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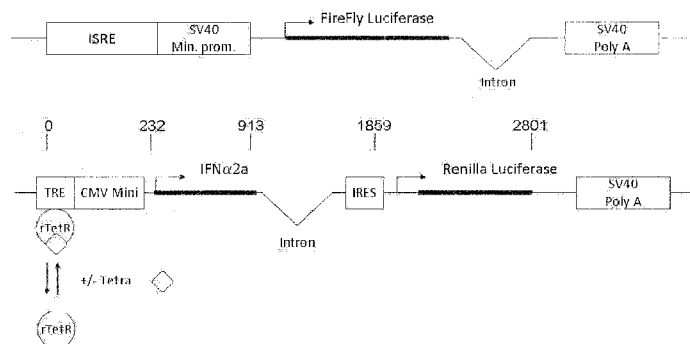
Fig. 14

(57) Abstract: The present invention provides a cell for use in a one- step cell-based assay for an extracellular ligand (e.g., I FNα) that initiates a ligand-specific signal at the nucleus of the cell and for neutralizing antibodies against the extracellular ligand. The cell-based one-step assay allows both the extracellular ligand concentration and the neutralizing antibody titer to be quantified in a single sample (e.g., serum) without the need for sample dilution and addition of exogenous extracellular ligand.

CELL, METHOD AND KIT FOR CONDUCTING AN ASSAY FOR NEUTRALIZING ANTIBODIES

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of priority from U.S. provisional application no. 61/033,621, filed March 4, 2008, the entire content of which is herein incorporated by reference.

BACKGROUND OF THE INVENTION

Field of the Invention

[0002] The present invention relates to a reporter gene assay, and to the cells and kit for conducting such an assay.

Description of the Related Art

[0003] Cell surface proteins permit intracellular transduction of extracellular signals. Cell surface proteins provide eukaryotic, as well as prokaryotic, cells a means to detect extracellular signals and transduce such signals intracellularly in a manner that ultimately results in a cellular response or a concerted tissue or organ response. Cell surface proteins, by intracellularly transmitting information regarding the extracellular environment via specific intracellular pathways induce an appropriate response to a particular stimulus. The response may be immediate and transient, slow and sustained, or some mixture thereof. By virtue of an array of varied membrane surface proteins, eukaryotic cells are exquisitely sensitive to their environment.

[0004] Extracellular signal molecules, such as cytokines, growth factors, certain hormones, vasodilators and neurotransmitters, exert their effects, at least in part, via interaction with cell surface proteins. For example, some extracellular signal molecules cause changes in transcription of

target gene via changes in the levels of secondary messengers,
such as cAMP. Other signals indirectly alter gene expression by
activating the expression of genes, such as immediate-early genes
that encode regulatory proteins, which in turn activate
expression of other genes that encode transcriptional regulatory
proteins. Other extracellular signal molecules cause activation
of latent cytoplasmic signal transducers and activators of
transcription (STAT) protein that enhance the transcription of
specific sets of genes.

[0005] Cell surface receptors and ion channels are among the
cell surface proteins that respond to extracellular signals and
initiate the events that lead to this varied gene expression and
response. Ion channels and cell surface-localized receptors are
ubiquitous and physiologically important cell surface membrane
proteins. They play a central role in regulating intracellular
levels of various ions and chemicals, many of which are important
for cell viability and function.

Cell Surface Receptors

[0006] Cell surface-localized receptors are membrane spanning
proteins that bind extracellular signalling molecules or detect
changes in the extracellular environment and transmit the signal
via signal transduction pathways to effect a cellular response.
Cell surface receptors bind circulating signal molecules, such as
cytokines, growth factors and hormones, etc., as the initiating
step in the activation of numerous intracellular pathways.
Receptors are classified on a structural basis or on the basis of
the particular type of pathway that is induced. Among these
classes of receptors are classes of cytokine receptors which
include those that bind growth factors and have intrinsic
tyrosine kinase activity, such as the heparin binding growth
factor (HBGF) receptors, the immunoglobulin receptor superfamily,
the hematopoietin/cytokine receptor superfamily, the nerve-growth

factor receptor superfamily, other receptor tyrosine or serine kinases, and those that couple to effector proteins through guanine nucleotide binding regulatory proteins, which are referred to as G protein coupled receptors and G proteins, respectively.

[0007] Cytokines are intercellular messengers which coordinate communication between cells within a particular tissue, for example, antibody and T cell immune system interactions, and serve to modulate or modify the biological response. They are pleiotropic and have a broad spectrum of biological effects on more than one type of cell or tissue. The receptors for cytokines are broadly grouped into two classes, where the Class I cytokine receptors include receptors that bind various interleukins (IL-2, IL-3, IL-4, IL-6, IL-7, IL-9, IL-11, IL-12, IL-15), erythropoietin (EPO), growth hormone (GH), granulocyte colony stimulating factor (G-CSF), granulocyte macrophage colony stimulating factor (GM-CSF), leukemia inhibitory factor (LIF), and ciliary neurotrophic factor (CNTF), TNFα, TGFβ, Fas-ligand, and the Class II cytokine receptors include receptors that bind interferon (IFN) α/β, IFNγ, and IL-10.

Interferon receptors

[0008] Human interferons (IFNs) are a family of homologous helical cytokines composed of three distinct classes: type I, type II, and type III based on nucleotide and amino acid sequence homology. Human Type I IFNs consist of IFN-α, IFN-β, IFN-ε, IFN-κ, and IFN-ω. Human IFN-α includes a group of closely related proteins encoded by at least 12 functional IFN-α genes. IFN-β, IFN-ε, IFN-κ, and IFN-ω, are encoded by single more distantly related genes. Type II IFN, or IFNγ, is encoded by an unrelated gene and binds to a distinct cell surface receptor (De Maeyer et al., 1988; Pestka et al., 1987 and Diaz et al., 1993). Recently,

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a novel group of interferons designated IFN-λ or type III IFNs
has been described. The group has three members IFN-λ1, IFN-λ2,
and IFN-λ3 also termed interleukin-29 (IL-29)(λ1), and IL-28A/B
(λ2/3). (Sheppard et al., 2003; and Ank et al., 2006).

[0009] Type I IFNs bind to a common receptor, as shown by
their ability to cross-compete for receptor binding (Pestka et
al., 1987; Branca et al., 1981; and Merlin et al., 1985). The
Type 1 interferon receptor has the largest number of natural
ligands, some 14 in all, of all known cytokine receptors.
Binding of interferons to their cell surface receptor represents
the initial and probably most specific step in the IFN signaling
pathway.

[0010] The Type I IFN receptor is composed of two
transmembrane glycoproteins, IFNAR1 and IFNAR2 (Uze et al., 1990;
Novick et al., 1994; Lutfalla et al., 1995; Domanski et al.,
1995), which are rapidly tyrosine-phosphorylated following IFN
binding (Platanias et al., 1994; Constantinescu et al., 1994; and
Abramovich et al., 1994). Both subunits belong to the class II
cytokine receptor superfamily (Bazan et al., 1990 and Thoreau et
al., 1990) and are required for high affinity ligand binding and
the establishment of biological activity (Langer et al., 1996 and
Domanski et al., 1996). Class II cytokine receptors are
distinguished from Class I receptors on the basis of the pattern
of the conserved pairs of cysteine residues that are thought to
form disulfide bonds.

[0011] The Type II IFN (IFN γ) receptor is composed of two
transmembrane glycoproteins, IFNGR1 and IFNGR2 that are
preassembled at the cell surface. Binding of IFN γ to its
receptor activates the tyrosine kinases Jak1 and Jak2 resulting
in tyrosine-phosphorylation and formation of a Stat1 homodimer.
The activated Stat1 homodimer is then translocated to the nucleus

where it binds to the GAS (Gamma Activated Sequence) resulting in transcriptional activation of IFN γ activated genes.

