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Quantitative RT-PCR as an alternative to late-stage bioassays for vascular endothelial growth factor

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ABSTRACT

We have investigated the use of quantitative reverse transcription-polymerase chain reaction (qRT-PCR) as an alternative to a selection of late-stage functional bioassays for determination of the potency of preparations of vascular endothelial growth factor (VEGF). Responses were measured in cultures of human umbilical vein endothelial cells (HUVECs). Late-stage responses measured were cell survival and proliferation, and production of interleukin-8 (IL-8), interleukin-6 (IL-6), and tissue factor. The dose–response range was similar across the assays, increasing from 2 ng/mL VEGF and reaching a maximum between 30 ng/mL and 125 ng/mL VEGF. A number of VEGF-induced mRNA species demonstrated dose–response curves suitable for VEGF potency determination. IL-8 mRNA induction after 45 min incubation with VEGF, which showed maximal responses between 15.6 ng/mL and 62.5 ng/mL VEGF, was selected for further characterization. This gene-expression bioassay was robust across a range of cell seeding densities and could be used for samples processed immediately following incubation with VEGF and for cell lysates stored at −80 °C for 3 months. We also compared this gene-expression bioassay and the assays of late-stage responses in the potency measurement of the inhibitors of VEGF activity, anti-VEGF monoclonal antibody MAB293, and a VEGF soluble receptor VEGFsR1 preparation. We present a critical evaluation of the use of qRT-PCR in assaying the potency of VEGF and its inhibitors, and of the potential of this platform for measuring the potency of other biological therapeutics.

1. Introduction

Bioassays are generally the only means of determining the potency of biological products for therapeutic, diagnostic, or research use, and are a regulatory requirement in the specifications for release for the majority of biological therapeutic products. Cell-based *in vitro* bioassays for potency are widely used in the biopharmaceutical industry [1,2] and many involve measurement of late-stage responses such as cell proliferation or production of a specific protein. Late-stage responses generally require 1–4 days incubation of the cells after exposure to the test product [3,4] with maintenance of a stable and sterile culture environment during this period. Some responses, such as phosphorylation, spatial translocation of signalling elements, and modifications in patterns of gene expression, occur very rapidly after binding of the biologically active products to their receptors. Measurement of such so-called early responses can reduce the period of cell incubation to hours or minute. One early response in native signal transduction pathways is the induction or inhibition of endogenous gene promoter elements, so we have investigated measurement of changes in endogenous gene expression as an alternative to a range of bioassays measuring various late-stage responses to vascular endothelial growth factor (VEGF, also known as VEGF-A).

VEGF is a secreted glycoprotein and alternative splicing of the human VEGF gene results in several isoforms, the most common of which is that of 165 amino acid residues, VEGF 165, used in this study. VEGF has many biological activities of clinical interest, including vasculogenesis, angiogenesis, lymphangiogenesis, vascular maintenance, and neurotrophic and neuroprotective activities [5,6]. Assay of VEGF activity and VEGF inhibitors underpins a variety of current drug development programs, including therapeutic angiogenesis and bone repair [7] and inhibition of VEGF activity in the treatment of cancers and other conditions involving pathological development of blood vessels (for a review, see Ref. [8]). Many of the inhibitors of VEGF activity which have potential therapeutic applications, such as monoclonal antibodies and soluble receptors, are themselves biological molecules and so require bioassays for their potency determination.

Here we describe several different bioassays we developed for determining the potency of VEGF and inhibitors. Each of the assay systems has particular advantages and disadvantages. In addition to late-stage bioassays we showed the use of a gene-expression

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assay platform for measurement of the biological activity of VEGF and inhibitors, based on the detection of gene induction using quantitative reverse transcription-polymerase chain reaction (qRT-PCR). This type of assay has recently been shown to be capable of measuring the biological activity of interferon and of neutralizing antibodies in patient serum [9]. Our results, together with those of Bertolotto et al., demonstrate the potential of this method as a rapid bioassay for measurement of the potency of a range of therapeutic biological products but show that assay conditions have to be adapted to the cell system being used and the induced gene selected for the readout.

2. Materials and methods

2.1. Human umbilical vein endothelial cells (HUVEC) cell culture and VEGF

Low passage "primary" HUVECs were purchased from TCS Biologicals (Buckingham, UK), maintained at 37 °C, 5% CO_2, in endothelial growth medium EGM-2 (Cambrex, Wokingham, UK), and passaged by trypsinization of the adherent cell layer every 3–4 days when approaching confluence. Frozen stocks were made and resuscitated as required. Cells were used up to approximately 50 population doublings from the initial passage in our laboratory. Recombinant human $VEGF_{165}$ used was NIBSC reference reagent 01/424 or WHO reference reagent 02/286 [1]. VEGF soluble receptor-1 (VEGFsR1) was donated by R&D Systems (Minneapolis, USA). Anti-VEGF monoclonal antibody (MAB293) was purchased from R&D systems.

2.2. VEGF assays

HUVECs were plated in Nunc 96-well tissue culture plates (Life Technologies, Paisley, Scotland). The edge rows and columns were not used, but were filled with 200 μL medium, as were any other unused wells. VEGF, VEGFsR1 and MAB293 solutions and dilution curves were prepared in RPMI 1640 with L-glutamine (Life Technologies) with 0.2% (v/v) fetal bovine serum (FBS) (Life Technologies or Sigma). For assays involving VEGFsR1 or MAB293, the solutions were preincubated with the appropriate VEGF solution for 30 min at 37 °C before addition of the mixture to the cells. The final volume per well, after all additions, was 200 μL.

2.2.1. AlamarBlue and cell number (crystal violet staining) assays

VEGF solutions, preincubated, where appropriate, with VEGFsR1 or MAB293, were added to 96-well plates in a total volume of 150 μL. HUVEC suspensions were freshly prepared by trypsinization and resuspension in RPMI + 0.2% (v/v) FBS. Cell concentration and viability were determined by counting using a hemocytometer and trypan blue staining. Cell concentration was adjusted by addition of RPMI + 0.2% (v/v) FBS to 400,000 cells/mL and 50 μL of the cell suspension was added to appropriate wells to give a total of 20,000 cells per well in a final volume of 200 μL. VEGF, VEGFsR1, and MAB293 concentrations shown in figures are the final concentrations incubated with the cells. Plates were incubated (37 °C, 5% CO_2) for 48 h. Then, either (a) the cells were fixed with 1% glutaraldehyde and stained with 0.1% crystal violet in 200 mM Tris, pH 6.8 [10], or (b) 40 μL alamarBlue™ (Serotec) was added per well, followed by incubation for a further 15–16 h, after which the absorbance was read as the difference $A_{570\,nm} - A_{600\,nm}$.

