Review

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Bioanalysis

Applications of cell-based bioassays measuring the induced expression of endogenous genes

Cell-based bioassays are used to determine the biological activity of complex biotherapeutic products, to assign potency and to assure the quality and consistency of the manufacturing process. Clinically, these assays are used to assess bioactivity in patient samples, particularly for the detection of antidrug neutralizing antibodies. Owing to their versatility, cellular assays that measure endogenous gene expression by quantitative reverse transcription PCR offer a rapid and automatable alternative to assays measuring functional, late-stage responses. Notably, detection of immediate early gene expression represents a direct response of the cell to receptor ligation by the biotherapeutic. We review current developments in the use of this approach and demonstrate its application to the detection of receptor-binding autoantibodies using, as a case study, the detection of autoantibodies to the thyroid-stimulating hormone receptor.

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Biotherapeutic medicines, including products such as recombinant proteins, monoclonal antibodies and nonprotein products derived from living organisms, are used in the management of a wide range of conditions including rheumatoid arthritis, multiple sclerosis, Crohn's disease, infertility and cancers.

Produced by engineered cells or bacteria, or purified from a biological source such as blood, urine or animal tissue, biotherapeutic products are larger and more complex than chemically synthesized medicines and their biological activity is dependent on the nature of the source material, manufacturing conditions, downstream processing and formulation. Such complexity and heterogeneity necessitates the comprehensive characterization of biotherapeutic medicines in order to achieve consistent quality, better safety and efficacy during drug development and production, as mandated by regulatory agencies [1–3].

In vitro cell-based biological assays usually rely on the quantification of a measurable response to a biotherapeutic preparation in a clonal cell line in order to determine biological potency relative to a standard preparation. Being a critical requirement for biotherapeutic product development, biological assays are used to assess the bioactivity of a ligand of interest in a clinical specimen, for example, to monitor therapy or to detect neutralizing antibodies (NAbs) through inhibition of the response. Here, we provide an overview of the range of responses measured in such assays and discuss the advantages of developing biological assays that measure the induction of endogenous genes in responsive cell lines using quantitative reverse-transcription PCR (qRT-PCR). Furthermore, we describe the potential applications of this approach, notably, in the quality control of biological medicines, the detection of NAbs in patient serum and the assessment of receptor-stimulating autoantibodies that characterize some autoimmune disorders.

Cell-based bioassays

The development of a cell-based biological assay requires the identification or engineering of a responsive cell line in which the nature, magnitude and the stability of the response reflects the mode of action, dose

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Key terms

Biological assay or bioassay: A procedure to determine the biological activity of a substance relative to a standard preparation.

Potency: A measure of the biological activity of a substance in a bioassay.

Quantitative reverse-transcription PCR: The measurement of gene expression by the quantitation of mRNA through the synthesis of a representative population of cDNA and the amplification of a specific sequence of DNA by the PCR.

Immediate early gene: Genes which are transcribed rapidly in response to a stimulus without prior protein synthesis.

Neutralizing antibody: An antibody that inhibits or neutralizes the biological effect of an antigen.

Immunogenicity: The ability of a substance to induce an immune response.

and exposure time of the ligand [4]. The mode of action of the biotherapeutic is readily demonstrated in bioassays that measure a late-stage response such as cell proliferation, antiviral activity or protein expression. Examples include the proliferative response of the human acute myeloid leukemic cell line, UT-7, to stimulation with erythropoietin (EPO) [5] or the expression of the human leukocyte antigen, HLA-DR, in the human colorectal adenocarcinoma cell line, COLO 205, in response to IFN-γ [6]. Commonly, however, a detectable response requires incubating cells with the product for several days, prolonging the requirement for maintaining a sterile environment and increasing the risk of imprecision due to evaporation and edge effects. In addition, assays involving clinical specimens may be susceptible, over such extended incubation times, to interference from nonspecific factors in the clinical sample.

Early-stage responses occur rapidly after the binding of the biotherapeutic product to the receptor, providing measurable responses such as receptor phosphorylation, the generation of second messenger molecules and modifications in patterns of gene expression. Examples include the use of antiphosphotyrosine antibodies to detect phosphorylation of the IGF-1 receptor [7] and the measurement of cAMP accumulation to assess thyroid-stimulating hormone (TSH) bioactivity in the rat thymocyte cell line, FRTL-5 [8,9]. The signal amplification of such intracellular signaling cascades has led to the wide adoption of reporter gene assays, whereby a cell line is engineered to express a readily detectable reporter gene such as firefly luciferase, driven by a promoter that is responsive to the applied stimulus. Such responses can significantly reduce the incubation time to hours or minutes, and convenient detection further decreases the time to result. However, this approach requires substantial commitment to the development and maintenance of transfected cell lines and many such lines are not then made widely available.

The need to improve sensitivity, reliability and speed has driven advancements in biological assays, exemplified by the development of bioassays to quantify the bioactivity of preparations of therapeutic type I interferons (IFN) described in the case study, below.

Case study: advances in biological assays for recombinant IFN-β

Recombinant IFNs are used to treat infectious and autoimmune disease, and in the treatment of some cancers [10]. The bioactivity of preparations of IFNs is quantified using a variety of cell-based biological assays measuring responses such as antiviral activity, inhibition of cell proliferation and immunomodulation [11].

The conventional biological assay for IFN-β is the cytopathic effect bioassay in which samples are preincubated with cells that express the IFN type I receptor to stimulate an antiviral response. Subsequent infection with a virus allows quantification of this response by measuring cell viability. Although assays based on various cell line and virus combinations have been developed, the inhibition of the replication of encephalomyocarditis virus in the human lung carcinoma cell line, A549, is the established method to determine IFN-β bioactivity [12]. However, the assay is laborious and time consuming, and requires prolonged periods of sterile culture and the facilities to handle cultures of live virus. The inherent variability in infection and replication of the virus can also lead to poor assay reproducibility.

In an effort to simplify bioassays of IFN and to improve reproducibility, alternative cell-based bioassays for potency measurements have been developed. Files and co-workers demonstrated that the expression of the cytoplasmic GTPase, MxA, in A549 cells in response to stimulation with type I IFN could be used to assess IFN bioactivity and to detect anti-IFN NAbs [13]. Subsequently, and in response to lack of available anti-MxA antibodies, assays quantifying mRNA encoding MxA were developed [14–16]. Other genes, such as the endogenous early IFN-inducible 6–16 gene (*IFI6*) have also been shown to be induced in a dose-responsive manner to IFN-β and also to IFN-α1a stimulation [17]. The 6–16 gene is induced through activation of the Jak–STAT3 signaling pathway and there is evidence that IFN-induced 6–16 expression is associated with both antiviral and antiapoptotic processes that agree with the known biological mode of action of IFN products [18,19]. Reporter gene assays minimize the process-

ing steps following IFN treatment of cells by providing a readily detectable signal through the expression of a reporter gene such as luciferase under the control of a inducible promoter in a IFN type I responsive cell line. Several such systems have been developed [20–22], as reviewed by Meager [23], and the use of division-arrested frozen cell lines allows some wider use of these mainly proprietary cell lines [24].