[0012] Type III interferons bind to a unique receptor comprising the IL-28Rα, which is specific for chain the IFN-λs, and the IL-10Rβ chain which is also part of the receptors for IL-10, IL-22, and IL-26 (Ank et al, 2006).

[0013] In contrast to other cytokine receptors, particularly the IFN-γ receptor, neither IFNAR1 nor IFNAR2 alone bind to IFNα or IFNβ with an affinity comparable to the heterodimer. Despite the fact that IFNAR2 plays a prominent role in ligand binding, IFNAR1 contributes to IFN binding by increasing the affinity of the receptor complex (4-10 fold) relative to that of IFNAR2 alone. IFNAR1 also modulates the specificity of ligand binding relative to that observed with IFNAR2 alone (Cohen et al., 1995; Russell-Harde et al., 1995; Cutrone et al., 1997; and Cook et al., 1996). IFNAR1 has a larger extracellular domain than most other class II cytokine receptors, composed of 4 immunoglobulin-like subdomains separated by di- or tri-proline motifs which can be divided into two tandem repeats (Novick et al., 1994; Lutfalla et al., 1992; and Uzé et al., 1995).

[0014] Human, murine and bovine IFNAR1 have been cloned and expressed in human and murine cells. Studies performed with transfected cells show that IFNAR1 plays a central role in ligand binding, cellular responses to IFNs and in the induction of the biological activities of the Type I interferons (Novick et al., 1994; Abramovich et al., 1994; Uzé et al., 1992; Mouchel-Vielh et al., 1992; Lim et al., 1993; Cleary et al., 1994; Constantinescu et al., 1995; Hwang et al., 1995; Vandenbroek et al., 1995; and Colamonici et al., 1994). The IFN receptor also determines the high degree of species specificity characteristic of the IFNs. Thus, transfection of mouse cells with IFNAR1 and IFNAR2 renders mouse cells sensitive to human type I IFNs since both human and

mouse cells share a common signaling pathway and common IFN responsive elements in the promoter regions of IFN regulated genes. Furthermore, the intracellular domain of IFNAR1 has been shown to play a key role in the transduction of the signal initiated at the cell surface to the nucleus following binding of Type I interferons (Basu et al., 1998). Targeted disruption of the IFNAR1 gene results in the loss of the antiviral response to Type I IFNs demonstrating that this receptor polypeptide is an essential component of the receptor complex and that both IFNAR1 and IFNAR2 subunits are required for IFNα and IFNβ signaling (Vandenbroek et al., 1995; Muller et al., 1994; Fiette et al., 1995; Steinhoff et al., 1995; and van den Broek et al., 1995).

[0015] Binding of type I interferon to the receptor complex activates two Janus kinases, Tyk2 and JAK1, which mediate the tyrosine phosphorylation and activation of two latent cytoplasmic transcription factors STAT1 and STAT2 which form a complex (ISGF3) with a p48 DNA binding protein, interferon responsive protein 9 (IRF 9), which is translocated to the nucleus to promote specific gene transcription (Fu et al., 1992; Schindler et al., 1992; Darnell et al., 1994; Ihle et al, 1995; and Taniguchi, 1995). Both Tyk2 and STAT2 are constitutively associated with the membrane proximal region of the IFNAR1 chain, while JAK1 and STAT1 are physically associated with IFNAR2 and all four factors are rapidly activated during IFNα stimulation (Lutfalla et al., 1995; Bazan, 1990; Basu et al., 1998; Barbieri et al., 1994; Velazquez et al., 1995; Uddin et al., 1995; Yan et al., 1996(a) and 1996(b).

[0016] Binding of type III IFNs to their cell-surface receptor also activates the ISGF3 complex suggesting that type III IFNs also activate a number of genes in common with type I IFNs (Ank et al., 2006).

Pattern Recognition Receptors

[0017] Key populations of cells including dendritic cells
(DCs) distributed throughout the peripheral tissues act as
sentinels capable of recognizing infectious agents through
pattern-recognition receptors (PRR). These include the Toll-like
receptor (TLR) family of cell surface and endosomal membrane
receptors (Uematsu and Akira, 2007) and the retinoic acid-
inducible gene I (RIG-I)-like cytosoloic receptor proteins RIG-I,
MDA5, and LGP2 (Yoneyama and Fujita, 2007). Thirteen members of
the TLR family have been identified in mammals (Uematsu and
Akira, 2007). Each TLR mediates a distinctive response in
association with different combinations of four Toll/IL-1
receptor (TIR) domain-containing adaptor proteins (MyD88, TRIF,
TIRAP/MAL, and TRAM). All the TLRs except TLR3 interact with
MyD88. TLR3, which recognizes single-stranded or double-stranded
viral RNA, is localized in the endosomes of myeloid DCs and
requires acidification of vesicles for activation. TLR3 signals
via TRIF and activates TBK1/IKKe which phosphorylates the
interferon regulatory factor 3 (IRF3) and NFkB, resulting in
production of IFNβ (Hemmi et al, 2004, Perry et al., 2004). The
RIG-I-like receptor proteins are DExD/H box RNA helicases two of
which, RIG-I and MDA5, carry caspase activation and recruitment
domain (CARD)-like motifs at the N-terminus (Yoneyama and Fujita,
2007). The CARD domain interacts with IPS-1 resulting in
activation of IRF3 and NFkB and production of IFNβ. Thus,
activation of PRRs leads to the production of pro-inflammatory
cytokines including type I IFNs and activation of the innate
immune response.

[0018] Dendritic cells signal principally through TLRs while
RIG-I-like receptors predominate in other cell types. Two major
DC sub-sets can be distinguished in man, CD11c(+) monocyte
derived myeloid DCs, present in most tissues, and CD11c(-)

plasmacytoid DCs (pDCs), present principally in lymph nodes. Plasmacytoid DCs are the principal producers of type I IFNs in response to viruses (Steinmann and Hemmi, 2006). Plasmacytoid DCs express high levels of TLR7/8 and TLR9 that recognize single-stranded RNA (ssRNA) and CpG DNA respectively (Diebold et al., 2004, Heli et al., 2004). Hemmi et al., 2000). Activation of both TLR7/8 and TLR9 leads to the formation of a complex with MyD88 and phosphorylation of IRF7 and production of high levels of type I IFNs (Uematsu and Akira, 2007).

