation, normal T cell expressed and secreted
RNases	ribonucleases
rRNA	ribosomal RNA
RT-PCR	reverse transcription followed by PCR
SEM	standard error of the mean
TAE	Tris-acetate-EDTA buffer (Section 3.2.1.1)
TEMED	N,N,N',N'-tetramethylethylenediamine
TGF-β	Transforming Growth Factor-β
Th	T Helper cell
Th1 or Th2	type 1 or 2 T Helper cell, respectively
TNF	Tumour Necrosis Factor

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9 REFERENCES

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