Cell-based bioassays based on the measurement of endogenous, early gene expression

Cell-based bioassays have been developed that measure endogenous immediate early gene expression stimulated by the ligand of interest and quantified by measurements of mRNA by qRT-PCR. Using this technique, the amplification of target mRNAs, represented as reverse-transcribed cDNA fragments, are detected in real time as the PCR reaction progresses [25,26]. Clinical applications of qRT-PCR technology include the detection and quantification of pathogens [27], the prenatal detection of chromosomal abnormalities [28] and in oncology, the detection, diagnosis and stratification of disease [29,30].

Immediate early genes, also known as primary response genes, are expressed in response to stimulation without the need for *de novo* protein synthesis, as reviewed by Fowler *et al.* [31]. Their function is to direct the response of the cell to the stimulus and many encode transcription factors and are proto-oncogenes. Thus, immediate early gene expression represents a response that is attributable to the nature and duration of an applied stimulus and, if also dose responsive, has the potential to provide a rapid and robust measurable response in a cell-based biological assay as depicted in Figure 1. Here, we review proof-of-concept, cell-based bioassays that quantify specific immediate early genes by qRT-PCR for use in the assessment of biotherapeutic potency and stability and in the monitoring of neutralizing antibody levels in patient samples. In addition, we demonstrate that this approach can be applied to the detection and measurement of receptor-binding autoantibodies in autoimmune disease.

Assessment of potency & the quality control of biotherapeutic medicines

The potency of a biotherapeutic product is a measure of its bioactivity relative to a standard preparation in a biological assay. Determination of potency is a regulatory requirement in order to ensure the correct clinical dose and to assess stability, batch-to-batch consistency and the effect of any changes to the manufacturing process. The measurement of endogenous early gene expression in such biological assays has been demonstrated for potency measurements of VEGF and its inhibitors [32]. In this study, the expression of IL-8 mRNA was detected in human umbilical vein endometrial cells after stimulation with VEGF for 45 min. The expression of IL-8 mRNA was dose responsive and the potency values obtained were comparable with bioassays that measured later responses such as cell proliferation and the production of IL-8, IL-6 and tissue factor proteins. As well as reduced total assay time, the authors demonstrated that this approach showed improved flexibility and had the potential for automation and assay interruption to enable the archiving of samples. Thus, this approach could be used during the production of a biotherapeutic medicine as an in-process assay to monitor changes in product quality and performance.

Silva and co-workers also demonstrated that this approach could contribute to the quality assessment of biotherapeutic preparations using, as a case study, an assessment of preparations of IFN-α-2a and IFN-α-2b that had been stored under accelerated stress conditions [33]. Using qRT-PCR, the quantification of mRNA encoding MxA in the human lung carcinoma cell line, A549, detected loss of bioactivity in preparations of IFN-α-2a and IFN-α-2b stored at 37°C for 9 weeks.

The 6–16 gene was also shown to be induced in a dose-dependent manner in A549 cells stimulated for 4 h with IFN-α and IFN-β thereby allowing both the bioassay and qRT-PCR detection to be performed in one day [17]. Comparable results were also obtained using branched DNA technology, which uses a series of hybridization steps to anchor the mRNA of interest onto a solid support to permit further hybridization steps to bind an enzyme-conjugated probe for detection. Although expensive, this technology measures the mRNA directly without the need for extraction, reverse transcription and amplification.

Detection of NAbs

NAbs are part of an adverse drug reaction in which the biotherapeutic product elicits an immune response resulting in the generation of antidrug antibodies. Antidrug antibodies may be binding antibodies that do not interfere with the biological activity of the biotherapeutic medicine but can alter its pharmacokinetic profile, or they may be NAbs. NAbs inhibit the bioactivity of the product, either through binding to epitopes at the active site or through steric disruption of receptor binding [34,35]. Factors that can contribute to the immunogenicity of a biotherapeutic preparation include contaminants, impurities, glycosylation profile or aggregation state [36] and an assessment of

