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Technical Brief

Retinal VEGF mRNA measured by SYBR Green I fluorescence: A versatile approach to quantitative PCR

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Purpose: To determine whether continuous monitoring of SYBR Green I fluorescence provides a reliable and flexible method of quantitative RT-PCR. Our aims were (i) to test whether SYBR Green I analysis could quantify a wide range of known VEGF template concentrations, (ii) to apply this method in an experimental model, and (iii) to determine whether 20 existing primer pairs could be used to quantify their cognate mRNAs.
Methods: Real-time quantitative PCR was performed using a LightCycler rapid thermal cycler (Roche). Retinal VEGF mRNA levels were measured in a murine model of oxygen-induced retinopathy during vaso-obliterative and hypoxic phases.
Results: This technique was able to detect as few as 10 control template copies, with quantitative data available routinely for 1000 or more copies. The levels of retinal VEGF mRNA expression followed the hypoxia-induced pattern determined previously by conventional methods. All gene-specific primer pairs which amplify a specific product by conventional PCR were successfully converted to SYBR Green analysis, including those for housekeeping genes glyceraldehyde phosphate dehydrogenase (GAPDH), cyclophilin, and acidic ribosomal phosphoprotein PO (ARP/36B4) and for 28S rRNA. In each case melting curve analysis and agarose gel electrophoresis confirmed the specificity of the amplification product.
Conclusions: The sequence-independent detection of DNA with SYBR Green I means that it can be used to quantify the amplification of any cDNA using gene-specific primers. This rapid and flexible method is ideally suited for researchers in vision science wishing to quantify mRNAs from many different genes because it does not require investment in gene-specific hybridization probes.

Reverse transcription PCR (RT-PCR) provides a powerful method for analyzing mRNA expression from small samples. However, because traditional methods rely on end-point analysis they are at best semi-quantitative due to variations in amplification efficiency in the later stages of the reaction. Attempts to make the analysis more quantitative, for example by inclusion of known competitive templates, have proved laborious and technically challenging. The use of fluorescent hybridization probes [1] has enabled real-time monitoring of the amplification reaction. When the probes are intact energy transfer between a fluorescent reporter dye and a quencher dye keeps the fluorescence emission low. Following hybridization with the amplification product the probe is either degraded [2] or the reporter and quencher dyes are separated, in both cases restoring the fluorescence of the reporter. The fluorescence generated is proportional the amount of product present. The cycle number at which the level of fluorescence rises above a background threshold value is inversely proportional to the log of the initial number of template copies [3].

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The use of hybridization probes in quantitative RT-PCR is now well established [4,5]. However, to date all hybridization probe formats (Taqman [2,6], Molecular beacons [7]and LightCycler probes etc.) require both synthesis and optimization of a specific probe for each gene of interest. This can be problematical and expensive [8]. An alternative method to monitor DNA amplification, independent of its sequence, involves the high affinity double-stranded DNA (dsDNA) binding dye SYBR Green I. This has negligible fluorescence in the absence of dsDNA but has a large fluorescence enhancement upon binding to dsDNA. The major advantage of this technique is that, in theory, it can be used with any primer pair provided that no products other than the specific one of interest are amplified: such non-specific PCR products would contribute to the SYBR Green I fluorescence and prevent accurate quantification.

The potential benefits of using SYBR Green I dye to continuously monitor PCR product formation have been demonstrated [9,10]. We describe how this method may be applied routinely to rapidly quantify the levels of specific mRNAs within small samples. The efficacy of a wide range of primers for use in real-time quantitative PCR with a LightCycler fluorescence temperature cycler was evaluated. We also sought to test the reliability of this method in the established experimental murine model of oxygen-induced retinopathy [11]. The

expression pattern for hypoxia-mediated upregulation of vascular endothelial growth factor (VEGF) in this model is well characterized [12-14] and therefore offers an excellent system for evaluation of this quantitative PCR technique in ocular tissues.

METHODS

Primers: Primers were designed using the GCG Prime program (Genetics Computer Group) available via the UK HGMP Resource Centre. The details of the amplification conditions and products and are shown in Table 1.