IL-8, IL-6, and tissue factor (TF) assays: HUVECs were seeded at 80,000 cells per well in 200 μL RPMI + 2% FBS in a 96-well plate and incubated (37 °C, 5% CO_2) for 15–16 h. The medium was then removed and the cell layer rinsed with 200 μL per well RPMI + 0.2% (v/v) FBS. VEGF solutions, preincubated, where appropriate with VEGFsR1 or MAB293, were added to give a final volume of 200 μL per well. Plates were incubated for between 0 h and 48 h. For secreted products, the supernatant was removed, aliquoted and assayed immediately, or after storage at −70 °C. IL-6 [11] and IL-8 were measured by the same ELISA method against the international standard for IL-6 (code 89/548) or IL-8 (code 89/520), using the appropriate polyclonal antibody preparations against IL-6 or IL-8, respectively. For measurement of cell-associated products, the cell layer was rinsed with Tris-buffered saline (TBS: 50 mM Tris, 150 mM NaCl, pH 7.4), and then subjected to three freeze–thaw cycles in 100 μL per well TBS. Tissue factor was measured by chromogenic assay measuring the conversion of factor X (NIBSC Reagent 98/754) to Xa in the presence of recombinant activated factor VII (1st international standard, code 89/688) and Ca^{2+} [12]. RecombiPlasTin®, recombinant tissue factor from ortho diagnostics (Raritan, NJ, US) at a dilution of 1:100 was used to define an activity of 1 U/mL.

2.3. VEGF gene-expression assay

VEGF treatment: HUVECs were grown in 24-well plates (0.5×10^6 cells, 1.25 mL per well) or 96-well plates (80,000 or 160,000 cells, 200 μL per well) in RPMI + 2% (v/v) FBS for 15–16 h under sterile conditions. All subsequent steps do not require sterile operating conditions. The medium was removed and the cell layer rinsed with 200 μL per well RPMI + 0.2% FBS. Cells were then exposed to VEGF (0–250 ng/mL) in RPMI + 0.2% (v/v) FBS for 20 min, 45 min, or 90 min. 45 min was selected as the standard incubation time. For experiments involving VEGFsR1 or MAB293, all of which were conducted using 96-well plates, VEGF was preincubated in RPMI + 0.2% (v/v) FBS with the appropriate VEGFsR1 (0–500 ng/mL) or MAB293 (0–1280 ng/mL) solutions for 30 min prior to addition of 200 μL per well to the cell layer and incubation for 45 min.

Isolation of total RNA: Total RNA was extracted from control (unstimulated) and VEGF-stimulated HUVECs, using RNAeasy spin-column technology with a DNase incubation step to remove residual genomic DNA according to the manufacturers instructions (Qiagen, Crawley, UK). RNA was also extracted from samples that had been exposed to VEGF and then immediately lysed in lysis buffer (Qiagen) and stored at −80 °C for 3 months to determine if archiving of sample plates at −80 °C was a viable option for storage. RNA was isolated from either 0.5×10^6 cells (24-well plates) or 80,000–160,000 cells per well (96-well plates) and quantified using a Nanodrop spectrophotometer. cDNA was synthesized simultaneously from 250 ng RNA using MMLV-RT Superscript II (Promega). Briefly, oligo$(dT)_{18}$ (1 μg) and random 10-mers (1 μg) were added to the RNA (10 μL), and the mixture was heated (70 °C, 5 min) to remove secondary RNA structure and then cooled on ice. Dithiothreitol (10 mM), dATP, dCTP, dTTP, and dGTP (all 0.5 mM, Promega), recombinant ribonuclease inhibitor (80 units, RNAsin, Promega), MMLV-RT (200 units), and diethyl pyrocarbonate-treated water were added to make the final volume 20 μL, and the mixture was incubated at 42 °C for 50 min. MMLV-RT was inactivated by heating at 70 °C for 15 min. The cDNA was diluted 5- or 10-fold with tRNA (10 μg/mL) or water and used immediately in PCR reactions or stored at −20 °C for future use. An aliquot of RNA was not reverse-transcribed and was diluted with tRNA and stored at −80 °C to be used to check for genomic contamination (minus RT control).

qPCR: PCR reactions were set up using a CAS automated liquid handling system (Corbett Research UK, Cambridge) with intron-spanning primers specific for the gene of interest and a house-keeping gene (GAPDH). Primer sequences for human FosB, c-Fos, TF, and GAPDH were as previously published [13]. Human IL-6 primer sequences were forward: 5′-AGG AGA CTT GCC TGG TGA

Table 1
PCR Primer annealing temperatures and amplified product sizes

Gene	Annealing temperature (°C)	Product size (bp)
FOSB	54	249
cFos	52	247
TF	55	252
IL-6	55	196
IL-8	55	180
GAPDH	55	251

AA-3′; reverse: 5′-CAG GGG TGG TTA TTG CAT CT-3′. Human IL-8 primer sequences were forward: 5′-GTG CAG TTT TGC CAA GGA GT-3′; reverse: 5′-CTC TGC ACC CAG TTT TCC TT-3′. PCR reactions contained 2× Sensimix (Quantace, London UK), 50× SYBR green, forward, and reverse primers (500 nM final concentration), and either sample cDNA (one-third dilution) or standard DNA (generated from a purified PCR product of the gene of interest). PCRs were performed on a Rotorgene 6000 (Corbett Research UK, Cambridge) and included 40 cycles consisting of 95 °C denaturation for 0 s, annealing for 10 s at T_{ann} (Table 1), and a 72 °C extension phase for 10 s. Fluorescence measurements were taken at the end of the 72 °C extension phase. The amplification product of each primer pair was subjected to melting point analysis and subsequent gel electrophoresis to ensure specificity of amplification. Data were analysed using two-standard curve and delta–delta ct software and gene expression was quantified relative to the expression of GAPDH (a house-keeping gene whose expression had previously been shown not to change with VEGF treatment in HUVECs) to normalise for differences in tissue loading.

3. Results

3.1. VEGF assays

Preliminary investigations had identified various responses of HUVECs as possible readouts for potency assays for VEGF and, consequently, for assays for inhibitors of VEGF activity [14]. In this study, a range of responses and assay parameters, including VEGF dose ranges, cell seeding densities and incubation times, were investigated for their potential in measuring the biological activity of VEGF and VEGF inhibitors.