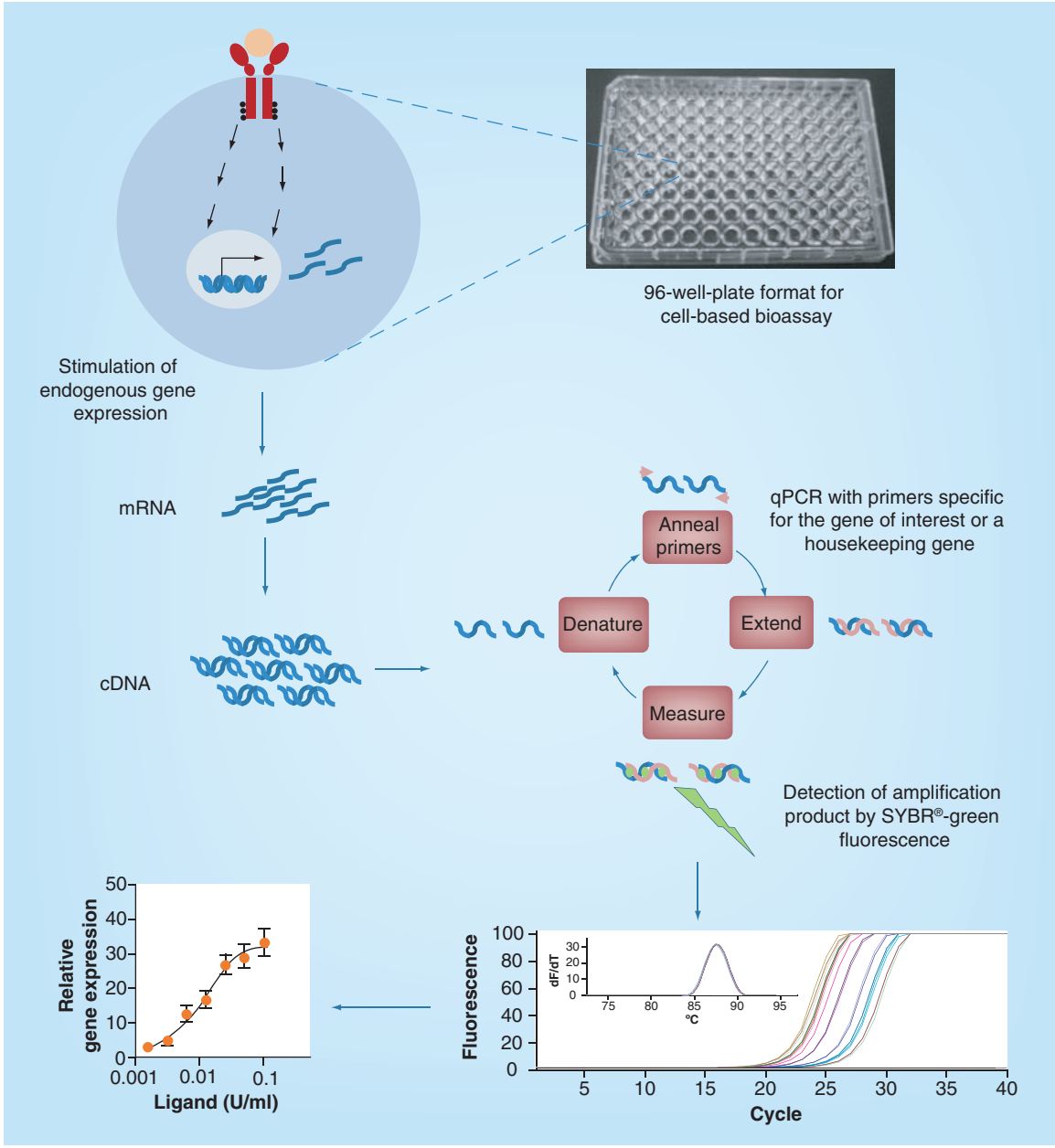

Figure 1. Cell-based bioassays based on the measurement of endogenous gene expression by quantitative real-time PCR using primers specific for the gene of interest. Gene expression is quantified by the increase in fluorescence signal with cycle number. The melt curve (insert, bottom right) of the rate of change of fluorescence with temperature as the DNA denatures demonstrates the amplification of a single PCR product. The gene expression of each sample, normalized to the expression of a housekeeping gene, is expressed as fold change over control, unstimulated cells.
qPCR: Quantitative real-time PCR.

immunogenicity is required for the approval of a new biotherapeutic medicine [37,38]. Hence, the establishment of cell-based bioassays to detect NAbs is an essential component during preclinical and clinical development and after regulatory approval, particularly when introducing changes to the manufacturing process, formulation or route of administration. Clinically, detection of NAbs in patient serum is important for diagnosis and monitoring of treatment. Detection requires the quantification of a reduction in the bioactivity of the biotherapeutic in a cell-based bioassay.

Severe adverse reactions can occur if NAbs cross-react with the endogenous equivalent of the biotherapeutic product, as seen in the development of pure red cell aplasia in patients with NAbs to therapeutic, recombinant, human EPO [39]. These are quantified

by the reduction in EPO bioactivity as determined by a cell proliferation bioassay, commonly detected by measuring the incorporation of ^{3}H-thymidine into newly synthesized DNA [40–42]. False positives occur if nonspecific serum factors interfere with cell proliferation over the 3-day incubation period and strategies to investigate these matrix effects have been developed. These include the construction of the proprietary, murine 32D-EPOR cell line [40], which will proliferate in response to either EPO or IL-3. Serum containing anti-EPO NAbs will inhibit proliferation in response to EPO but cells will still proliferate in response to IL-3. Conversely, the presence of nonspecific inhibitory factors in a patient sample will inhibit the proliferation of 32D-EPOR cells in response to both EPO and IL-3. An alternative approach to investigating false-positive samples is to deplete NAb from serum samples using Protein G and Protein L, which will restore EPO-stimulated cell proliferation if the inhibition is mediated by NAb but not if it is due to nonspecific factors [43]. Addressing matrix effects has been highlighted as a key consideration in a recent White Paper on the design of NAb assays [44].

A NAb assay based on measuring endogenous, early gene expression greatly decreases the time that cells are in contact with patient serum from days to hours or minutes. This was demonstrated by the rapid, EPO-stimulated induction of the immediate early gene, *EGR1*, in the EPO-sensitized, human acute myeloid leukemic cell line, UT-7/EPO [45]. The induction of *EGR1* was inhibited by preincubating cells with serum from a patient with antibody-mediated pure red cell aplasia and the inhibition could be reversed with a 50-fold increase in exogenous EPO [46]. Furthermore, the fold-change in *EGR1* gene expression upon stimulation was routinely greater than 50-fold. Such a robust cellular response allows a low stimulatory concentration to be selected for the detection of NAb in dilutions of patient sera. This increases the sensitivity of the assay, thereby allowing greater dilutions of patient serum, which could further reduce the effects of any nonspecific factors [47].

The chronic use of IFN-β biotherapeutics for the treatment of relapsing–remitting multiple sclerosis can also result in the generation of NAbs, with some recombinant treatments and routes of administration resulting in a reported prevalence of NAb positivity of greater than 40% [48–50]. To monitor patients, the biological assays for IFN-β products, described in the case study above, have been adapted for the detection of NAbs [21,24,51–54], including assays measuring IFN-stimulated accumulation of MxA mRNA by qRT-PCR. Bertolotto and colleagues demonstrated that bioassays measuring MxA mRNA were less variable than both the cytopathic effect and MxA protein-based assays for IFN-β [15]. The authors attributed this improved precision to the close correlation of receptor binding and the expression of the gene encoding MxA and the standard practice in qRT-PCR of normalizing the level of expression of the gene of interest to the level of expression of a housekeeping gene that minimizes imprecision resulting from cell counting, cell growth rates or cell clumping.

As the patents protecting many of the originator biotherapeutic products expire, standardized and widely available assays to detect NAb will be important as non-innovator products are developed, some of which may be marketed without undergoing such extensive regulatory evaluation as approved biosimilar or innovator products. Cell-based biological assays that measure endogenous, early gene expression in responsive cell lines provide a convenient approach to the assessment of immunogenicity of new biotherapeutic products or formulations without the extensive development required to establish cell lines that are stably transfected with receptors and reporter gene constructs.

Detection of stimulatory autoantibodies

Autoimmune diseases are characterized by the development of autoantibodies that bind cell surface or internal cellular components. Analysis of the serum autoantibody profile of patients with these conditions is integral to the clinical diagnosis, prognosis and monitoring of treatment. A number of conditions are characterized by autoantibodies that bind to cell surface receptors either agonistically or by blocking the action of an endogenous ligand.

Graves' disease is characterized by the development of circulating stimulatory autoantibodies directed against the TSH receptor (TSHR). Stimulatory autoantibodies (TSAb) mimic the action of TSH, activating the cAMP-dependent G-protein coupled TSHR, resulting in hyperthyroidism [55]. Additional complications of the condition include Graves' opthalmopathy [56] and Graves' dermopathy [57]. Patients may also develop blocking antibodies (TBAbs), which prevent the binding of TSH to TSHR and neutral antibodies, which recognize the TSHR without affecting the binding of TSH but that may contribute to antibody-mediated destruction of thyrocytes [58].