VEGF standard curve: The product amplified by the murine VEGF primers was purified using a Sephaglass BandPrep kit (Amersham Pharmacia Biotech UK Ltd, Little Chalfont, UK) and quantified spectrophotometrically. PCR amplification was performed with template dilutions ranging from 10^1 to 10^6 copies and the fluorescence signal was obtained at 85 °C, just below the Tm of the product and above the Tm of the primer dimers (Figure 1A).

Neonatal murine model of proliferative retinopathy: Animal care guidelines comparable to those published by the Institute for Laboratory Animal Research (Guide for the Care and Use of Laboratory Animals) were employed. Postnatal day 7 C57/BL6 mice were exposed to 80% oxygen for 5 days and then returned to room air as described previously [11,15]. This model causes hyperoxia-induced closure of the central retinal capillary beds and leads to retinal hypoxia upon return to room air. Eyes were enucleated from mice at the end of the high oxygen period and at 12 and 24 h after return to room air.

RNA extraction and cDNA synthesis: Retinas were dissected from at least 5 different mice and immediately snap-frozen in liquid nitrogen. The frozen retinas were then pulverized, resuspended in Trizol reagent (Life Technologies Ltd, Paisley, UK), homogenized using a Qiashredder (Qiagen Ltd, Crawley, UK), and RNA extracted according to the manufacturer's protocol. Residual DNA was removed by treatment with 5 units of DNase I (Clontech Laboratories, Basingstoke, UK) at 37 °C for 45 min followed by inactivation at 65 °C for 10 min. 2 µg of RNA was reverse transcribed with Superscript II reverse transcriptase (Life Technologies Ltd, Paisley, UK). The cDNA was diluted five-fold prior to PCR amplification.

Real-time quantitative PCR: Real-time PCR was performed using a LightCycler rapid thermal cycler system (Roche Diagnostics Ltd, Lewes, UK) according to the manufacturer's instructions. Reactions were performed in a 20 µl volume with 0.5 µM primers and $MgCl_2$ concentration optimized between 2-5 mM. Nucleotides, Taq DNA polymerase, and buffer were included in the LightCycler-DNA Master SYBR Green I mix (Roche Diagnostics Ltd, Lewes, UK). A typical protocol took approximately 15 min to complete and included a 30 s denaturation step followed by 40 cycles with a 95 °C denaturation for 0 s, 55 °C annealing for 5 s, and 72 °C extension for 5 s. Extension periods varied with specific primers depending on the length of the product (~1 s/25 bp). Detection of the fluorescent product was carried out either at the end of the 72 °C extension period or after an additional 2 s step at 2 °C below the product Tm. To confirm amplification specificity the PCR products from each primer pair were subjected to a melting curve analysis and subsequent agarose gel electrophoresis (Figure 2).