TNF receptors

[0019] Tumor necrosis factor alpha (TNF-α) is a multifunctional cytokine that exerts pleiotropic effects on different cell types. TNF-α is synthesized as pro-TNF, a 26 kDa membrane bound protein, which is released upon cleavage of its pro domain by TNF-converting enzyme (TACE) to yield a 17 kDa protein consisting of 157 amino acids that exists as a homotrimer in solution. TNF-α bind to two distinct receptors TNFR-1 (p55) and TNFR2 (p75). TNFR1 contains a death domain (absent from TNFR2) which is involved in the induction of apoptosis. Binding of the TNF-α homotrimer to TNFR-1 results in trimerization of TNFR-1 and the silencer of death domain (SODD) is released. The TNFR-associated death domain (TRADD) binds to the death domain of TNFR-1 and recruits the adaptor proteins, receptor interacting protein (RIP), TNFR-associated factor 2 (TRAF-2), and the Fas-associated death domain (FADD). TNFR-1 signals apoptosis, by FADD binding pro-caspase-8 the activation of which leads to induction of a protease cascade resulting in apoptosis. TNFR-1 signals survival by recruitment of TRAF-2 which inhibits apoptosis via the cytoplasmic inhibitor of apoptosis protein (cIAP). One of the principal signaling pathways triggered by recruitment of TRAF-2 and RIP to the TNFR-1 receptor complex is the NF-κB pathway which

transduces a signal to the nucleus culminating in transcriptional activation of a number of TNF target genes (Schwamborn et al., 2003). NF-κB is a ubiquitous transcription factor induced by a number of cytokines (including IFNγ, IL2, IL5 and IFNα2). NF-κB is involved in the regulation of numerous genes involved in processes including, the inflammatory response, apoptosis, cancer, neuronal survival, and innate immunity. Activation of NF-κB is controlled principally at the posttranscriptional level by degradation of the inhibitory subunit IκB of the p55/p65/IκB complex present in the cytoplasm. Activating stimuli such as TNFα activate a kinase complex composed of two IκB-specific kinases (IKKα and IKKβ) and a modulatory subunit (NEMO or IKKγ). This leads to phosphorylation of the inhibitory subunit, which is then ubiquitinylated and degraded via the proteasome. This triggers translocation of NF-κB into the nucleus, where it initiates transcription by binding to regulatory sequences (NF-κB recognition/binding sequences) present in the promoter region of NF-κB target genes.

G-coupled receptors

[0020] The G protein transmembrane signaling pathways consist of three proteins: receptors, G proteins and effectors. G proteins, which are the intermediaries in transmembrane signaling pathways, are heterodimers and consist of α, β and γ subunits. Among the members of a family of G proteins the α subunits differ. Functions of G proteins are regulated by the cyclic association of GTP with the α subunit followed by hydrolysis of GTP to GDP and dissociation of GDP.

[0021] G protein coupled receptors are a diverse class of receptors that mediate signal transduction by binding to G proteins. Signal transduction is initiated via ligand binding to the cell membrane receptor, which stimulates binding of the

receptor to the G protein. The receptor G protein interaction
releases GDP, which is specifically bound to the G protein, and
permits the binding of GTP, which activates the G protein.
Activated G protein dissociates from the receptor and activates
the effector protein, which regulates the intracellular levels of
specific second messengers. Examples of such effector proteins
include adenyl cyclase, guanyl cyclase, phospholipase C, and
others.

Growth Factors and Growth Factor Receptors

[0022] Polypeptide growth factors are modulators of cell
proliferation and differentiation whose biological functions are
mediated by the interaction of the growth factor with cell
surface receptors and subsequent alterations in gene expression.
Growth factors bind to specific receptors and appear to induce
tyrosine phosphorylation and c-fos mRNA synthesis. In addition,
at least some growth factors, such as platelet-derived growth
factor (Yeh et al., 1987) and heparin-binding growth factor-2 or
basic fibroblast growth factor (Bouche et al., 1987), are
translocated to the nucleus.

[0023] Activation of growth factor receptors by interaction
with specific growth factors or with agents such as phorbol
mistric acetate (PMA) activates protein kinase C, which is a
family of phospholipid- and calcium-activated protein kinases.
This activation results in the transcription of an array of
proto-oncogene transcription factor encoding genes, including c-
fos, c-myc and c-jun, proteases, protease inhibitors, including
collagenase type I and plasminogen activator inhibitor, and
adhesion molecules, including intercellular adhesion molecule I.
Protein kinase C activation antagonizes growth factor activity by
the rapid phosphorylation of growth factor receptors, which
thereby decreases tyrosine kinase activity. Growth factors and
other mitogens that induce cell proliferation and cell growth are

believed to play a role in tumor growth, which often carry identifiable cell surface receptors specific for growth factors and other extracellular signals.

[0024] The interaction of nerve growth factor (NGF) with its receptor is typical of the array of responses such an extracellular signal induces. NGF is a polypeptide growth hormone that is necessary for differentiation and growth of the neural crest-derived sensory neuron. NGF binds to its specific cell surface receptor and is retrogradely transported to the cell body (Changelian et al., 1989). This initiates a cascade of intracellular events, culminating in a differentiated phenotype. PC12 cells, which are a rat pheochromocytoma cell line, are used as a model for the study of NGF-mediated differentiation. When treated with NGF, PC12 cells change from replicating adrenal-chromaffin-like cells to nonreplicating, electrically excitable sympathetic-neuron-like cells.

[0025] Concomitant with the phenotypic changes, there is induction and expression of specific genes. Binding of NGF to PC12 cells induces the immediate and rapid expression of certain genes, including the c-fos, NGF1-A and NGF1-B genes, which are referred to as early genes. Such early genes are believed to encode transcriptional regulators. The NGF-1A gene product contains tandemly repeated "zinc finger" domains that are characteristic of DNA-binding proteins, and the NGF1-B protein is homologous to members of the glucocorticoid receptor family and, thus, may function as a ligand-dependent modulator of transcription. The c-fos gene product, FOS appears to function as a transcriptional regulatory molecule.

The c-fos Gene and Related Genes

[0026] As discussed above, induction of expression of the c-fos gene is an event that is common to a number of response

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pathways that are initiated by the activity of a variety of cell surface proteins.

[0027] The c-fos gene product, FOS, associates with the transcription activator JUN, which is the product of the c-jun gene, to form a complex that forms a transcription activation complex, AP-1. Transcription of both c-fos and c-jun is induced rapidly and transiently following stimulation. The induced mRNAs accumulate for 1-2 hours in the cytoplasm where the FOS and JUN proteins, which are short-lived, are translated and then translocated to the nucleus to form a heterodimeric protein complex that binds to the DNA regulatory element, the AP-1 binding site.

[0028] The c-fos and c-jun genes are members of gene families that encode proteins that participate in the formation of heterodimeric complexes that interact with AP-1 binding sites. Transcription factor AP-1 is composed of several protein complexes whose concentrations change upon cell stimulation. These complexes specifically interact with a seven-base core nucleotide sequence motif, that is known to be a relatively common constituent of both positive and negative transcriptional regulatory elements and that is required for both basal and induced levels of gene expression.

[0029] The gene products, FOS and JUN cooperate in the regulation of target genes that underlie many cellular and adaptive responses to the environment. They are involved in a number of neurophysiological processes.

[0030] Thus, c-fos induction involves distinct second messenger pathways that act via separate regulatory elements and that differentially modify, the resulting gene product, FOS, which in turn interacts in different ways with differentially modified JUN protein. Therefore, a multitude of extracellular events induce expression of a small number of inducible proteins

that form an array of protein complexes that can differentially bind to DNA regulatory elements that contain AP-1 binding sites. Therefore, numerous cell surface proteins can act via overlapping transduction pathways and transduce extracellular signals that ultimately induce a variety of responses.

[0031] There are many assays that may rely on *in vivo* activity in a living cell line. One example is a cell line having an Interferon Stimulatory Response Element (ISRE) connected to a luciferase gene, or another reporter gene, so that when the cell line is subjected to the presence of interferon as an extracellular signal, the signal transduction activity of endogenous interferon cell surface receptors produces a signal that activates the ISRE, which then causes transcription of the luciferase gene. Thus, the activity of luciferase in creating light can be measured and is related to the amount of interferon which is present in the sample, and which is proportional to the amount of interferon over a particular range (Lallemand et al., 1996).

[0032] Lleonart et al. (1990) described a reporter gene assay for Type I interferon based on monkey Vero cells transfected with Type I interferon inducible mouse Mx promoter linked to the human growth hormone (hGH) gene as the reporter gene. This Type I interferon assay was developed further by transfecting monkey Vero cells with a plasmid carrying the luciferase reporter gene under the control of the Type I interferon inducible mouse Mx1 promoter (Canosi et al., 1996).