The detection of TSAb is used to distinguish Graves' disease from other hyperthyroid conditions and to establish the severity and risk of recurrence

Key term

Autoantibody: An antibody which binds to the cells or tissues of the individual in which it is produced.

during treatment [59,60]. Current, solid-phase assays to detect TSHR autoantibodies use competitive binding technology in which serum from autoantibody positive patients prevents the binding of either TSH (second-generation bioassays) or a TSHR-stimulatory monoclonal antibody, M22, (third-generation bioassays) to immobilized TSHR [61–63]. The sensitivity and specificity of the second- and third-generation of TSAb assays has prompted proposals for the wider use of these tests in clinical practice [64,65]. Binding assays, however, do not distinguish between TSAbs and TBAbs. This is important both for accurate diagnosis and in cases such as during pregnancy, when the distinction between TSAb and TBAb has clinical consequences [66,67].

Early approaches to measuring stimulation of the TSHR used radioimmunoassays to detect cAMP activity in the rat thyrocyte cell line, FRTL-5 [8,9], or in cell lines stably transfected with TSHR [68,69]. These late-stage bioassays were cumbersome and expensive and have now been replaced with reporter gene assays using CHO cells stably transfected with both the TSHR and a reporter gene construct comprising the luciferase reporter gene controlled by a cAMP response element [70,71]. The reporter gene assay has been refined further by the development of a cell line expressing a chimeric TSHR/rat LH receptor that has a comparable TSAb-stimulated cAMP response as the native receptor but has a decreased background response to TSH [72].

We report here, a proof-of-concept study demonstrating that measurement of endogenous early gene expression by qRT-PCR in the widely available TSH-sensitive cell line, FRTL-5, can be used to detect TSAb. As described in Figure 2A & B, both TSH and preparations containing TSAb were shown to stimulate the dose-dependent expression of the *Nr4a1* gene after an incubation of 2 h and quantification by qRT-PCR. To normalize for differences in tissue loading, *Nr4a1* gene expression was quantified relative to the expression of a housekeeping gene, rat β-actin (*Actb*), the expression of which had been shown previously not to change with TSH-treatment of FRTL-5 cells. The *Nr4a1* (*Nur77, NGFI-B*) gene [73,74] is a member of the NR4A subgroup of immediate early genes that are expressed in response to changes in the cellular environment induced by physiological and physical stimuli (as reviewed by Maxwell and Muscat [75]). The *Nr4a1* gene encodes a ligand-dependent, nuclear transcription factor that has homology with the cytoplasmic thyroid/steroid hormone receptors [76]. Expression of *Nr4a1* was shown to be induced by TSH through a cAMP-dependent mechanism in canine thyrocytes [77]. In the FRTL-5 cell line, Calebiro and coworkers used microarray analysis to demonstrate the induction of *Nr4a1* after stimulation of cells with TSH for 30 min [78].

The TSAb preparations investigated using this bioassay were the first and second WHO International Standards (IS) for TSAb, coded 90/672 and 08/204, respectively, which were established for the calibration of TSAb assays. The first WHO IS, 90/672, contained TSAb from a plasmapheresed human donor with Graves' disease. It was assigned a unitage of 100 mIU/ampoule following a collaborative study in which participating laboratories measured both receptor-binding and thyroid-stimulating activities. The second WHO IS, 08/204, which replaced the first WHO IS in 2010, comprised an ampouled preparation of the human TSAb monoclonal antibody, M22. The second WHO IS was calibrated in terms of the first WHO IS, 90/672, by receptor-binding assays and assigned a potency of 113 mIU/ampoule [79]. However, when determined by TSAb bioassays, the geometric mean potency of second WHO IS, 08/204, in terms of the first IS, 90/672, was 242 mIU/ampoule. This twofold difference suggests that the patient-derived, first WHO IS contains a heterogenous population of autoantibodies with stimulating, neutral and inhibitory functions. When tested in the cell-based bioassay measuring endogenous *Nr4a1* gene expression, a similar ratio of bioactivity between the first and second IS was observed (Figure 2B). The lower limit of detection for the monoclonal TSAb was around 0.4 mIU/ml, which compares with current TSAb binding assays and bioassays [61].

To ensure that the response was not due to background serum levels, FRTL-5 cells were incubated with pooled human serum at concentrations up to 50% (v/v) (Figure 2C). Increasing human serum concentrations resulted in minimal (<fivefold) stimulation of *Nr4a1* gene expression, indicating that the dilution of patient serum samples at 1:2, as used in current TSAb assays, would not have an adverse effect on the specificity of the assay. Furthermore, TSH-stimulated *Nr4a1* gene expression was inhibited in a dose-responsive manner by preincubation of FRTL-5 cells with 1–1000 ng/ml K1–70 [80], a human monoclonal TBAb, confirming that stimulation of the TSHR is required for the induction of *Nr4a1* gene expression (Figure 2D).

Here, we provide proof of principle that the measurement of endogenous, early gene expression by qRT-PCR can be used to detect stimulatory activity to the TSHR in patient samples without the need to develop an engineered cell line. This approach could be readily applied to the detection of agonistic autoantibodies for other conditions such as systemic scle-