HUVEC survival and proliferation in serum-poor medium (RPMI 1640 + 0.2% (v/v) FBS) could be measured by staining of viable cells with crystal violet (Fig. 1A) or by measuring cell metabolic activity by alamarBlue reduction (Fig. 1B). Direct counting by hemocytometer of trypsinized cells (data not shown) confirmed that differences in alamarBlue reduction and crystal violet staining reflected differences in HUVEC cell number. 48 h was selected as the incubation time with VEGF and 15–16 h as the further incubation with alamarBlue. The amplitude of the measured response increased from around 2 ng/mL VEGF, reaching a maximum between 30 ng/mL and 125 ng/mL and decreasing at higher VEGF concentrations. A twofold dilution series from 250 ng/mL to 1 ng/mL therefore, gave 5–7 responses points on the rising part of the dose–response curve.

As alternative readouts, VEGF-stimulated production of IL-6, IL-8, and tissue factor was investigated. Secreted product was measured in the cell supernatant and cell-associated product was measured by lysis of the washed cells after three freeze–thaw cycles. For IL-6 and IL-8, the majority of the product was found in the supernatant while the majority of the tissue factor was found with the lysed cells. For IL-8 and IL-6, the levels in the cell supernatant continued to increase up to the maximum incubation time measured, i.e. 48 h (Fig. 2A and B, respectively). The cell-associated IL-8 peaked at 3 h and then declined (not shown). The cell-associated IL-6 levels were too low to assess over the time course. Cell-associated tissue factor peaked at 5 h (dose–response at 5 h shown in Fig. 2C) and then declined. Secreted tissue factor levels were too low to measure over the time course. 5 h was selected as the incubation time with VEGF for the IL-6, IL-8 (supernatant) and tissue factor (cell-associated) assay systems. This combined adequate amplitude of signal with logistical convenience for completion of the ELISA or chromogenic step of the assay within the working day if required. Supernatants or washed cells (at any stage of the freeze–thaw cycles) could be stored at −70 °C for later processing. The dose–response curves for the IL-6, IL-8, and tissue factor assay systems were similar to those of the alamarBlue and crystal violet systems, rising from around 2 ng/mL and peaking between 31.2 ng/mL and 125 ng/mL. The levels of IL-8 secreted were higher and less variable than those of IL-6, so IL-6 was not routinely used as readout. These assay systems were used to assay inhibitors of VEGF activity, including antithrombin, a neutralizing anti-VEGF antibody (MAB293) and VEGFsR1. Examples of inhibition of VEGF activity by MAB293, measured by tissue factor production, and inhibition of VEGF activity by VEGFsR1, measured by alamarBlue reduction, are shown in Figs. 5A and 6A, respectively.

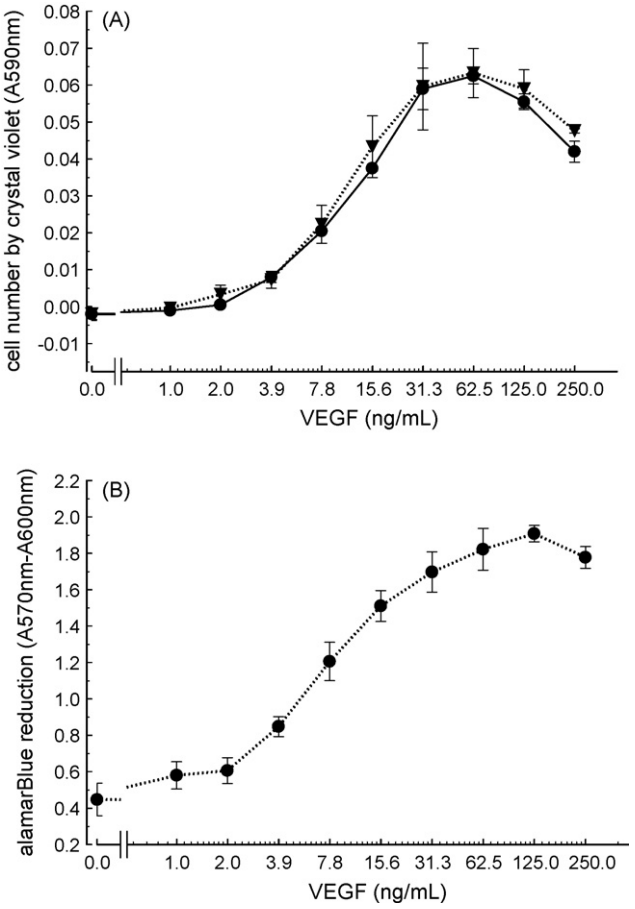

Fig. 1. (A) VEGF-stimulated survival of HUVECs measured by crystal violet staining. The dose–response curve can be used to measure the relative potency of samples of VEGF as illustrated by this comparison of a stressed sample with a reference preparation. HUVECs were incubated for 48 h with VEGF (1st WHO reference reagent, code 02/286) either from an ampoule held at the normal storage temperature of −20 °C (circle solid) or from an ampoule stored at +20 °C for 20 months (triangle down solid). The cells were then fixed and stained with crystal violet solution. Data points represent the mean values of three wells (±S.D.) (B) VEGF activity measured by reduction of alamarBlue™. HUVECs were incubated with VEGF for 48 h before addition of the redox dye alamarBlue™ for a further 16 h. The reduction of alamarBlue™, measured as the difference in absorbance at 570 nm compared with 600 nm ($A_{570\,nm} - A_{600\,nm}$), indicates the metabolic activity of the cell population. Data points represent the mean values of three wells (±S.D.).