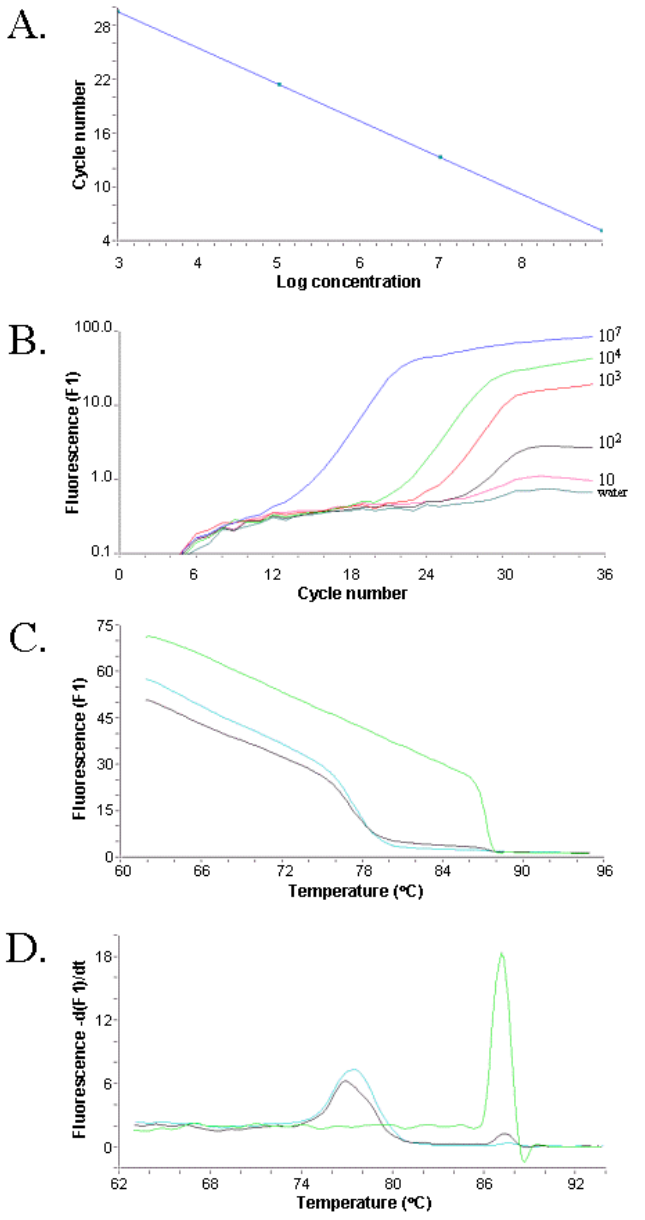

Figure 1. Amplification of VEGF dilution series. Panel **A** shows an example of a linear standard curve from 10^3 to 10^9 copies of VEGF (r=-1, mean squared error=0.000181). A plot of fluorescence against cycle number (**B**) demonstrates discrimination down to 10 copies of VEGF template DNA. To achieve this sensitivity it is necessary to measure fluorescence at several degrees below the Tm of the specific product. This avoids fluorescence from primer dimers, which may be generated at high cycle numbers in samples with very few or no target sequences (black=100 and light blue=water). These background products are indicated by the peaks present at lower temperatures in the first derivative plot (**D**) derived from the melting curve analysis (**C**).

TABLE 1. OLIGONUCLEOTIDE PRIMER SEQUENCES AND RAPID AMPLIFICATION CONDITIONS

Gene M/H* (Accession)	Primer sequences(5'-3') Forward and Reverse	$MgCl_2$ (mM)	Anneal temp	Product bp & Tm
28S rRNA (M/H)	TTGAAAATCCGGGGGAGAG	4	54	100
X00525, M11167	ACATTGTTCCAACATGCCAG			87.1(M)86.9(H)
AGE-R3 (MAC-2) (H)	GTTGCCTTCCACTTTAACCC	4	54	181
M35368	CATTCACTGCAACCTTGAAG			81.3(H)
ARP (36B4) (M/H)	CGACCTGGAAGTCCAACTAC	4	53	109
X15267, M17885	ATCTGCTGCATCTGCTTG			82.4(M)80.5(H)
Cathepsin-D (M)	ACAACAGTGACAAGTCCAG	3	55	321
X53337	TGTTCAGGTAGAAGGAGAAG			91.2(M)
Caveolin (M/H)	TACAAGCCCAACAACAAG	5	55	209
U07645/Z18951	ACAGTGAAGGTGGTGAAG			87.1(H)
Cyclin D2 (M)	CTTCCAAGCTGAAAGAGACC	4	53	111
M83749	TACCCAACACTACCAGTTCC			85.5(M)
Cyclophilin (H)	CTCCTTTGAGCTGTTTGCAG	4	55	325
Y00052	CACCACATGCTTGCCATCC			86.0(H)
GAPDH (M)	AACGACCCCTTCATTGAC	4	57	191
M32599	TCCACGACATACTCAGCAC			86.6(M)
HIF (M/H)	ACAAGTCACCACAGGACAG	3	52	168
AF003695/U22431	AGGGAGAAAATCAAGTCG			83.1(M)
Laminin B1 (H)	ACATTGACACGACAGACCC	4	56	231
NM_002291/M61951	GAAGACACAAGCACTGACC			88.2(H)
LR67 (M/H)	TCACTAACCAGATCCAAGC	4	55	350
J02870, X15005	CTTGGTCACAGCCTTCTC			88.9(M)83.1(H)
MAC 1 (M)	GATGAGACAAAGAACAACACAC	4	55	208
X07640	TGAAGAACCTCTGAGCATCC			86.2(M)
NRL (M)	AGTCCCTTGGCTATGGAATATG	4	54	147
L14935	ACTGAAGGTGGGTGAAGGAG			87.5(M)
PDGFA (M/H)	GTCCAGGTGAGGTTAGAGG	4	57	210/273*
M29464, X03795	CACGGAGGAGAACAAAGAC			88.8(M)
PDGFB (M/H)	AAGCACACGCATGACAAG	5	55	109
X02811	GGGGCAATACAGCAAATAC			87.5(M)
PlGF (M)	ACAGAAGTGGAAGTGGTG	4	56	503
X80171	GGCTAATAAATAGAGGGTAGG			87.2(M)
Rhodopsin (M)	ACACCACTCAACTACATCC	5	57	463
M55171 (U22180)	CGTAGCAGAAGAAGATGAC			90.3(M)
VEGF (M)	TTACTGCTGTACCTCCACC	4	55	189
S38083	ACAGGACGGCTTGAAGATG			87.2(M)
VEGF (H)	CGAAACCATGAACTTTCTGC	4	55	302
M32977	CCTCAGTGGGCACACACTCC			88.5(H)
VEGFD (M)	ACCTCCTACATCTCCAAAC	3	56	422
D89628	TCCAGACTTTCTTTGCAC			86.0(M)