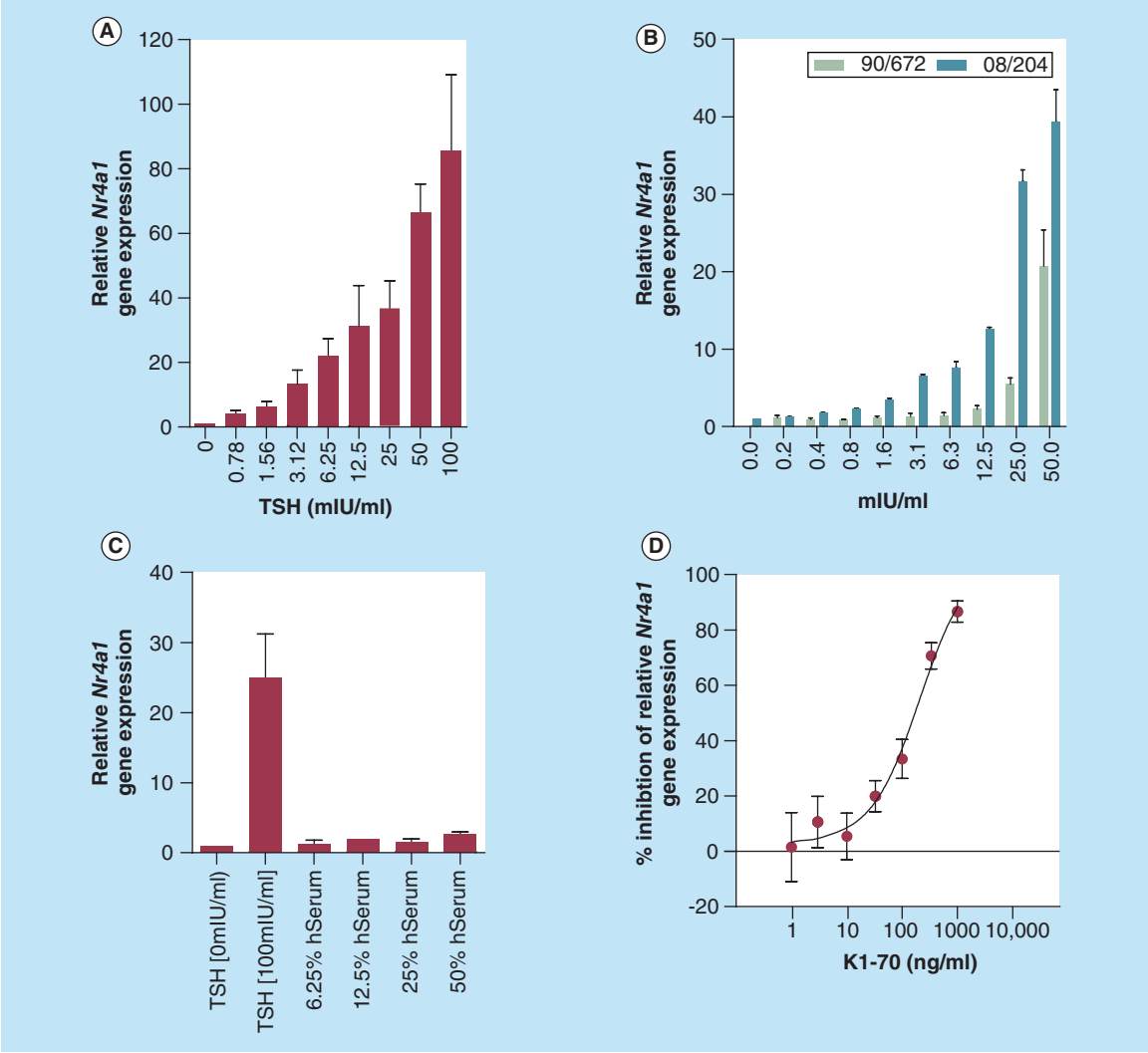

Figure 2. Dose-responsive, immediate early gene expression stimulated by thyroid-stimulating hormone or thyroid-stimulating antibodies. The *Nr4a1* gene was expressed in FRTL-5 cells stimulated for 2h with **(A)** 0–100 mIU/ml TSH (NIBSC code 03/192) and **(B)** 0–50 mIU/ml of the human TSAb monoclonal antibody M22 (NIBSC code 08/204) and 0–50 mIU/ml TSAb from a plasmapheresed human donor (NIBSC code 90/672) as measured by quantitative reverse-transcription PCR and presented as the fold change of normalized *Nr4a1* expression over unstimulated FRTL-5 cells. **(C)** *Nr4a1* gene expression was not stimulated by 0–50% (v/v) pooled normal human serum. **(D)** Preincubation of FRTL-5 cells with 1–1000 ng/ml K1–70, a TSHR blocking mAb, inhibited TSH-stimulated expression of the *Nr4a1* gene. Details of the biological assay, RNA preparation and quantitative RT-PCR can be found in the Supplementary Material.
TSH: Thyroid stimulating hormone.

rosis, which is characterized by stimulatory autoantibodies to the platelet derived growth factor receptor [81,82], dilated cardiomyopathy in which autoantibodies to the β_1-adrenergic receptor are involved [83,84] and preeclampsia, which is associated with the development of antibodies that bind and activate the major angiotensin II type I receptor [85,86].

Conclusion & future perspective

The use of endogenous gene expression as a measurable readout in a cell-based biological assay has potential for use in both biotherapeutic development and quality control, and in the monitoring of patients who have developed NAbs to a biotherapeutic medicine or autoantibodies with receptor binding activity. The use of qRT-PCR to detect gene expression in clinical samples (whole blood, peripheral blood mononuclear

Key term

Endogenous gene expression: The transcription of genes that are present in the genome of the cell and which have not been inserted or modified.

cells and isolated T cells) is becoming accepted in clinical laboratories to detect biomarkers of diseases such as cancers, cardiac conditions and autoimmune disorders. Detection of α-fetoprotein mRNA in peripheral blood has been suggested to correlate with the clinical stage of hepatocellular carcinoma [87,88] and CE-marked kits are available for α-fetoprotein mRNA detection. The FDA-approved Allomap® (XDx Inc., Brisbane, Australia) kit measures the expression of 20 genes in peripheral blood mononuclear cells by qRT-PCR to identify patients with a low probability of heart transplant rejection. Although in both these examples, the biomarkers are not expressed through cell stimulation, *ex vivo*, these demonstrate that approved procedures have been developed for the accurate measurement of mRNA.

The approach described in this review requires the careful selection of a responsive cell line and the identification of a dose-responsive gene, as well as optimization of the assay and qRT-PCR conditions. However, without the need to generate cell lines stably-transfected with a receptor and a reporter gene construct, the length of time required to develop an assay is considerably reduced. As such, this approach could be beneficial during the preclinical development of a biotherapeutic medicine, to assess the impact of changes to manufacturing processes and biotherapeutic formulations on bioactivity and immunogenicity. Furthermore, the immediate early genes induced by a biotherapeutic medicine will be an early and direct response of the cell to the stimulation, thereby truly representing the mode of action of the biotherapeutic in the chosen cell line.

The use of qRT-PCR to measure endogenous early gene expression provides sensitive and reproducible measurements of bioactivity and stability. The standard practice of normalizing the expression of the gene of interest to that of a housekeeping gene corrects for any variations in cell density in each sample. Measurements of bioactivity, stability and neutralization are comparable to alternative early and late-stage bioassays but do not require stably-transfected cell lines or the use of virus or radioactivity that are commonly required for late-stage assays. Already, the technology presented here provides biological assays that can be performed in one day. Further improvements to both total assay time and hands-on operator time can be made using kits that obviate the need for a separate RNA isolation step (e.g., Ambion® Cells-to-CT™; Life Technologies, Paisley, UK and FastLane Cell DNA; Qiagen, Crawley, UK) and also, by the use of closed, automated, high-throughput systems that comply with regulatory requirements.

Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: www.future-science.com/doi/suppl/10.4155/BIO.14.98

Executive summary

Cell-based bioassays

- Cell-based bioassays to determine the bioactivity of a biotherapeutic product often measure late-stage responses such as cell proliferation, antiviral activity and protein synthesis.

Bioassays based on the measurement of endogenous, early gene expression

- Immediate early genes are expressed rapidly in response to the binding of a biotherapeutic product to its receptor and can be measured by quantitative reverse-transcription PCR.
- A dose-responsive, immediate early gene provides a readout in a bioassay that does not involve the use of live virus, radioactivity or the construction of stably-transfected reporter cell lines.

Assessment of potency & the quality control of biotherapeutic medicines

- Assays based on the measurement of endogenous gene expression can be used to characterize a biotherapeutic medicine.
- Such assays can be developed rapidly and allow in-process archiving of samples prior to measurement.

Detection of neutralizing antibodies

- Bioassays to detect neutralizing antibodies measure a reduction in bioactivity but can be affected by interfering factors in patient serum resulting in false positives.
- A rapid and sizeable fold change in endogenous gene expression could allow the use of greater dilutions of serum and a reduction in the time it is applied to cells.