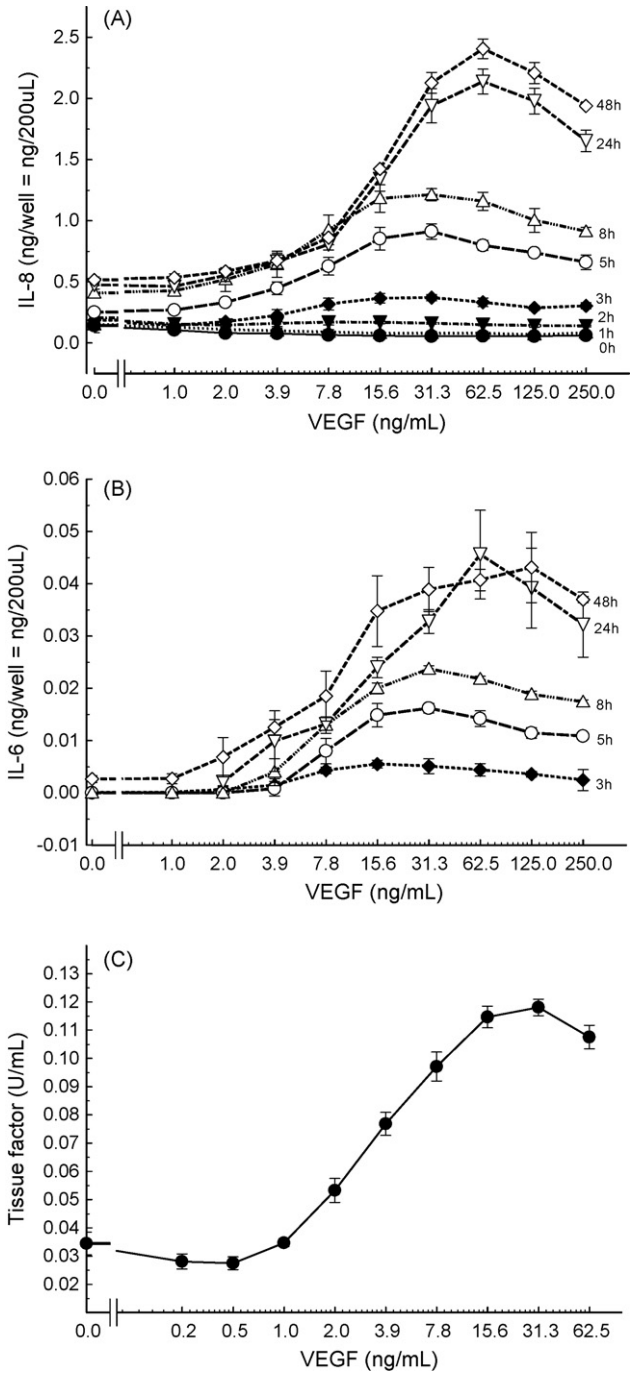

Fig. 2. VEGF-stimulated cytokine and tissue factor production by HUVECs. Dose–response curves were measured for VEGF-stimulation of production of a selection of proteins. Levels of secreted protein were measured in the cell culture medium and intracellular levels were measured by rinsing and then lysing the cells. (A) The time course of the secretion of IL-8 into the cell culture medium, which represented the majority of the IL-8 produced. IL-8 was measured by in-house ELISA. (B) The time course of the secretion of IL-6 into the cell culture medium, which represented the majority of the IL-6 produced. IL-6 was measured by in-house ELISA. Levels of secreted IL-6 were lower than IL-8 and could not be detected before 3 h. (C) The cell-associated tissue factor at 5 h, the time point at which maximum levels were reached. Secreted tissue factor levels were much lower. Tissue factor activity was measured by a chromogenic assay as described in Section 2. Data points in Fig. 2 represent the mean values of three wells (±S.D.).

3.2. Gene-expression bioassays

A selection of genes was screened for a VEGF dose-dependent response. These included the early response genes FosB and cfos, reported to be rapidly induced by VEGF in HUVECs [13], and genes for the induced cytokines, IL-6, IL-8, and TF [14]. The expression of other early response genes such as members of the Jun family of transcription factors, including c-Jun, JunB, and JunD, were not investigated since it has been reported to that VEGF has no effect on the expression of these genes in HUVECs [13]. Gene expression was determined using real-time qPCR with all data normalized against the expression of GAPDH as a reference gene. Dose-dependent increases in the levels of all VEGF-responsive mRNA species were detected after 45 min exposure to VEGF (Fig. 3) with the highest increases in cfos mRNA levels at 20 min. Increases over control (zero dose VEGF) mRNA levels ranged from 3-fold (IL-6) to 17-fold (TF) after 45 min. Maximum increases in mRNA levels were obtained with 15.6 ng/mL or 62.5 ng/mL VEGF, i.e. over a similar dose range to the alamarBlue and cytokine release assays described previously (Figs. 1 and 2). With the exception of TF and FosB, mRNA levels of all gene targets decreased markedly after 90 min exposure to VEGF. IL-8 was selected as the gene target for all future assays since it demonstrated greater induction and a less variable dose–response than other gene targets after 45 min exposure to VEGF. Although initial experiments were performed in 24-well plates (0.5×10^6 cells per well), the 96-well plate format is a more optimal platform for complete automation of cell treatment, cell lysis and RNA extraction. To ensure that changing the cell density/cell number did not prevent the assay of IL-8 gene induction and subsequent mRNA quantification, IL-8 gene induction in response to VEGF was investigated further in cells seeded in 24-well plate and 96-well plate format. HUVECs seeded in 24-well plates at 0.5×10^6 cells per well or in 96-well plates at 80,000 cells per well or 160,000 cells (single sample generated from two pooled wells) were stimulated with VEGF (0–250 ng/mL). Measurements of IL-8 mRNA demonstrated that dose–response curves were obtained at all three cell densities (Fig. 4A) with similar maximal responses (approximately 13-fold) whether RNA was extracted from a single well of a 96-well plate (80,000 cells) or a single well of a 24-well plate (0.5×10^6 cells). The responses at both these cell concentrations were almost linear in the range 0.5–32 ng/mL VEGF (most reproducibly linear in the range 1–16 ng/mL) and this was consistent between repeated assays of HUVECs assayed from single wells of a 96-well plate (Fig. 4B). Although, the linear range of IL-8 gene inductions in cells where RNA was extracted from two pooled wells of a 96-well plate was over a reduced range (0.5–8 ng/mL) in comparison to the other cell densities, this would not preclude the use of this cell density for a functional assay for VEGF. RNA extracted using a 96-well plate-based vacuum manifold extraction protocol (SV96, Promega) also demonstrated approximate linearity across this dose range but the maximal responses were no more than 6.5-fold and the dose–responses were less uniform than those obtained with manual RNA extraction methods (data not shown). As a result, this vacuum-based RNA extraction protocol was not used routinely. From the data shown, the limit of detection of the gene-expression bioassay is 0.5 ng/mL VEGF, based on the three standard-error limits for the 0 ng/mL and 0.5 ng/mL doses and the limit of quantitation is 1 ng/mL. From inspection of the data shown in Fig. 4B, the dose–response is linear between 1 ng/mL and 16 ng/mL with good reproducibility, as demonstrated by the error bars on the figure. It should be noted that this study is limited to an assessment of the feasibility of using qRT-PCR as an alternative potency assay. More advanced equipment permitting greater replication of doses, as described in Section 4, would give a higher degree of precision