[0033] A further type of interferon reporter gene assay was developed by Hammerling et al. (1998) who used a human glioblastoma cell line transfected with a reporter gene construct of glial fibrillary acidic protein (GFAP) promoter and an *E. coli* β-galactosidase (lacZ) reporter gene. In this particular assay, it is the reduction/inhibition of β-galactosidase expression by

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either human Type I or Type II interferon in a selective and dose dependent manner that is measured.

[0034] Therapeutic proteins and in particular recombinant biopharmaceuticals represent an important and growing class of therapeutic agents. The safety and efficacy of therapeutic proteins can be severely impaired, however, by their immunogenicity. In addition to affecting pharmacokinetics, pharmacodynamics, bioavailability, and efficacy, anti-drug antibodies can also cause immune complex disease, allergic reactions and in some cases severe autoimmune reactions (Casadevall et al., 2002; and Neumann et al., 2000). It is widely accepted that injection of foreign proteins into humans can elicit an immune reaction leading to the production of binding and in some cases neutralizing antibodies (NAbs). Neutralizing antibodies block the biological activity of a biopharmaceutical either by binding directly to an epitope within or close to the active site of the protein or to an epitope that prevents binding of the drug to a cell surface receptor. It is becoming increasingly apparent, however, that repeated injection of recombinant homologues of authentic human proteins, such as interferon beta (IFNβ) or erythropoietin especially when aggregated or partially denatured, can result in a break in tolerance to self-antigens leading to the production of NAbs (Schellekens, 2008). This is of particular concern in the treatment chronic diseases such as certain forms of cancer and autoimmune disease. This can result in the failure of the patient to respond to therapy and may even prove to be life threatening in the case of NAbs that cross react with an essential non redundant endogenous protein such as erythropoietin (Casadevall et al., 2002) or megakaryocyte growth and developmrnt factor, MGDF (Neumann et al., 2000). Assessment of immunogenicity is therefore an important component of the evaluation of drug safety

in both pre-clinical and clinical studies and is a prerequisite for the development of less immunogenic and safer biopharmaceuticals. Monitoring patients for the presence of NAbs to biopharmaceuticals and the correlation of immunogenicity with clinical data is key for determining the safety of treatment and for the interpretation of clinical data.

[0035] The results of a number of large randomized clinical studies have shown that interferon beta (IFNβ) reduces the frequency and severity of clinical relapses, slows disease progression, and improves the quality of life in patients with relapsing-remitting multiple sclerosis (RRMS) (Clerico et al., 2007; and McCormick et al., 2004). Repeated treatment with recombinant IFNβ, however, can cause a break in immune tolerance to self-antigens in some patients, resulting in the production of neutralizing antibodies (NAb) to the recombinant protein homologue (Hartung et al., 2007; Noronha, 2007; and Namaka et al., 2006). Appearance of NAbs is associated with both reduced pharmacodynamics (induction of IFNβ responsive gene products; Deisenhammer et al., 2004), and a reduced clinical response determined by either magnetic resonance imaging (MRI) or disease progression (Hartung et al., 2007; Noronha, 2007; and Namaka et al., 2006). The frequency and titers of anti-IFNβ antibodies vary as a function of the type of IFNβ preparation used to treat the patient, as well as the frequency and route of administration. Although direct comparisons among many of the studies is difficult due to the use of different neutralization assays and standards, comparative studies have shown that IFNβ-1b is more immunogenic than IFNβ-1a (Bertolotto et al., 2002) possibly due to the lower specific activity of IFNβ-1b and hence the higher protein mass injected (Antonetti et al., 2002). Amino acid differences, lack of glycosylation of recombinant IFNβ-1b compared with the native protein or currently licensed forms of

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IFNβ-1a, or formulation characteristics may also contribute to the immunogenicity of IFNβ-1b (Giovannoni, 2004).

[0036] Two principal approaches are used to quantify anti-drug NAbs: the constant antigen method in which a constant amount of drug (e.g., IFN) is mixed with serial dilutions of serum, and the constant antibody method in which a fixed dilution of serum is mixed with varying concentration of drug. In both cases the titration end-point is usually taken as the median of the maximum and minimum values of the dose-response curve which is defined as one laboratory unit (LU). NAb titer is usually determined using the Kawade method of calculation that determines the serum dilution that reduces drug activity from 10 to 1 LU/ml (Grossberg et al., 2001a and 2001b). Residual drug activity is usually determined using a cell-based assay. Such assays are notoriously difficult to standardize and are at best semi-quantative due to the absence of appropriate standards for anti-drug NAbs.

[0037] Current methods for detecting the presence of neutralizing antibodies to IFNα or IFNβ are based on the inhibition of IFN activity determined using either antiviral bioassays (Grossberg et al., 2001a and 2001b) or induction of an IFN induced protein (Deisenhammer et al., 2004). Bioassays based on the ability of IFNs to inhibit virus replication 1) are imprecise and require skilled operators in order to obtain reproducible results, 2) only two fold or greater differences can be detected, 3) give variable results, and 4) take several days to complete. Measurement of the induction of an IFN-induced antiviral protein such as MxA requires use of cell lines or peripheral blood, and subsequent evaluation of protein levels by ELISA or measurement of MxA mRNA levels (Deisenhammer et al., 2004).

[0038] A highly sensitive and reproducible method for quantifying type I IFN activity has recently been developed,

based on human pro-monocytic U937 cells, transfected with the
firefly luciferase reporter-gene controlled by an IFN responsive
chimeric promoter (Lallemand et al., 2008), which allows IFN
activity to be determined selectively with a high degree of
precision, and within a few hours. Treatment of these cells
(PIL5) with the anti-mitotic drug vinblastin allows cells to be
stored frozen for prolonged periods without loss of IFN
sensitivity or the need for cell cultivation and avoids assay
variation associated with cell proliferation (Lallemand et al.,
2008). Although this assay overcomes many of the limitations of
conventional cell-based neutralization assays or other reporter-
gene assays (Lam et al., 2008) for the determination of IFN
activity or for the quantification of anti-IFN Nabs, it remains
relatively labor intensive. Thus, quantification of anti-IFN NAbs
requires serial dilutions of the serum sample to be tested, a
simultaneous IFN dose-response curve, and positive and negative
controls to be included in each assay as well as the availability
of reference reagents.

[0039] Bioassays for TNF-α are based on the ability of TNFα to
induce apoptosis in susceptible cells such as mouse L929 cells,
usually in the presence of actinomycin D. Such assays are
imprecise and difficult to use for the determine of NAbs to TNFα
antagonists such as Infliximab, Adalimumab or etanercept (Meager
A, 2006).

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admission that such document is pertinent prior art, or
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SUMMARY OF THE INVENTION

[0041] The present invention provides a cell for use in assaying for antibodies against an extracellular ligand that initiates a ligand-specific signal at the nucleus of the cell. The cell according to the present invention contains (1) a first DNA construct having a sequence that includes a first set of one or more transcription control elements, which is inducible by the ligand, and also encodes a first measurable tag (first reporter gene product), whose expression is driven by the first set of one or more transcription control elements when induced by the presence of the ligand and (2) a second DNA construct having a sequence that includes a second set of one or more transcription control elements different from the first, a DNA segment encoding a second measurable tag (second reporter gene product) whose expression is driven by the second set of one or more transcription control elements, and on a separate cistron a segment encoding a ligand, whose expression is also driven by the second set of one or more transcription control elements.