Detection of stimulatory autoantibodies

- Stimulatory autoantibodies contribute to autoimmune diseases, such as Graves' disease in which antibodies stimulate the thyroid-stimulating hormone receptor.
- Dose-reponsive induction of the immediate early gene, *Nr4a1*, in the rat thyrocyte cell line, FRTL-5, could be used to detect thyroid stimulating autoantibodies.

Future perspective

- Further improvements to reagents and equipment, including the use of automated technologies, lend support to this approach becoming a valid alternative to current procedures.

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References

Papers of special note have been highlighted as:
• of interest; •• of considerable interest.

1 Robinson CJ, Jones C. Quality control and analytical techniques for biopharmaceuticals. *Bioanalysis* 3(1), 81–95 (2011).

2 Chirino AJ, Mire-Sluis A. Characterizing biological products and assessing comparability following manufacturing changes. *Nat. Biotechnol.* 22(11), 1383–1391 (2004).

3 European Medicines Agency. Specifications: Test Procedures and Acceptance Criteria for Biotechnological/Biological Products. CPMP/ICH/365/96. www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2009/09/WC500002824.pdf

4 United States Pharmacopeia. Design and Development of Biological Assays, Chapter 1032 (2012). www.ipqpubs.com/wp-content/uploads/2010/06/USP_1032.pdf

•• **Provides guidance for the delivery of robust and reliable biological assays.**

5 Komatsu N, Nakauchi H, Miwa A *et al.* Establishment and characterization of a human leukemic cell line with megakaryocytic features: dependency on granulocyte-macrophage colony-stimulating factor, interleukin 3, or erythropoietin for growth and survival. *Cancer Res.* 51(1), 341–348 (1991).

6 Gibson UEM, Kramer SM. Enzyme-linked bio-immunoassay for IFN-g by HLA-DR induction. *J. Immunol. Methods* 125(1–2), 105–113 (1989).

7 Sadick MD, Intintoli A, Quarmby V *et al.* Kinase receptor activation (KIRA): a rapid and accurate alternative to end-point bioassays. *J. Pharm. Biomed. Anal.* 19(6), 883–891 (1999).

8 Kasagi K, Konishi J, Iida Y *et al.* A sensitive and practical assay for thyroid-stimulating antibodies using FRTL-5 thyroid cells. *Acta Endocrinol. (Copenh.)* 115(1), 30–36 (1987).

9 Vitti P, Chiovato L, Lopez G *et al.* Measurement of TSAb directly in serum using FRTL-5 cells. *J. Endocrinol. Invest.* 11(4), 313–317 (1988).

10 Muller F. Overview of the clinical applications of type I interferons. In: *The Interferons: Characterization and Application.* Meager A (Ed.). Wiley-VCH, Verlag GmbH & Co. KG, Weinheim, Germany, 277–308 (2006).

11 Meager A. Biological assays for interferons. *J. Immunol. Methods* 261(1–2), 21–36 (2002).

12 WHO Expert Committee on Biological Standardization. *Standardization of Interferons. World Health Organ Tech. Rep. Ser.* 725, 28–64 (1985).

13 Files JG, Gray JL, Do LT *et al.* A novel sensitive and selective bioassay for human type I interferons. *J. Interferon Cytokine Res.* 18(12), 1019–1024 (1998).

14 Pachner A, Narayan K, Price N *et al.* MxA gene expression analysis as an interferon-beta bioactivity measurement in patients with multiple sclerosis and the identification of antibody-mediated decreased bioactivity. *Mol. Diagn.* 7(1), 17–25 (2003).

15 Bertolotto A, Sala A, Caldano M *et al.* Development and validation of a real time PCR-based bioassay for quantification of neutralizing antibodies against human interferon-beta. *J. Immunol. Methods* 321(1–2), 19–31 (2007).

16 Aarskog NK, Maroy T, Myhr KM *et al.* Antibodies against interferon-beta in multiple sclerosis. *J. Neuroimmunol.* 212(1–2), 148–150 (2009).

17 Moore M, Meager A, Wadhwa M *et al.* Measurement of neutralising antibodies to type I interferons by gene expression assays specific for type 1 interferon-inducible 6–16 mRNA. *J. Pharm. Biomed. Anal.* 49(2), 534–539 (2009).

18 Zhu H, Zhao H, Collins CD *et al.* Gene expression associated with interferon alfa antiviral activity in an HCV replicon cell line. *Hepatology* 37(5), 1180–1188 (2003).

19 Cheriyath V, Glaser KB, Waring JF *et al.* G1P3, an IFN-induced survival factor, antagonizes TRAIL-induced apoptosis in human myeloma cells. *J. Clin. Invest* 117(10), 3107–3117 (2007).

20 Farrell R, Kapoor R, Leary S *et al.* Neutralizing anti-interferon beta antibodies are associated with reduced side effects and delayed impact on efficacy of interferon-beta. *Mult. Scler.* 14(2), 212–218 (2008).

21 Lam R, Farrell R, Aziz T *et al.* Validating parameters of a luciferase reporter gene assay to measure neutralizing antibodies to IFNbeta in multiple sclerosis patients. *J. Immunol. Methods* 336(2), 113–118 (2008).

22 Larocque L, Bliu A, Xu R *et al.* Bioactivity determination of native and variant forms of therapeutic interferons. *J. Biomed. Biotechnol.* 2011, 174615 (2011).

23 Meager A. Cell-based assays for the detection of neutralizing antibodies to interferon beta (IFN-β) and tumor necrosis factor alpha (TNFα) inhibitors. In: *Detection and Quantification of Antibodies to Biopharmaceuticals.* Tovey M (Ed.). John Wiley & Sons, Inc., Hoboken, NJ, USA. 133–156 (2011).

24 Lallemand C, Meritet JF, Erickson R *et al.* Quantification of neutralizing antibodies to human type I interferons using division-arrested frozen cells carrying an interferon-regulated reporter-gene. *J. Interferon Cytokine Res.* 28(6), 393–404 (2008).

25 Bustin SA. Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. *J. Mol. Endocrinol.* 25(2), 169–193 (2000).

26 Kubista M, Andrade JM, Bengtsson M *et al.* The real-time polymerase chain reaction. *Mol. Aspects Med.* 27(2–3), 95–125 (2006).

27 Sibley CD, Peirano G, Church DL. Molecular methods for pathogen and microbial community detection and characterization: current and potential application in diagnostic microbiology. *Infect. Genet. Evol.* 12(3), 505–521 (2012).

28 Raymond FL, Whittaker J, Jenkins L *et al.* Molecular prenatal diagnosis: the impact of modern technologies. *Prenat. Diagn.* 30(7), 674–681 (2010).

29 Matsuda K, Sugano M, Honda T. PCR for monitoring of minimal residual disease in hematologic malignancy. *Clin. Chim. Acta* 413(1–2), 74–80 (2012).