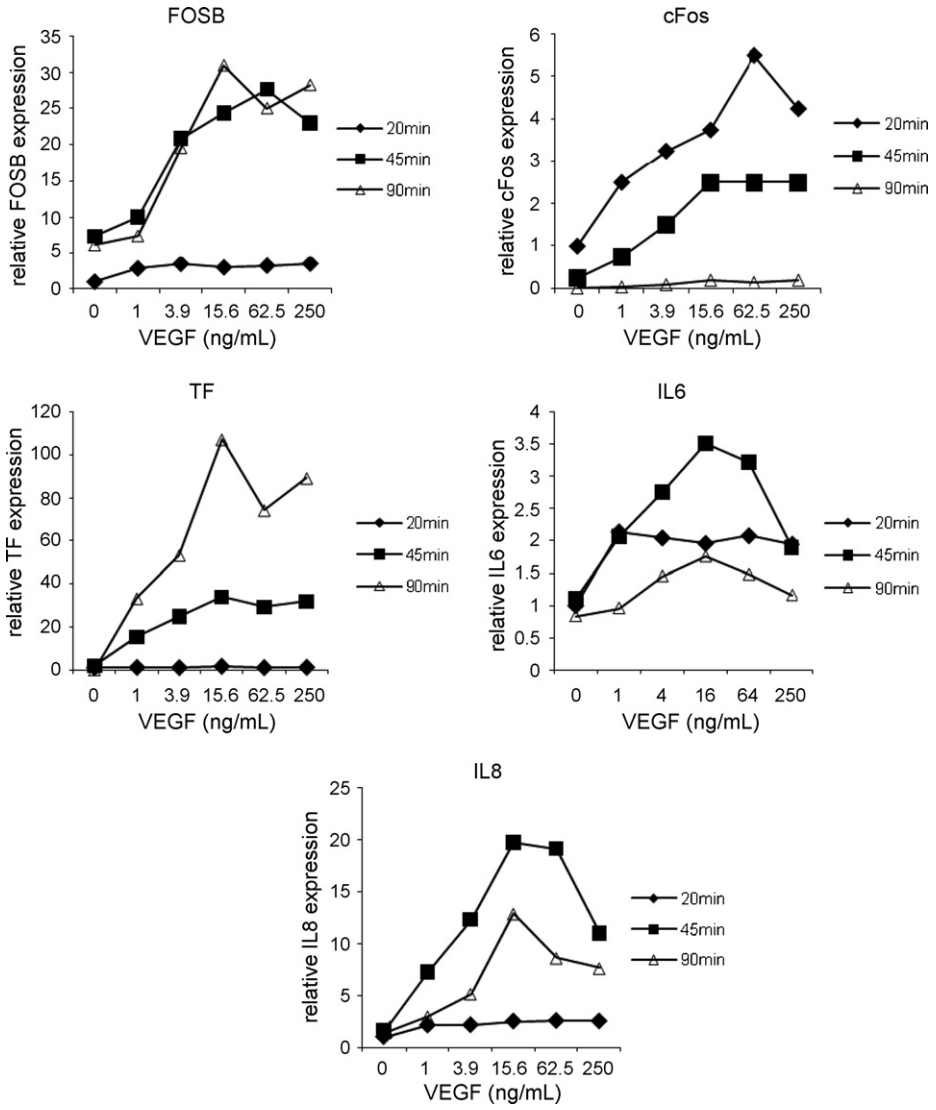

Fig. 3. Screen for VEGF-responsive target genes. A range of genes responsive to VEGF (0–250 ng/mL) stimulation in HUVECs (0.5×10^6 cells seeded in 24-well plates) were investigated to determine the time-course of gene induction and also the maximal fold-increase over basal mRNA expression. Cells were exposed to VEGF concentrations for 20 min, 45 min, and 90 min and gene expression was expressed relative to the zero dose VEGF (20 min incubation).

for the potency measurements and an optimized, fully validated protocol would be expected to show improved performance characteristics.

3.3. Assay of VEGF monoclonal antibody and VEGF soluble receptor 1 activities

MAB293 and VEGFsR1 were both shown to inhibit VEGF activity. A range of doses of MAB293 or VEGFsR1 was preincubated with a fixed dose of VEGF prior to addition to HUVECs. The VEGF concentration was selected to (a) give a sufficiently large response to permit measurement of different amplitudes of inhibition and (b) avoid the plateau region of the VEGF dose–response curve.

Inhibition of VEGF activity by MAB293 was measured by the production of tissue factor (Fig. 5A) or stimulation of IL-8 gene expression (Fig. 5B). VEGF (7.8 ng/mL) was preincubated with MAB293 (0–1280 ng/mL). In both assay systems, the maximal inhibition resulted in a signal indistinguishable from that in the absence of added VEGF (TF Fig. 2C; IL-8 mRNA, Fig. 4B). In both assay systems, maximal inhibition was achieved between 80 ng/mL and 160 ng/mL MAB293, with 50% inhibition between 20 ng/mL and 40 ng/mL.

Inhibition of VEGF activity by VEGFsR1 was measured by alamarBlue reduction (Fig. 6A) and IL-8 gene expression (Fig. 6B). Using the alamarBlue reduction readout, VEGFsR1 doses ranging from 0 ng/Ml to 500 ng/mL were assessed at various fixed VEGF concentrations. Inhibition of alamarBlue reduction to the level observed in the absence of added VEGF was obtained at 250 ng/mL VEGFsR1 in 7.8 ng/mL and 15.6 ng/mL VEGF, and at 500 ng/mL in 31.2 ng/mL VEGF. For the bioassay measuring IL-8 gene expression, a concentration of 7.8 ng/mL VEGF was selected, with VEGFsR1 ranging from 0 ng/mL to 1000 ng/mL. The two-bioassay systems showed similar dose–response curves for the inhibition by VEGFsR1 of 7.8 ng/mL VEGF, with maximal inhibition obtained at 250 ng/mL VEGFsR1. Half-maximal inhibition was obtained between 31.25 ng/mL and 61.25 ng/mL in the alamarBlue system and around 125 ng/mL in the IL-8 mRNA system. This difference between the two assay systems in the absolute values for the VEGFsR1 concentration at half-maximal inhibition was not regarded as large when considered in the context of the inter-assay variation observed in the responses of a single assay system, as described further in the Section 4.