The length of the specific amplification products and their approximate Tm are indicated (these varied ±0.3 °C depending on the template). *Primers may be suitable for use with murine (M) or human sequences (H). Accession numbers are given for the gene sequences from which primers are derived (certain sequences contain mismatches with the primers but have been shown to be amplified effectively). The asterisk ("*") marks an alternatively spliced product.

The quantification data were analyzed with the LightCycler analysis software as described previously [10]. The baseline of each reaction was equalized by calculating the mean value of the five lowest measured data points for each sample and subtracting this from each reading point. Background fluorescence was removed by setting a noise band. The number of cycles at which the best-fit line through the log-linear portion of each amplification curve intersects the noise band is inversely proportional to the log of copy number [3].

RESULTS

Specific amplification: Melting curve analysis demonstrated that each of the primer pairs described in Table 1 amplified a single predominant product with a distinct Tm as shown for VEGF in Figure 2. Once the predicted length of each product had been confirmed by agarose gel electrophoresis (Figure 2) the Tm was used to identify specific products in subsequent analyses.

Sensitivity: The PCR product amplified by the murine VEGF(M) primers was quantified and used as a template for amplification at a range of 10^1 to 10^6 copies. A linear standard curve could routinely be generated from a starting copy number of 1000 or above (Figure 1A). With fluorescence measured at 2 °C below Tm as few as 10 template copies could be distinguished from background levels (Figure 1B) because any signal from primer dimers was eliminated (Figure 1C,D).

Multiple primer pairs: Following optimization of $MgCl_2$ concentration almost all the gene-specific primer pairs tested amplified a single product (Table 1). The lengths and melting curve analyses of the products amplified from the housekeeping genes GAPDH (M32599), cyclophilin (Y00052), and ARP (X15267) are shown in Figure 3. There was rarely significant primer dimer formation within the number of cycles required for quantification from a range of experimental samples.

Retinal VEGF gene expression: Real-time PCR was used to quantify the levels of VEGF mRNA in the retinas of neonatal mice immediately following exposure to 80% oxygen (t=0) and at 12 and 24 h after return to room air (t=12 and t=24). To enable accurate analysis of relative gene expression between separate runs the quantitative values for each time point were expressed relative to the values at the other times. Analysis of three separate experiments revealed a two-fold increase in VEGF levels between t=0 and t=24 ($p<0.01$), Figure 4. Levels of the housekeeping gene ARP did not vary significantly between treatments while 28S rRNA was slightly reduced at t=12.