30 Lianidou ES, Markou A. Circulating tumor cells in breast cancer: detection systems, molecular characterization, and future challenges. *Clin. Chem.* 57(9), 1242–1255 (2011).

31 Fowler T, Sen R, Roy AL. Regulation of primary response genes. *Mol. Cell* 44(3), 348–360 (2011).

32 Burns CJ, Silva MM, Gray E *et al.* Quantitative RT-PCR as an alternative to late-stage bioassays for vascular endothelial growth factor. *J. Pharm. Biomed. Anal.* 47(3), 460–468 (2008).

33 Silva MM, Lamarre B, Cerasoli E *et al.* Physicochemical and biological assays for quality control of biopharmaceuticals: interferon alpha-2 case study. *Biologicals* 36(6), 383–392 (2008).

34 Ponce R, Abad L, Amaravadi L *et al.* Immunogenicity of biologically-derived therapeutics: assessment and interpretation of nonclinical safety studies. *Regul. Toxicol. Pharmacol.* 54(2), 164–182 (2009).

35 Wadhwa M, Thorpe R. Guidance on immunogenicity assessment of biologically derived therapeutic proteins: a european perspective. In: *Detection and Quantification of Antibodies to Biopharmaceuticals.* Tovey M (Ed). John Wiley & Sons, Inc., NJ, USA, 243–268 (2011).

36 Singh SK. Impact of product-related factors on immunogenicity of biotherapeutics. *J. Pharm. Sci.* 100(2), 354–387 (2011).

37 European Medicines Agency: *Guideline on Immunogenicity Assessment of Biotechnology-Derived Therapeutic Proteins. EMEA/CHMP/BMWP/14327/2006*. EMEA, London, UK (2007).

38 US FDA. *Guidance for Industry. Assay development for immunogenicity testing of therapeutic proteins*. US FDA, Rockville, MD, USA (2009).

39 Casadevall N, Nataf J, Viron B *et al.* Pure red-cell aplasia and antierythropoietin antibodies in patients treated with recombinant erythropoietin. *N. Engl. J. Med.* 346(7), 469–475 (2002).

40 Wei X, Swanson SJ, Gupta S. Development and validation of a cell-based bioassay for the detection of neutralizing antibodies against recombinant human erythropoietin in clinical studies. *J. Immunol. Methods* 293(1–2), 115–126 (2004).

41 Kelley M, Cooper C, Matticoli A *et al.* The detection of anti-erythropoietin antibodies in human serum and plasma: part II. Validation of a semi-quantitative ^{3}H-thymidine uptake assay for neutralizing antibodies. *J. Immunol. Methods* 300(1–2), 179–191 (2005).

42 Thorpe R, Swanson SJ. Current methods for detecting antibodies against erythropoietin and other recombinant proteins. *Clin. Diagn. Lab Immunol.* 12(1), 28–39 (2005).

43 Sanchez S, Barger T, Zhou L *et al.* Strategy to confirm the presence of anti-erythropoietin neutralizing antibodies in human serum. *J. Pharm. Biomed. Anal.* 55(5), 1265–1274 (2011).

44 Gupta S, Indelicato SR, Jethwa V *et al.* Recommendations for the design, optimization, and qualification of cell-based assays used for the detection of neutralizing antibody responses elicited to biological therapeutics. *J. Immunol. Methods* 321(1–2), 1–18 (2007).

•• **Describes the key considerations and recommended approaches to the measurement of neutralizing antibodies.**

45 Komatsu N, Yamamoto M, Fujita H *et al.* Establishment and characterization of an erythropoietin-dependent subline, UT-7/EPO, derived from human leukemia cell line, UT-7. *Blood* 82(2), 456–464 (1993).

46 Ferguson J, Bird C, Wadhwa M *et al.* Detection of neutralizing antibodies to erythropoietin by inhibition of rHuEPO-stimulated EGR1 gene expression in the UT-7/EPO cell line. *J. Immunol. Methods* 387(1–2), 191–198 (2013).

47 Finco-Kent D, Grenham A. Standardization and validation of cell-based assays for the detection of neutralizing anti-drug antibodies. In: *Detection and Quantification of Antibodies to Biopharmaceuticals.* Tovey M (Ed). John Wiley & Sons, Inc., NJ, USA, 243–268 (2011).

48 Farrell RA, Giovannoni G. Measuring and management of anti-interferon beta antibodies in subjects with multiple sclerosis. *Mult. Scler.* 13(5), 567–577 (2007).

49 Creeke PI, Farrell RA. Clinical testing for neutralizing antibodies to interferon-beta in multiple sclerosis. *Ther. Adv. Neurol. Disord.* 6(1), 3–17 (2013).

• **Comprehensive review of IFN-β immunogenicity in patients with multiple sclerosis, the measurement of anti-IFN-β neutralizing antibodies and their clinical significance.**

50 Sorensen PS. Neutralizing antibodies against interferon-beta. *Ther. Adv. Neurol. Disord.* 1(2), 125–141 (2008).

51 Meager A. Measurement of interferon activities. In: *The Interferons: Characterization and Application.* Meager A (Ed). Wiley-VCH, Verlag GmbH & Co. KGaA, Weinheim, Germany, 339–374 (2006).

52 Pungor E Jr, Files JG, Gabe JD *et al.* A novel bioassay for the determination of neutralizing antibodies to IFN-beta1b. *J. Interferon Cytokine Res.* 18(12), 1025–1030 (1998).

53 Seo YJ, Kim GH, Kwak HJ *et al.* Validation of a HeLa Mx2/Luc reporter cell line for the quantification of human type I interferons. *Pharmacology* 84(3), 135–144 (2009).

54 Lallemand C, Tovey MG. A novel one-step cell-based assay for quantification of neutralising antibodies to biopharmaceuticals. In: *Detection and Quantification of Antibodies to Biopharmaceuticals.* Tovey M (Eds). John Wiley & Sons, Inc., NJ, USA, 383–398 (2011).

55 Prabhakar BS, Bahn RS, Smith TJ. Current perspective on the pathogenesis of Graves' disease and ophthalmopathy. *Endocr. Rev.* 24(6), 802–835 (2003).

56 Douglas RS, Gupta S. The pathophysiology of thyroid eye disease: implications for immunotherapy. *Curr. Opin. Ophthalmol.* 22(5), 385–390 (2011).

57 Fatourechi V. Thyroid dermopathy and acropachy. *Best. Pract. Res. Clin. Endocrinol. Metab* 26(4), 553–565 (2012).

58 Morshed SA, Ando T, Latif R *et al.* Neutral antibodies to the TSH receptor are present in Graves disease and regulate selective signaling cascades. *Endocrinology* 151(11), 5537–5549 (2010).

59 Davies TF, Ando T, Lin RY *et al.* Thyrotropin receptor-associated diseases: from adenomata to Graves disease. *J. Clin. Invest* 115(8), 1972–1983 (2005).