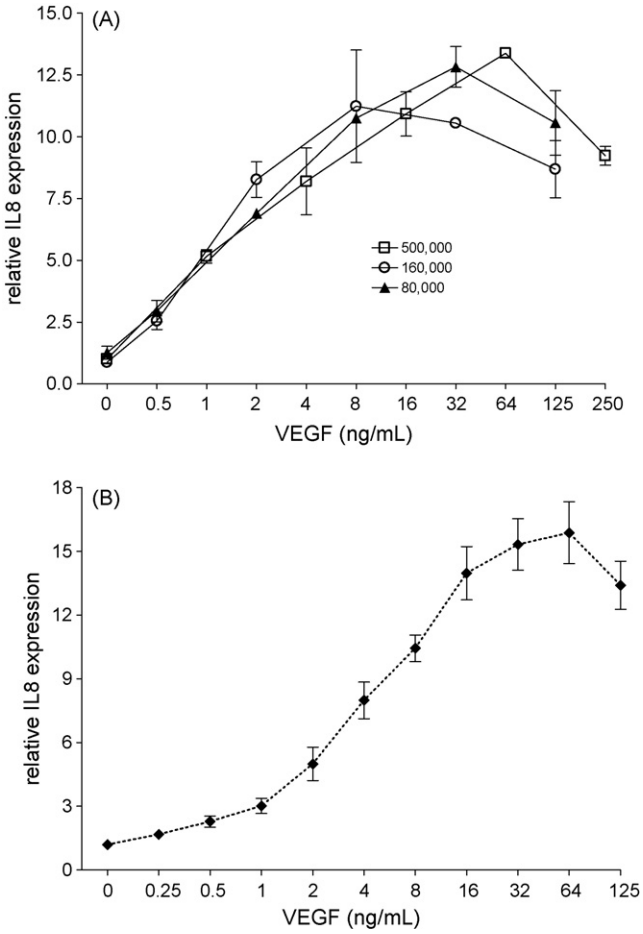

Fig. 4. Comparison of cell number on VEGF dose–responses. (A) HUVECs seeded in 24-well plates at 500,000 cells per well or in 96-well plates at 80,000 cells per well or 160,000 cells (single sample generated from two pooled wells) were stimulated with VEGF (0–250 ng/mL). (B) Representative VEGF dose–response in HUVECs seeded at 80,000 cells per well in a 96-well plate. HUVECs were exposed to the VEGF doses as indicated for 45 min. All samples were between three and six assays and points represent the mean values (±S.D.).

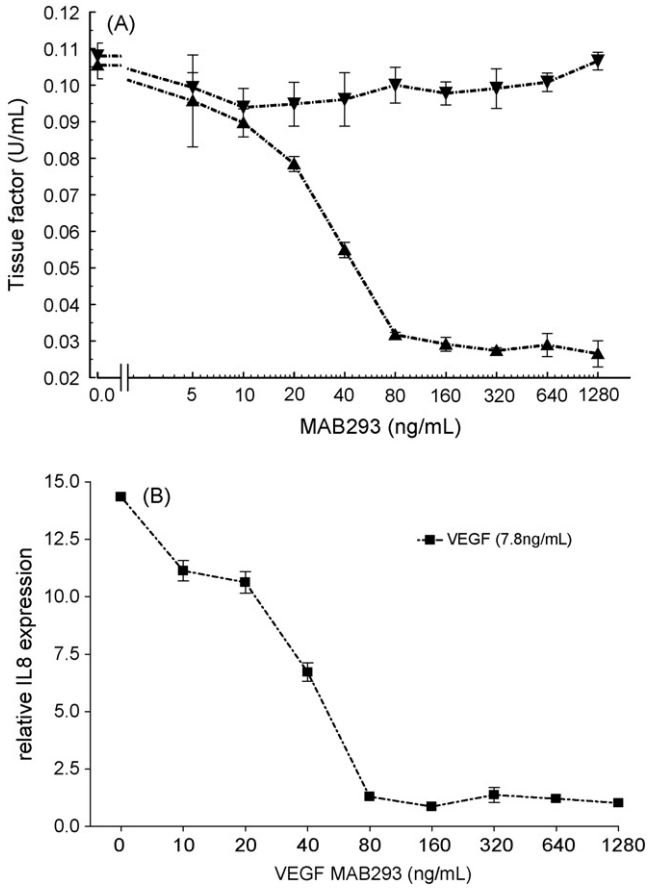

Fig. 5. VEGF monoclonal antibody (MAB293) inhibits VEGF-stimulated tissue factor production and IL-8 gene expression. HUVECs treated with 7.8 ng/mL VEGF were exposed to increasing concentrations of MAB293 (0–1280 ng/mL) and the inhibition of VEGF activity was assayed by tissue factor production (A: (▲); points represent the mean values (±S.D.) for four wells of a 96-well plate) and IL-8 gene expression (B: (■); points represent the mean values (±S.D.) from three to five assays). The specificity of this monoclonal antibody effect was also determined using a non-VEGF specific monoclonal antibody (A:(▼); points represent the mean values (±S.D.) for four wells of a 96-well plate).

3.4. Storage of assay samples for later processing

For the assay measuring crystal violet staining of the HUVECs, 96-well plates were frequently stored at room temperature, in the dark, for a few days following the fixation by glutaraldehyde, rinsing and subsequent drying, and before addition of the crystal violet solution. For the tissue factor chromogenic assays, and IL-6 and IL-8 ELISA assays, plates containing the lysed cell contents or the cell culture supernatants were routinely stored at −70 °C for several weeks. This storage for later processing caused no detectable difference compared with samples that were processed immediately. The only point in the alamarBlue protocol at which plates can be stored is following the incubation with alamarBlue, which offers little advantage. Plates stored at 4 °C for a few days showed a small further increase in alamarBlue reduction (data not shown) and freezing of plates was not investigated.

To investigate the possibility of interrupting the qRT-PCR assay protocol and storing samples for later processing, two 96-well plates containing VEGF-treated HUVECs were lysed in parallel as previously. One plate was immediately processed for RNA isolation and cDNA synthesis and the second plate of HUVECs (stabilised in lysis buffer) was immediately transferred to −80 °C for storage. After 3 months at −80 °C, RNA was extracted from VEGF-treated HUVECs and the expression of IL-8 mRNA was analysed as previously and compared to the expression of IL-8 mRNA in VEGF-treated HUVECs where RNA was isolated immediately after treatment (Fig. 7). Prior to retrotranscription into cDNA, the RNA was initially quantified from both sets of samples by UV spectrophotometry and was found to have exhibited no degradation after storage at −80 °C (data not shown). Analysis of VEGF dose–responses from both sets of samples demonstrated a good correlation in terms of overall profile, maximal-fold stimulation over basal and linearity of response.