DISCUSSION

Primer pairs that are effective in conventional PCR amplification or designed using appropriate software are readily converted for LightCycler SYBR Green I analysis. One run, with between 3-5 mM $MgCl_2$ and at the predicted or previously determined annealing temperature, was generally sufficient to optimize conditions for each primer pair. The great majority of our primers (>90%) amplified a single, specific product therefore fluorescence from SYBR Green I binding to primer dimers or misprimed products did not present a problem. At the high cycle numbers required to detect low template con-

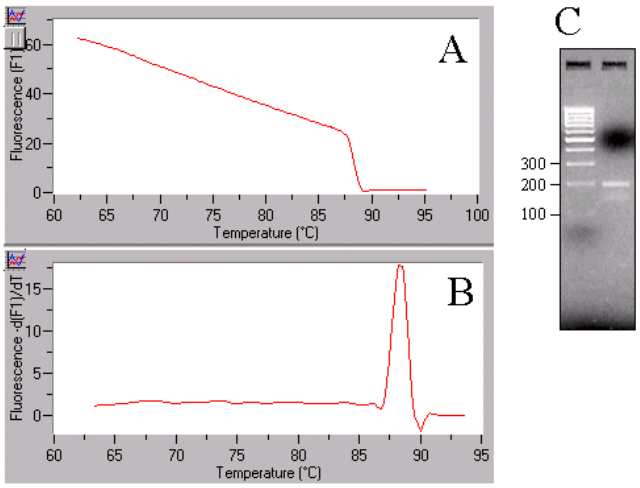

Figure 2. Specific amplification of VEGF. (**A**) A melt curve analysis of a VEGF amplification reaction demonstrating the gradual reduction in fluorescence as temperature increases. The rapid fall off at 87 °C indicates the presence of a specific product that melts at this temperature. The Tm of this product can be visualized more clearly as a peak in a first derivative plot (**B**). Agarose gel electrophoresis analysis (**C**) demonstrates that this peak corresponds to a single band of the size predicted 189 bp.

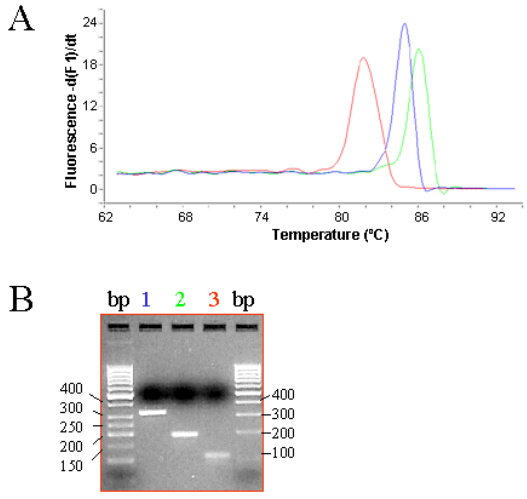

Figure 3. Housekeeping genes. Panel **A** shows the distinct melting curves of the amplification products from three housekeeping genes: green, GAPDH (M32599); blue, cyclophilin (Y00052); and red, ARP (X15267). In panel **B**, the same reactions were analyzed by agarose gel electrophoresis and revealed single amplification products of the predicted sizes (Lane 1. cyclophilin, 349 bp; Lane 2. GAPDH, 191 bp; and Lane 3. ARP, 109 bp).

centrations, for example <1000 copies of VEGF template (Figure 1), primer dimers were formed in addition to specific product. The fluorescence from these dimers may be eliminated by measuring the fluorescence at a temperature above their Tm but several degrees below that of the specific product [10], for example 85 °C for VEGF (M). Under these conditions the dimers will be single-stranded and therefore not bind SYBR Green I dye. While this technique effectively eliminates small amounts of primer dimers, enabling quantification of specific products, it should not be used to mask large amounts of short nonspecific products whose amplification might compete with that of the specific product.

Using dilutions of purified PCR product as template it was possible to routinely quantify down to 1000 copies and detect as few as 10 copies (Figure 1B). This level of sensitivity should be sufficient for most research applications; the cDNAs from the genes we examined were quantitatively amplified from all experimental samples analyzed. Indeed, it often proved beneficial to dilute the cDNA template by up to 10 fold, thereby reducing variations in initial background fluorescence, which despite baseline adjustment could affect quantification. Real-time PCR enables quantification by analysis of the log-linear phase of amplification and on-line monitoring enables the reaction to be stopped once past this phase, reducing background which might occur during further non-essential cycles.