60 Zophel K, Roggenbuck D, Schott M. Clinical review about TRAb assay's history. *Autoimmun. Rev.* 9(10), 695–700 (2010).

• **Concise overview of the advances in bioassays to detect thyroid-stimulating antibodies and the clinical applications of these assays.**

61 Smith BR, Bolton J, Young S *et al.* A new assay for thyrotropin receptor autoantibodies. *Thyroid* 14(10), 830–835 (2004).

62 Hermsen D, Broecker-Preuss M, Casati M *et al.* Technical evaluation of the first fully automated assay for the detection of TSH receptor autoantibodies. *Clin. Chim. Acta* 401(1–2), 84–89 (2009).

63 Schott M, Hermsen D, Broecker-Preuss M *et al.* Clinical value of the first automated TSH receptor autoantibody assay for the diagnosis of Graves' disease (GD): an international multicentre trial. *Clin. Endocrinol. (Oxf.)* 71(4), 566–573 (2009).

64 Matthews DC, Syed AA. The role of TSH receptor antibodies in the management of Graves' disease. *Eur. J. Intern. Med.* 22(3), 213–216 (2011).

65 Tozzoli R, Bagnasco M, Giavarina D *et al.* TSH receptor autoantibody immunoassay in patients with Graves' disease: improvement of diagnostic accuracy over different generations of methods. Systematic review and meta-analysis. *Autoimmun. Rev.* 12(2), 107–113 (2012).

66 Hovens GC, Buiting AM, Karperien M *et al.* A bioluminescence assay for thyrotropin receptor antibodies predicts serum thyroid hormone levels in patients with *de novo* Graves' disease. *Clin. Endocrinol. (Oxf.)* 64(4), 429–435 (2006).

67 Gaberscek S, Zaletel K. Thyroid physiology and autoimmunity in pregnancy and after delivery. *Expert Rev. Clin. Immunol.* 7(5), 697–706 (2011).

68 Morgenthaler NG, Pampel I, Aust G *et al.* Application of a bioassay with CHO cells for the routine detection of stimulating and blocking autoantibodies to the TSH-receptor. *Horm. Metab Res.* 30(3), 162–168 (1998).

69 Vitti P, Elisei R, Tonacchera M *et al.* Detection of thyroid-stimulating antibody using Chinese hamster ovary cells transfected with cloned human thyrotropin receptor. *J. Clin. Endocrinol. Metab.* 76(2), 499–503 (1993).

70 Watson PF, Ajjan RA, Phipps J *et al.* A new chemiluminescent assay for the rapid detection of thyroid stimulating antibodies in Graves' disease. *Clin. Endocrinol. (Oxf.)* 49(5), 577–581 (1998).

71 Jordan NJ, Rinderle C, Ashfield J *et al.* A luminescent bioassay for thyroid blocking antibodies. *Clin. Endocrinol. (Oxf.)* 54(3), 355–364 (2001).

72 Lytton SD, Li Y, Olivo PD *et al.* Novel chimeric thyroid-stimulating hormone-receptor bioassay for thyroid-stimulating immunoglobulins. *Clin. Exp. Immunol.* 162(3), 438–446 (2010).

73 Hazel TG, Nathans D, Lau LF. A gene inducible by serum growth factors encodes a member of the steroid and thyroid hormone receptor superfamily. *Proc. Natl. Acad. Sci. USA* 85(22), 8444–8448 (1988).

74 Milbrandt J. Nerve growth factor induces a gene homologous to the glucocorticoid receptor gene. *Neuron* 1(3), 183–188 (1988).

75 Maxwell MA, Muscat GE. The NR4A subgroup: immediate early response genes with pleiotropic physiological roles. *Nucl. Recept. Signal.* 4, e002 (2005).

76 Fahrner TJ, Carroll SL, Milbrandt J. The NGFI-B protein, an inducible member of the thyroid/steroid receptor family, is rapidly modified posttranslationally. *Mol. Cell Biol.* 10(12), 6454–6459 (1990).

77 Pichon B, Jimenez-Cervantes C, Pirson I *et al.* Induction of nerve growth factor-induced gene-B (NGFI-B) as an early event in the cyclic adenosine monophosphate response of dog thyrocytes in primary culture. *Endocrinology* 137(11), 4691–4698 (1996).

78 Calebiro D, Filippis TD, Lucchi S *et al.* Selective modulation of protein kinase A I and II reveals distinct roles in thyroid cell gene expression and growth. *Mol. Endocrinol.* 20(12), 3196–3211 (2006).

79 Burns C, Tiplady R, Rigsby P. *WHO International Collaborative Study of the Proposed 2nd International Standard for Thyroid Stimulating Antibody. Expert Committee on Biological Standardization WHO/BS/10. 2142*. World Health Organization, Geneva, Switzerland (2010).

80 Evans M, Sanders J, Tagami T *et al.* Monoclonal autoantibodies to the TSH receptor, one with stimulating activity and one with blocking activity, obtained from the same blood sample. *Clin. Endocrinol. (Oxf.)* 73(3), 404–412

81 Svegliati S, Olivieri A, Campelli N *et al.* Stimulatory autoantibodies to PDGF receptor in patients with extensive chronic graft-versus-host disease. *Blood* 110(1), 237–241 (2007).

82 Baroni SS, Santillo M, Bevilacqua F *et al.* Stimulatory autoantibodies to the PDGF receptor in systemic sclerosis. *N. Engl. J. Med.* 354(25), 2667–2676 (2006).

83 Holthoff HP, Zeibig S, Jahns-Boivin V *et al.* Detection of anti-beta1-AR autoantibodies in heart failure by a cell-based competition ELISA. *Circ. Res.* 111(6), 675–684 (2012).

84 Bornholz B, Weidtkamp-Peters S, Schmitmeier S *et al.* Impact of human autoantibodies on beta1-adrenergic receptor conformation, activity, and internalization. *Cardiovasc. Res.* 97(3), 472–480 (2013).

85 Xia Y, Kellems RE. Receptor-activating autoantibodies and disease: preeclampsia and beyond. *Expert. Rev. Clin. Immunol.* 7(5), 659–674 (2011).

•• **Useful review of disease conditions resulting from autoimmune conditions involving receptor activation.**

86 Herse F, Lamarca B. Angiotensin II type 1 receptor autoantibody (AT1-AA)-mediated pregnancy hypertension. *Am. J. Reprod. Immunol.* 69(4), 413–418 (2013).

87 Matsumura M, Shiratori Y, Niwa Y *et al.* Presence of alpha-fetoprotein mRNA in blood correlates with outcome in patients with hepatocellular carcinoma. *J. Hepatol.* 31(2), 332–339 (1999).

88 Ijichi M, Takayama T, Matsumura M *et al.* Alpha-Fetoprotein mRNA in the circulation as a predictor of postsurgical recurrence of hepatocellular carcinoma: a prospective study. *Hepatology* 35(4), 853–860 (2002).