4. Discussion

The complex structure of biological molecules means it is difficult, often impossible, to characterize a preparation sufficiently to predict its biological activity using physicochemical methods alone, so testing in a suitable bioassay system is generally necessary. For the majority of therapeutic biological products, a bioassay to measure biological activity is a regulatory requirement as part of the specifications [15]. Bioassays therefore, play an important role in the quality assurance and quality control of biological products. Cell-based assays, which have been reported as the functional bioassay systems most, widely used in the biopharmaceutical

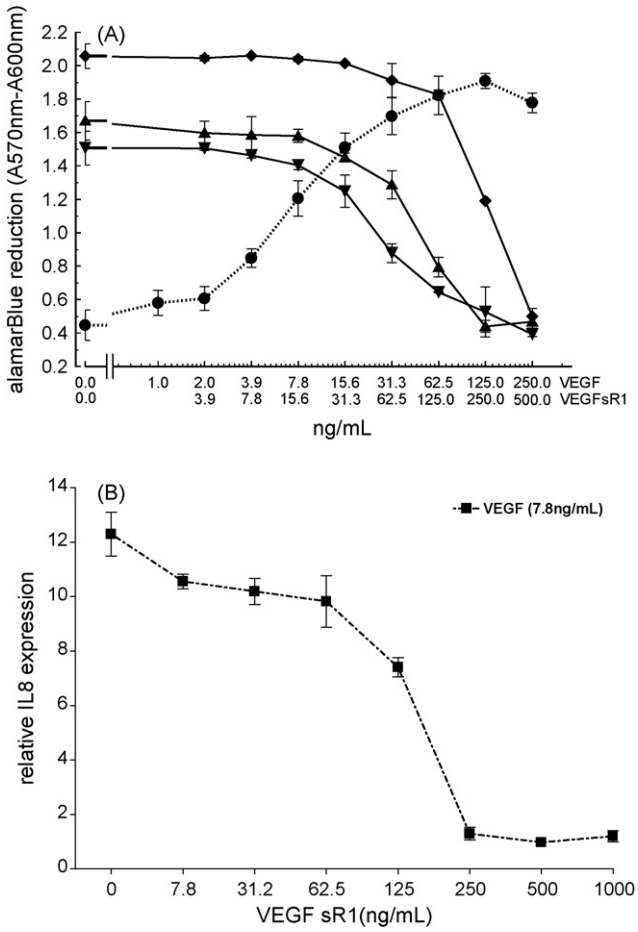

Fig. 6. VEGF sR1 inhibits VEGF-stimulated cell survival and IL-8 gene expression. HUVECs treated with 7.8–31.25 ng/mL VEGF were exposed to increasing concentrations of VEGF sR1 (0–1000 ng/mL). VEGF-stimulated cell survival, measured by reduction of redox dye alamarBlue™, and VEGF-stimulated IL-8 gene expression were determined. (A) The inhibitory effect of VEGF sR1 on VEGF-stimulated cell survival was assessed at three VEGF concentrations, 7.8 ng/mL (▼), 15.6 ng/mL (▲), and 31.25 ng/mL (♦). These results are from the same experiment (same culture plate, same cell, and VEGF stock) as Fig. 1B (dose–response shown on graph; ●). Points represent the mean values for three wells of a 96-well plate (±S.D.). (B) The inhibitory effect of VEGF sR1 on VEGF-stimulated IL-8 gene expression was assessed at a single concentration of VEGF (7.8 ng/mL). Points represent the mean values (±S.D.) from three to five assays.

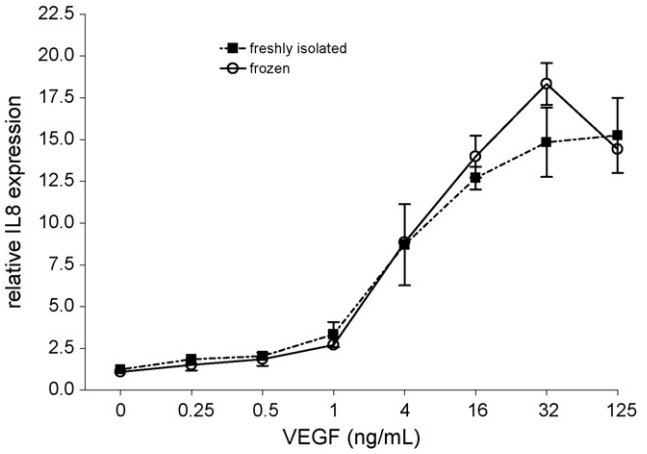

Fig. 7. IL-8 gene-expression analysis in response to VEGF stimulation in stored samples. HUVECs were exposed to increasing concentrations of VEGF (0–125 ng/mL) and RNA was immediately isolated (fresh; ■);or the RNA was stabilised *in situ* in lysis buffer, immediately transferred to −80 °C, and then isolated after 3 months storage (frozen; ○). Assays were performed in duplicate and points represent mean values (±S.D.).

industry [1,2], offer a range of measurable responses. The so-called late-stage responses, such as change in cell number, secretion of a specific protein or metabolism of a substrate, have the disadvantage of generally requiring incubation of the cells for a period of several hours to several days following exposure to the test product. Not only does this mean a delay in determination of the activity of the test product, but also it require maintenance of a stable and sterile cell culture environment for an extended period. One alternative to late-stage responses is the use of reporter gene assays, where an exogenous gene, such as firefly luciferase, that will permit a convenient readout, is inserted behind a promoter driven by the normal signal transduction pathway. Such systems can reduce the incubation times to hours or minute, and with a convenient readout such as luminescence, can provide a potency determination with little further delay. A disadvantage of reporter gene assays is the requirement for creation and maintenance of a stably transfected cell line.

Recently, there has been interest in the development of novel bioassays based on quantitative measurements of specific mRNA species. One study has described an approach using branched DNA technology that is able to identify erythropoietin (EPO) or anti-EPO neutralizing antibody activities in serum [16]. This technique utilizes nucleic acid hybridization to measure the expression of an EPO-stimulated gene in the UT-7 leukemia cell line and demonstrates improved sensitivity and specificity compared to assays based on cell proliferation. In the study reported here, we have demonstrated the use of qRT-PCR technology to measure the biological activity of VEGF and of biological inhibitors of VEGF as an alternative to a selection of late-stage bioassay systems. We showed that several genes induced by VEGF provide possible readouts, and selected the induction of IL-8 mRNA for further characterization.