[0042] The present invention also provides a kit containing a plurality of the cell according to the present invention, which kit is used for determining in a sample the level of an extracellular ligand that initiates a ligand-specific signal at the nucleus of the cell or of a neutralizing antibody against either the extracellular ligand or an antagonist of the extracellular ligand. Additionally, the present invention further provides a method for determining such a level in a sample.

BRIEF DESCRIPTION OF THE DRAWINGS

[0043] Figure 1 is a schematic flow diagram of the steps performed in the recent "iLite" cell-based assay and in the "NanoLite" one-step assay according to the present invention.

[0044] Figure 2 is a schematic illustration of an embodiment in which the ligand (cytokine) and the Renilla luciferase (RL) reporter gene are expressed from the same promoter. The Renilla luciferase remains in the cell while the expressed cytokine is secreted, interacting with a receptor for the cytokine ligand which initiates signal transduction to drive expression of firefly luciferase (FL) from a cytokine ligand-responsive promoter. The presence of neutralizing antibodies (NAb) for the cytokine prevents the cytokine from interacting with its cell surface receptor and results in a corresponding reduction in the activity of the cytokine (as determined by the relative activity of the cytokine-responsive firefly luciferase reporter, FL1/RL (control) > FL2/RL).

[0045] Figure 3 is a schematic illustration of two separate constructs, an ISRE/SV40 minimal promoter driving the expression of the firefly luciferase reporter gene and a cytomegalovirus (CMV) promoter driving the expression of both interferon α2a (IFNα2a) and the Renilla luciferase reporter gene.

[0046] Figure 4 is a schematic illustration of two separate constructs, an ISRE/SV40 minimal promoter driving the expression of firefly luciferase gene reporter and the minimal immediate early promoter of cytomegalovirus (CMV) driving the expression of both interferon α2a (IFNα2a) and Renilla luciferase gene reporter, but with the order of IFNα2a and Renilla luciferase expression reversed from that shown in Fig. 3.

[0047] Figure 5 is a schematic illustrations of two separate constructs, an ISRE/SV40 minimal promoter driving expression of the firefly luciferase reporter gene, and a tetracycline (Tet)-responsive element (TRE)/the CMV immediate early minimal promoter driving the expression of the Renilla luciferase reporter gene and IFNα2a (Tet-On). In the absence of tetracycline or doxycycline, the reverse Tet repressor (rTetR) binds to the TRE,

silencing transcription. Also depicted is the binding of the reverse tetracycline transactivator (rtTA) to the TRE following addition of tetracycline or doxycycline leading to activation of transcription.

[0048] Figure 6 is a schematic illustrations of two separate constructs; an ISRE/SV40 minimal promoter driving expression of the firefly luciferase reporter gene, and a tetracycline (Tet) responsive element (TRE)/CMV immediate early minimal promoter driving the expression of the Renilla luciferase reporter gene and IFNβ (Tet-On).

[0049] Figure 7 is a schematic illustration of two separate constructs for a gene reporter assay for anti-TNFα NAb, a 5x tandem repeat of the canonical NFκB recognition site/SV40 minimal promoter driving the expression of firefly luciferase gene reporter, and a Tet-responsive element (TRE)/CMV immediate early minimal promoter driving the expression of both TNFα and the Renilla luciferase gene reporter (Tet-On).

[0050] Figure 8 is a schematic illustration of three separate constructs for a gene reporter assay for anti-TNFα antagonist NAbs: a 5x tandem repeat of the canonical NFκB recognition site/SV40 minimal promoter driving the expression of Renilla luciferase reporter gene; a Tet-responsive element (TRE)/CMV immediate early minimal promoter driving the expression of both TNFα and the CBRLuc reporter gene (Tet-On); and a chimeric mifepristone inducible promoter driving transcription of the TNFα antagonist and the CBG68Luc reporter gene. The mifepristone-inducible chimeric promoter consists of the GAL4-UAS and the TATA sequence from the Adenovirus E1b minimal promoter that is transcriptionally silent in the absence of activation. The Gal4 DNA binding domain which binds the regulatory protein to the GAL4-E1b promoter and the truncated human progesterone receptor ligand binding domain (hPR-LBD) which undergoes conformational

change when it binds the progesterone antagonist mifepristone are
expressed from a minimal TK promoter on the vector. Thus, upon
addition of mifepristone, the antagonist binds to the hPR-LBD
region of the vector causing a conformational change in the
regulatory protein resulting in transcription of the TNFα
antagonist and the CBG68Luc reporter gene.

[0051] Figure 9 is a schematic illustration of two separate
constructs for a gene reporter assay for anti-erythropoietin
(EPO) NAb, a 5x tandem repeat of the signal transducer and
activator of transcription #5 (STAT5)/SV40 minimal promoter
driving the expression of the firefly luciferase reporter gene,
and a Tet-responsive element (TRE)/CMV immediate early minimal
promoter driving the expression of both EPO and the Renilla
luciferase reporter gene (Tet-On).

[0052] Figure 10 shows a schematic representation of a
luciferase reporter gene construct where luciferase expression is
under the control of a chimeric promoter containing an interferon
sensitive response element (ISRE) from the ISG15 gene and a
minimal SV40 promoter.

[0053] Figure 11 shows a schematic representation of an
enhanced green fluorescent protein (EGFP-1) reporter gene
construct where EGFP-1 expression is under the control of a
chimeric promoter containing an ISRE from the ISG15 gene and a
minimal SV40 promoter.

[0054] Figure 12 is a graph showing the titration curve of
anti-IFNα neutralizing antibodies using the method of the present
invention.

[0055] Figure 13 is a graph of the relative luminescence units
(RLU) observed in assaying for neutralizing antibodies to IFNα
according to the method of the present invention in cells
untransfected or transfected with the pIRES IFNA2 RL as the
second DNA molecule.

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[0056] Figure 14 is a schematic illustration of two separate constructs for the One-step assay in the Example herein below. The abbreviations are as follows: ISRE: Interferon Sensitive Response Element; SV40 Min. Prom: SV40 minimal promoter; Intron: Intron from the human β-globulin gene; SV40 Poly A: SV40 polyadenylation site; Firefly Luciferase: Coding region of the firefly luciferase gene; TRE: tetracycline responsive element; rTetR: Reverse tetracycline repressor; Tetra: Tetracycline; CMV Mini: CMV minimum promoter; IFNa2a: Signal peptide and coding region of the human interferon alpha2a gene; IRES: Internal ribosomal entry site; and Renilla Luciferase: Coding region of the Renilla luciferase gene

[0057] Figures 15A and 15B are graphs showing the effect of doxycycline centration on the expression of Firefly and Renilla luciferase activity in the One-step assay. One-step assay cells (PIL5C2.2) were treated with varying concentrations of doxycycline as described in the Materials and Methods and incubated overnight in duplicate with doxycycline alone or together with a 1/1,000 dilution of a polyclonal anti-human IFNα antibody as indicated in the figure. The activities of both Firefly and Renilla luciferase determined sequentially in the same well using the Dual-Glo luciferase assay system as described in the Materials and Methods. The cells were then lysed by the addition of 75 μl/well of the Firefly luciferase substrate containing reagent, and FireFly luciferase activity was determined as described in the Materials and Methods. Renilla luciferase activity was then determined following addition in the same well of 50μl the Renilla luciferase substrate. The neutralizing activity of the NAb sample was then determined from the ratio of the activity of Firefly luciferase of the NAb containing sample (FL2) normalized relative to Renilla luciferase

expression (RL2) and Firefly luciferase activity of the control sample (FL1) normalized relative to Renilla luciferase expression of the control sample (RL1): (FL2/RL2)/(FL1/RL1). In Figs. 15A and 15B, RL represents Renilla luciferase and luc represents Firefly luciferase.