The levels of retinal VEGF mRNA in a murine model of oxygen-induced retinopathy were quantified using the LightCycler SYBR Green I method, with a combined run and analysis time of less than 30 min. The reliability of this method was demonstrated by the concurrence of the results obtained with those previously reported from laborious end-point RT-PCR analysis [13] or Northern blotting [12]. The primers described for amplification of GAPDH (M32599), cyclophilin (Y00052), and ARP (X15267) [16], shown in Figure 3, enable reliable quantification of the expression of these housekeeping genes and should prove useful controls in future experiments. Quantification is possible over a wide dynamic range, and it was therefore possible to measure levels of the highly abundant 28S rRNA. rRNA comprises the majority of total RNA, and therefore the concentration of 28S rRNA provides confirmation of spectrophotometric quantification of RNA samples and provides an alternative internal control to potentially variable housekeeping genes.

Although comparison of experimental samples with standards of known concentration or dilution yielded quantitative values we found that variations between runs obscured the very consistent relative expression levels between samples (Figure 4). To compare relative expression between the members of analogous sets of samples from repeat experiments it is therefore preferable to convert absolute values to relative terms. For most research applications it is this relative expression which is of interest. Expression levels may be normalized to those of "housekeeping" genes or rRNA amplified with the primer sequences described in this paper and elsewhere.

There are many techniques available for measuring mRNA levels, each with inherent advantages and disadvantages. Direct detection methods such as RNase protection assays (RPAs) and Northern blots are undoubtedly quantitative, but they are laborious and lack the sensitivity required to analyse small samples. While PCR does enable analysis of the small samples derived from clinical and experimental ocular specimens it is difficult to quantify, even with time-consuming competitive approaches.

The advent of fluorescent hybridization probes has enabled the development of real-time PCR, which combines the advantages of conventional PCR with a quantitative capability. While hybridization probes provide very high sensitivity due to low background fluorescence levels, there is a high cost for synthesis of each new gene-specific probe, which can also be problematical to design [8]. The sensitivity of SYBR Green I analysis is more than adequate for the majority of research applications, and its quantitative nature is demonstrated by the linear relationship between the log of template concentration and cycle number at which fluorescence rises above baseline (Figure 1). When the expression of a variety of genes is to be analyzed all that is required for each new gene is a specific primer pair (in the absence of purified template, dilutions of a specific cDNA sample may be used for relative quantification). The rapid cycle times and use of melt-curve analysis in place of agarose gel electrophoresis and ethidium bromide staining (or Southern hybridization with radiolabelled internal oligonucleotides) make the analysis considerably faster (<30 min) than conventional PCR. In conclu-

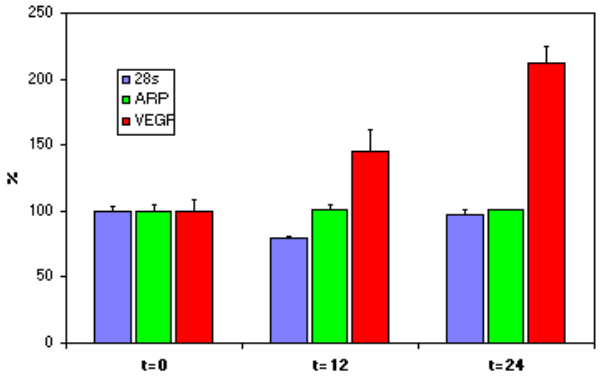

Figure 4. VEGF expression in a murine model of oxygen-induced retinopathy. Levels of VEGF and ARP mRNA and 28S rRNA were measured by real-time PCR in murine retina immediately after oxygen treatment (t=0) and 12 and 24 h after return to room air (t=12 and t=24). Each column represents the average of 3 amplification reactions (error bars represent standard deviation), performed on a single cDNA sample reverse transcribed from RNA prepared from a minimum of 8 pooled retinas from at least 4 mice derived from 2 or more litters. Values for t=0 were set to 100%. The level of ARP did not change significantly, 28S rRNA dropped slightly at t=12 while VEGF increased two-fold between t=0 and t=24 ($p<0.01$). Comparable results were obtained in a replicate experiment using a different set of mice.

sion, SYBR Green I analysis on the LightCycler fluorescence temperature cycler provides a flexible and rapid method for mRNA quantification.

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