Bioassay systems are complex and the response of the system tends to be sensitive to variables which it is impossible or impractical to control, such as batch-to-batch variation in certain media components or cell passage number. As a result, dose–response curves can vary between assays. This is the reason that potency measurements are not obtained as absolute values, such as ED50, but are measured relative to that of a reference preparation [17], as illustrated in Fig. 1A, where a stressed sample of VEGF is compared with the WHO reference reagent 02/286 for determination of its potency. If the reference and test preparations are sufficiently similar, then the response of each should be affected similarly by any variation in the assay system and the measured relative potency should remain constant. Use, over several years, of the various late-stage assays described in this report showed approximately fourfold variation in the ED50 for a given VEGF preparation within a given assay system and such variation is not unusual for bioassay systems. The variation in the VEGF dose–response curves between the various assay systems described in this report, including the qRT-PCR, is no greater than that observed between assays of one assay system. This underlines the importance of using an appropriate reference standard of a functionally similar material for measurement of potency of any biological, including inhibitors such as monoclonal antibodies and soluble receptors. The action of VEGF on cultures of HUVECs was chosen as the model system. VEGF is a molecule with several potential therapeutic applications (reviewed in Ref. [8]), and as is the case for a number of growth factors, inhibition of activity has therapeutic application. Therapeutic biological inhibitors of VEGF include the monoclonal antibodies Avastin [18] and Lucentis [19] which have been licensed for treatment of can-

cer and age-related macular degeneration. Furthermore, soluble receptors and related molecules are under investigation for a variety of pathological conditions. As model inhibitors of VEGF activity, we used the neutralizing monoclonal antibody, MAB293 (http://www.rndsystems.com/pdf/mab293.pdf), and a neutralizing soluble receptor VEGFsR1. Using the HUVECs, we had already investigated a number of bioassay systems, with different readouts, for the development of the WHO reference reagent for $VEGF_{165}$ [1] and in the study of inhibitors of VEGF pro-inflammatory and endothelial cell proliferation activities [14]. This provided the opportunity to compare the qRT-PCR assay with a range of different types of late-stage assay systems using the same cell stocks.

The total assay time required to obtain potency determination is the sum of the incubation time with the cells after dosing with the test products and the time required for any further processing of the samples. The incubation times for the various protocols were: qRT-PCR, 45 min; tissue factor chromogenic assay, IL-6 and IL-8 ELISA, 5 h; crystal violet, 48 h; alamarBlue, 63 h. While some variation on these incubation times would be possible, that required for the qRT-PCR is clearly much shorter. With only a 45-min incubation time, under normal laboratory conditions, it should be possible to perform the dosing without the need for a sterile environment. The crystal violet and alamarBlue protocols require no further processing whereas the other protocols do, bringing the total assay times to: qRT-PCR, 5 h; tissue factor chromogenic assay, 7 h; IL-6, and IL-8 ELISA, 11 h; crystal violet, 48 h; alamarBlue, 63 h. The overall assay time of the qRT-PCR protocol therefore, compared favorably with all the late-stage assay systems studied. Although very short cell incubation times of minute with immediate readouts can be obtained in assays involving induction of ion fluxes, such as proton flux [20], or Ca^{2+} flux [21], the use of these methods as potency assays is limited by suitability of cell lines and issues of reproducibility and ruggedness.

If measurements of critical quality and performance attributes of a product can be obtained sufficiently rapidly during the production process, this can allow adjustments to be made to the process to ensure final product quality. Bioassays generally take too long for this purpose unless the production process includes a holding step. However, with a 5-h assay time, biological activity measurement by qRT-PCR could be considered alongside the more conventional physicochemical analyses of process analytical technology.

All the assays in this study were performed manually, or with a small degree of automation, and were labor-intensive. However, if required, all of these protocols could be highly automated with existing commercial hardware and software. With currently available hardware, the throughput of the qRT-PCR is limited at the step of RNA extraction, where the capacity to automate RNA extraction is restricted to a single 96-well plate, per instrument, in a process requiring approximately 1 h. In contrast, in the other assay methods, up to ten 96-well plates were routinely processed simultaneously, and with automation, this throughput could be further increased. However, with the increasing interest in the use of qRT-PCR, it is probable that there will be a rapid development in the supporting hardware and this current limitation may soon be overcome.

The possibility of interrupting an assay protocol (a holding step) can be advantageous in allowing samples to be stocked so that they can be handled simultaneously, or, conversely, held until they can be processed through a downstream bottleneck. The qRT-PCR protocol allows samples to be held after lysis of the cells for 3 months at −80 °C and longer storage may be possible. Similarly, samples for analysis by IL-6 or IL-8 ELISA or tissue factor chromogenic assay can be stored as frozen cell supernatants or lysed cells, respectively. For the assay measuring crystal violet staining of the HUVECs, plates can be stored dry, at room temperature, in the dark, following the fixation by glutaraldehyde and rinsing. For the alamarBlue protocol, the only point at which the plates might be stored is immediately preceding the absorbance reading, which offers little advantage so was not investigated.

The relative costs of the different methods will depend strongly on the existing instrumentation, manpower, and assay requirements of a particular laboratory. The qRT-PCR methodology described here requires an initial outlay for dedicated instrumentation although real-time thermal cyclers are becoming increasingly common in general laboratories. The consumable costs for the qRT-PCR, tissue factor chromogenic assay and the cytokine ELISA protocols are higher than those for the alamarBlue and crystal violet assays. However, the qRT-PCR offers an assay platform that is easily adapted to other biological products. In general, once a responsive cell line has been found, there will be a wider choice of up- or down-regulated genes providing measurable changes in mRNA levels than there will be measurable changes in secreted protein or cell number. In addition, changes in the availability and consistency of the antibody preparations required for ELISAs can be a source of variation in assay performance.

All cell-based bioassays are subject to interference from factors like product and process-related impurities and environmental conditions. Since changes in gene expression occur more rapidly than changes in protein levels, the qRT-PCR assay permits short incubation times and this may avoid some of these sources of interference. A further and important advantage of qRT-PCR is the use of housekeeping genes to normalize the measured mRNA levels. This provides an internal control, thus, eliminating the effects of some sources of upstream variability. In addition, the qRT-PCR methodology offers the possibility of returning to archive samples of cDNA to measure the level of an alternative mRNA species. This could be of benefit in discovery or development and would permit the investigation of other responsive pathways and other readouts. It is possible to store cell supernatants or lysed cells for this purpose, but the quantity of solution stored limits the number of subsequent assays. Stored cDNA can be greatly diluted prior to PCR amplification, permitting a much larger number of subsequent assays.

Our study demonstrates the utility of qRT-PCR in measuring potency of biological therapeutics using the model biological products, VEGF, and biological inhibitors of VEGF, a neutralizing monoclonal antibody and a neutralizing soluble receptor. For the qRT-PCR assay, the dose range of VEGF and of the inhibitors was similar to that for a variety of other assay systems. Our results suggest that the qRT-PCR method described has potential for wide application as a bioassay for potency. However, our work and that of Bertolotto et al. [9], demonstrates that careful selection of both the most appropriate gene target, and investigation of the incubation time of the cells with the biological product of interest, is essential for the development of a useful bioassay. We are currently extending our investigation to other biological therapeutics and measuring biological activities in complex matrices.

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