[0058] Figures 16A and 16B are graphs showing the effect of doxycycline concentration on the expression of Firefly and Renilla luciferase activity in the One-step assay. One-step assay cells (PIL5C2.2) were treated with varying concentrations of doxycycline as indicated in the figure and incubated overnight in duplicate with doxycycline alone or together with a 1/10 or 1/100 dilution of the human serum indicated in the figure. The activities of both Firefly and Renilla luciferase determined sequentially in the same well using the Dual-Glo luciferase assay system as described in the Materials and Methods. The cells were then lysed by the addition of 75 µl/well of the Firefly luciferase substrate containing reagent, and FireFly luciferase activity was determined as described in the Materials and Methods. Renilla luciferase activity was then determined following addition in the same well of 50µl of the Renilla luciferase substrate. The neutralizing activity of the NAb sample was then determined from the ratio of the activity of Firefly luciferase of the NAb containing sample (FL2) normalized relative to Renilla luciferase expression (RL2) and Firefly luciferase activity of the control sample (FL1) normalized relative to Renilla luciferase expression of the control sample (RL1): (FL2/RL2)/(FL1/RL1).

[0059] Figure 17 is a graph showing the effect of doxycycline concentration on the expression of Firefly and Renilla luciferase activity in the One-step assay. One-step assay cells (PIL5C2.2) were treated with varying concentrations of doxycycline as described in the Materials and Methods and incubated overnight in

duplicate. The activities of both Firefly and Renilla luciferase determined sequentially in the same well using the Dual-Glo luciferase assay system as described in the Materials and Methods. The cells were then lysed by the addition of 75 µl/well of the Firefly luciferase substrate containing reagent, and FireFly luciferase activity was determined as described in the Materials and Methods. Renilla luciferase activity was then determined following addition in the same well of 50µl of the Renilla luciferase substrate.

[0060] Figure 18 is a graph showing the effect of varying concentration of doxycycline on the neutralization activities of human sera in the One-step assay. One-step assay cells (PIL5C2.2) were treated with varying concentrations of doxycycline as indicated in the figure and incubated overnight in duplicate with doxycycline alone or together with a 1:20 dilution of the human serum indicated in the figure. The activities of both Firefly and Renilla luciferase determined sequentially in the same well using the Dual-Glo luciferase assay system as described in the Materials and Methods. The cells were then lysed by the addition of 75 µl/well of the Firefly luciferase substrate containing reagent, and FireFly luciferase activity was determined as described in the Materials and Methods. Renilla luciferase activity was then determined following addition in the same well of 50µl of the Renilla luciferase substrate. The neutralizing activity of the NAb sample was then determined from the ratio of the activity of Firefly luciferase of the NAb containing sample (FL2) normalized relative to Renilla luciferase expression (RL2) and Firefly luciferase activity of the control sample (FL1) normalized relative to Renilla luciferase expression of the control sample (RL1): (FL2/RL2)/(FL1/RL1).

[0061] Figures 19A and 19B are graphs showing NAb quantification using a constant IFN concentration (100 IU/ml)

versus varying serum concentrations (Fig. 19A) or varying IFN centration versus constant serum concentration (1/100) (Fig. 19B). Serial dilutions of human serum were incubated in duplicate for 1 hour at 37°C followed by 2 hours at 4°C with a constant quantity (10 LU/ml) of a IFNα2 as described in the Materials and Methods (Figure 19A), or a constant dilution of serum (1:100) was incubated under the same conditions with serial dilutions of IFN (Figure 19B). Residual IFN activity was then assayed using the PIL5 gene-reporter assay as described in the Materials and Methods. The IFN preparation used in each neutralization test was also assayed simultaneously to determine its precise IFN activity in that day's assay. The lowest dilution of serum tested was also assayed alone for the presence of IFN activity or toxicity. Neutralizing titer was determined using the Kawade methodology (Grossberg et al., 2001b; and Lallemand et al., 2008) which determines the reciprocal of the antibody dilution that reduces IFN activity from 10 to 1.0 LU/ml and expressed as TRU/ml as described in the Materials and Methods. Neutralization titers were corrected for the actual number of LU/ml of IFN used in the neutralization assay from the value obtained in the simultaneous IFN titration.

[0062] Figures 20A-20C are graphs comparing determination of neutralizing titer using different methods/assays. The neutralizing titer of a series of human sera was determined by the constant antibody method using the reporter-gene assay and the results were compared with those obtained for the same sera determined using the one-step assay (Figure 20A). The neutralizing titer of the same series of human sera was determined by the constant IFN method using the CPE assay as described in the Materials and Methods and the results were compared with those obtained for the same sera determined using the reporter-gene assay and the constant antibody method (Figure

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20B). The neutralizing titer of the same series of human sera was determined by the constant IFN method using the CPE assay as described in the Materials and Methods and the results were compared with those obtained for the same sera determined using the one-step assay (Figure 20C).

DETAILED DESCRIPTION OF THE INVENTION

[0063] Conventional cell based assays for the quantification of neutralizing antibodies (NAbs) are imprecise, give variable results, and often require two or more days to complete. Furthermore, conventional cell-based assays require specialized personnel and biological containment facilities, are labor intensive, and difficult to automate. The use of division-arrested frozen cells transfected with a reporter gene controlled by a ligand-responsive chimeric promoter (WO 2004/039990 and US 2004/023517, incorporated herein by reference) in an assay for neutralizing antibodies would allow anti-ligand NAbs to be quantified with precision within hours. Although such an assay would overcome many of the limitations of conventional cell-based neutralization assays, it would still remain relatively labor intensive and require serial dilutions of both the test sample and ligand, positive and negative controls, and reference reagents, to be included in the assay. Furthermore, assay precision is adversely affected by loss of assay cells (or carry-over of ligand or NAb following serial dilution). Such assays also remain relatively difficult to automate.

[0064] The present invention avoids the limitations of the currently available assays as discussed above by developing a cell, and an assay for the quantification of neutralizing antibodies based on using such a cell, which has been engineered to express and secrete the ligand (extracellular ligand) of interest and a reporter gene transcribed from the same inducible

promoter. The cell also contains another reporter gene
controlled by a chimeric promoter which is ligand-responsive.
Expression of the former reporter product gene is strictly
proportional to the expression of the ligand and allows ligand
expression to be quantified (i.e., by determining the amount of
expressed reporter gene product). Expression of the latter
ligand-responsive reporter gene allows ligand activity to be
quantified as well. The presence of anti-ligand NAbs in the
immediate environment of the cell will neutralize a quantity of
extracellular ligand (secreted from the cell) proportional to the
neutralization capacity of the antibody, and thus prevent the
extracellular ligand from interacting with its specific cell
surface receptor (or with a pattern recognition receptor). This
will result in a corresponding reduction in the activity of the
extracellular ligand, and hence the expression of the ligand-
responsive reporter-gene, the activity of which can be
quantified. Figure 2 schematically illustrates this system using
levels of firefly luciferase (FL) and Renilla luciferase (RL)
activity.

[0065] The degree of reduction in the expression of the
ligand-responsive reporter gene in the presence or absence of the
NAb sample to be quantified will allow the relative neutralizing
titer of the sample to be quantified, relative to a given level
of expression of a different reporter gene transcribed from the
same promoter as the ligand.

[0066] The cell and the cell-based assay method (termed
"NanoLite" as opposed to the "iLite" assay method of
WO2004/039990 and US2004/023517) according to the present
invention, when used for assaying neutralizing antibodies, has
many advantages over the conventional cell-based assay (i.e.,
CPE) and even over the more recent "iLite" cell-based assay in
that it is essentially a one-step assay (where only undiluted

sample need to be added to the cells). Figure 1 presents a flow
diagram comparison between the recent iLite cell-based assay and
the NanoLite assay of the present invention, which clearly shows
that NanoLite involves less steps and less time to perform. It
should be noted that the NanoLite assay according to the present
invention, using the cell of the present invention, is a one-step
assay where, in contrast to the iLite or other conventional cell-
based assays, neither addition of ligand (cytokine) nor dilution
of the sample is required. Table 1 below further summarizes the
many advantages that the NanoLite assay method of the present
invention has over the CPE and iLite assays.

Table 1

	CPE	*iLite*	*Nano Lite*
Time (hours)	96	18	5
Reagents Required	+	+	-
Serial Dilutions	+	+	-
Positive Control	+	+	-
Negative Control	+	+	-
Ligand Standard Curve	+	+	-
Results/Cell Number	+	+	-
Maximum Samples/plate	10	10	96
HTS Automated	-	+/-	+

[0067] As contemplated by the present inventors, the cell of
the present invention, for use in assaying antibodies to an
extracellular ligand that initiates a ligand-specific signal at
the nucleus of the cell, contains at least (a) a first DNA
construct, which has a sequence that includes a first set of one
or more transcription control elements that is inducible by the
ligand, and a portion encoding a first measurable tag (i.e.,
reporter gene product) driven by the first set of one or more

transcription control elements, where the first tag can be detected when the first set of one or more transcription control elements is induced by the ligand, (b) a second DNA construct, which has a sequence that includes (i) a second set of one or more transcription control elements different from the first set, (ii) a DNA segment, driven by the second set of one or more measurable tag (i.e., second reporter gene product different from the first) which can be independently measured in the presence of the first tag, and vice versa, and (iii) on a separate cistron, a DNA segment encoding the ligand, also driven by the second set of one or more transcription control elements.

[0068] The cell according to the present invention may be any mammalian or avian cell line, with human cells most preferred. Preferred cell lines include but are not limited to, human promonocytic (i.e., U937), myeloid (i.e., U266R), T-cell lymphoma (i.e., Jurkatt), breast adenocarcinoma (i.e., MCF7) cell lines and mouse lymphoma (i.e., L120) and erythroid leukemia cell lines.

[0069] The extracellular ligand (or its antagonist/antibody), for which the titer of neutralizing antibodies thereto are determined in the method according to the present invention discussed below, is intended to encompass any therapeutic agent, such as therapeutic proteins, which activates (or blocks, in the case of an antagonist of/antibody against the extracellular ligand) the signal transduction activity of a cell surface protein, and for which neutralizing antibodies generated thereto in the mammalian subject treated with the therapeutic agent would be undesirable. The extracellular ligand may also encompass components of molecules or preparations such as live or attenuated virus or bacterial vaccines, which components interact with pattern recognition receptors. Preferred non-limiting examples of such an extracellular ligand include cytokines,

chemokines and growth factors, such as interferon-α, interferon-β, interferon-γ, erythropoietin (EPO), TNFα, interleukins, growth hormone, granulocyte colony stimulating factor (G-CSF), and granulocyte macrophage colony stimulating factor (GM-CSF); gonadotropins, insulin and other hormones; integrins; immunoglobulins (polyclonal, monoclonal, chimeric, humanized or single chain, etc.); and other proteins that interact with a cell surface molecule or with a pattern recognition receptor to transmit a signal to the nucleus. Non-limiting examples of antagonists (i.e., antibodies) of the extracellular ligand, which antagonist the neutralizing antibodies bind to, include TNFα antagonists such as Enbrel and Infliximab (a chimeric antibody), Adalimumab (a fully human antibody), and Etanercept (an IgG1Fc TNFp75 receptor fusion protein).

[0070] Neutralizing antibody (NAb) assays are clinically very important today because those patients being treated continuously for a chronic disease, such as remitting/relapsing MS treated with interferon β, cease obtaining benefit from treatment with the therapeutic agent once an immune response, in particular production of NAbs, has been mounted against the therapeutic agent by the patient. Thus, it is important to be able to detect when and if a patient has developed NAbs in order to stop treatment at that point. Also, it will prevent the possibility of adverse reactions such as anaphylactic shock and perfusion reactions, and allow the patient to be treated with an alternative effective therapy. Furthermore, NAb testing can provide considerable cost savings to the health care provider/insurer and to the patient by avoiding continued use of an ineffective and expensive biopharmaceutical.

[0071] The cell surface protein from which its signal transduction activity, in response to an extracellular signal from a therapeutic agent or protein, regulates the expression of

a reporter gene product can be any such cell surface protein that
is known to those of skill in the art or that may be identified
by those of skill in the art.   Exemplary cell surface proteins
include, but are not limited to, cell surface receptors and ion
channels.   Non-limiting examples of cell surface receptors
include cytokine receptors (e.g., receptors for Type I
interferon, Type II interferon, interleukins, growth hormone,
erythropoietin (EPO), granulocyte colony stimulating factor (G-
CSF), granulocyte macrophage colony stimulating factor (GM-CSF),
TNFα, TGFβ, Fas ligand, leukemia inhibitory factor (LIF), ciliary
neurotrophic factor (CNTF), etc.), growth factor receptors,
hormone receptors, T cell receptors, antigen receptors,
complement receptors, and neuroreceptors.   The reference text, J.
M. Cruse and Robert E. Lewis, Atlas of Immunology, CRC Press,
Washington, DC, 1999, which discloses many receptors involved in
immune response and immune system interactions is entirely
incorporated herein by reference.   Cell surface receptors also
include, but are not limited to, muscarinic receptors (e.g.,
human M2 (GenBank accession #M16404); rat M3 (GenBank accession
#M16407); human M4 (GenBank accession #M16405); human M5 (Bonner
et al., 1988); and the like); neuronal nicotinic acetylcholine
receptors (e.g., the α2, α3 and β2 subtypes); the rat α2 subunit
(Wada et al., 1988); the rat α3 subunit (Boulter et al., 1986);
the rat α4 subunit (Goldman et al., 1987); the rat α5 subunit
(Boulter et al., 1990); the rat β2 subunit (Deneris et al.,
1988); the rat β3 subunit (Deneris et al., 1989); the rat β4
subunit (Duvoisin et al., 1989); combinations of the rat α
subunits, β subunits and α and β subunits; GABA receptors (e.g.,
the bovine α1 and β1 subunits (Schofield et al., 1987); the
bovine α2 and α3 subunits (Levitan et al., 1988); the γ-subunit
(Pritchett et al., 1989); the β2 and β3 subunits (Ymer et al.,
1989); the δ subunit (Shivers, B.D., 1989); and the like);

glutamate receptors (e.g., receptor isolated from rat brain (Hollmann et al., 1989); and the like); adrenergic receptors (e.g., human β1 (Frielle et al., 1987); human α2 (Kobilka et al., 1987); hamster β2 (Dixon et al., 1986); and the like); dopamine receptors (e.g., human D2 (Stormann et al., 1990); rat (Bunzow et al., 1988); and the like); NGF receptors (e.g., human NGF receptors (Johnson et al., 1986); and the like); serotonin receptors (e.g., human 5HT1a (Kobilka et al., 1987); rat 5HT2 (Julius et al., 1990); rat 5HT1c (Julius et al., 1988); and the like).

[0072] The pattern recognition receptor from which its signal transduction activity, in response to an extracellular signal from a component(s) of a molecule or preparation such as a live or attenuated virus or bacterial vaccine regulates the expression of a reporter gene product, includes but is not limited to Toll-like receptors (TLR) cell surface or endosomal membrane receptors (Uematsu and Akira, 2007), or the retinoic acid-inducible gene 1 (GIG-I)-like cytosolic receptor proteins RIG-I, MDA5, and LGP2 (Yoneyama and Fujita, 2007) that recognize or interact with components of live or attenuated virus or bacterial vaccines. Evaluation of neutralizing antibodies generated in the mammalian subject treated with the vaccine is important in order to determine the degree of protection afforded by vaccination.

[0073] Thirteen members of the TLR family have been identified in mammals (Uematsu and Akira, 2007). Each TLR mediates a distinctive response in association with different combinations of four Toll/IL-1 receptor (TIR) domain-containing adaptor proteins (MyD88, TRIF, TIRAP/MAL, and TRAM). All the TLRs except TLR3 interact with MyD88. TLR3, which recognizes single-stranded or double-stranded viral RNA, is localized in the endosomes of myeloid DCs and requires acidification of vesicles for activation. TLR3 signals via TRIF and activates TBK1/IKKε which

phosphorylates the interferon regulatory factor 3 (IRF3) and
NFκB, resulting in production of IFN β (Hemmi et al, 2004, Perry
et al., 2004). The RIG-I-like receptor proteins are DExD/H box
RNA helicases two of which, RIG-I and MDA5, carry caspase
activation.

[0074] Ion channels include, but are not limited to, calcium
ion channels (e.g., human neuronal α2 subunit (see WO89/09834);
rabbit skeletal muscle α1 subunit (Tanabe et al. 1987); rabbit
skeletal muscle α2 subunit (Ellis et al., 1988); rabbit skeletal
muscle β subunit (Ruth et al., 1989); rabbit skeletal muscle γ
subunit (Jay et al., 1990); and the like); potassium ion channels
(e.g., rat brain (BK2) (McKinnon, D., 1989); mouse brain (BK1)
(Tempel et al., 1988); and the like); sodium ion channels (e.g.,
rat brain I and II (Noda et al., 1986); rat brain III (Kayano et
al., 1988); and others).

[0075] It will be appreciated by those of skill in the art
that the cell surface protein or pattern recognition receptor
discussed above is preferably endogenous to the cell of the
present invention. However, it will also be appreciated that the
cell surface protein or pattern recognition receptor may be
expressed from cloned DNA, such as to supplement the number of
pattern recognition receptors or the number of the cell surface
protein at the surface of the cell, or the cell surface protein
or pattern recognition receptor may be expressed from cloned DNA
but is a cell surface protein or pattern recognition receptor
that is heterologous to the host cell.

[0076] For signal transduction, the intracellular signal that
is transduced is initiated by the specific interaction of an
extracellular signal with a receptor or ion channel present on
the cell surface. This interaction sets in motion a cascade of
intracellular events, including a ligand-specific signal at the
nucleus of the cell, the ultimate consequence of which is a rapid

and detectable change in the expression of a gene product, which
in the cell of the present invention is preferably a reporter
gene product. The extracellular signal or effector molecule is
any compound or substance that acts as a ligand to specifically
alter the activity of a cell surface protein or pattern
recognition receptor. Examples of such signals include, but are
not limited to, molecules such as cytokines (i.e., interferons),
growth factors, hormones, endorphins, neurotransmitters,
acetylcholine, and mitogenic substances, such as phorbol myristic
acetate (PMA), that bind to cell surface receptors and ion
channels and modulate the activity of such receptors and
channels. Other examples include components of live and
attenuated virus and bacterial vaccines.

[0077] The DNA constructs carried by the cell according to the
present invention are DNA constructs that include a nucleotide
sequence encoding a reporter gene product operatively linked to
transcriptional control elements/sequences. Transcription of the
reporter gene is controlled by these sequences. The activity of
at least one or more of these control sequences is directly or
indirectly regulated by the cell surface protein or pattern
recognition receptor. The transcriptional control sequences
include but are not limited to promoters and other regulatory
regions, such as enhancer sequences and repressor and activator
binding sites, that modulate the activity of the promoter, or
control sequences that modulate the activity or efficiency of the
RNA polymerase that recognizes the promoter, or control sequences
that are recognized by effector molecules, including those that
are specifically induced by interaction of an extracellular
signal with a cell surface protein or a pattern recognition
receptor. For example, modulation of the activity of the
promoter may be affected by altering the RNA polymerase binding
to the promoter region, or, alternatively, by interfering with

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initiation of transcription or elongation of the mRNA. Such
sequences are herein collectively referred to as transcriptional
control elements or sequences. In addition, the constructs may
include sequences of nucleotides that alter translation of the
resulting mRNA, thereby altering the amount of reporter gene
product expressed.

[0078] A promoter that is regulated or mediated by the
activity of a cell surface protein or pattern recognition
receptor is a promoter whose activity changes when a cell is
exposed to a particular extracellular signal (ligand) by virtue
of the presence of cell surface proteins or pattern recognition
receptors whose activities are affected by the extracellular
signal. For example, the c-fos promoter is specifically activated
upon the specific interaction of certain extracellular signals,
such as growth hormones, with a cell surface protein, such as a
growth hormone receptor. In particular, the regulation of such
promoters by the cell surface protein, though indirect, occurs
within minutes of the interaction of the cell surface protein
with the extracellular signal. As used herein, operative linkage
refers to the linkage of a transcriptional control element, i.e.,
promoter, to a nucleotide coding sequence such that the
transcriptional control element is properly positioned for its
activity of binding RNA polymerase and initiating transcription
of the nucleotide coding sequence. Thus, a nucleotide coding
sequence in operative linkage with a promoter is downstream, with
respect to the direction of transcription, from the promoter, is
in the correct reading frame with respect to the transcription
initiation site and is inserted in a manner such that
transcription elongation proceeds through the nucleotide coding
sequence.

[0079] Suitable transcriptional control elements may be
obtained or derived from the transcriptional regulatory regions

of genes whose expression is rapidly induced, generally within minutes, of contact between the cell surface protein or pattern recognition receptor and the effector ligand that modulates the activity of the cell surface protein or pattern recognition receptor. Examples of such genes include, but are not limited to, the immediate early genes (Sheng et al., 1990), such as c-fos. Immediate early genes are genes that are rapidly induced upon binding of a ligand to a cell surface protein. The transcriptional control elements that are preferred for use in the DNA (reporter gene) constructs include transcriptional control elements from immediate early genes, elements derived from other genes that exhibit some or all of the characteristics of the immediate early genes, or synthetic elements that are constructed such that genes in operative linkage therewith exhibit such characteristics. The characteristics of preferred genes from which the transcriptional control elements are derived include, but are not limited to, low or undetectable expression in quiescent cells, rapid induction at the transcriptional level within minutes of extracellular simulation, induction that is transient and independent of new protein synthesis, subsequent shut-off of transcription requires new protein synthesis, and mRNAs transcribed from these genes have a short half-life. It is not necessary for all of these properties to be present.

[0080] Suitable promoters and transcriptional control elements include, but are not limited to, the cytomegalovirus promoter (CMV), the simian virus 40 (SV40) promoter and minimal promoters thereof, the vasoactive intestinal peptide (VIP) gene promoter (cAMP responsive; Fink et al., 1988); the somatostatin gene promoter (cAMP responsive; Montminy et al., 1986); the proenkephalin promoter (responsive to cAMP, nicotinic agonists, and phorbol esters; Comb et al. 1986); the phosphoenolpyruvate carboxy-kinase gene promoter (cAMP responsive; Short et al.,

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1986); the NGFI-A gene promoter (responsive to NGF, cAMP, and
serum; Changelian et al., 1989); the transcriptional control
elements obtained or derived from the c-fos gene; and others that
may be known to or prepared by those of skill in the art.

[0081] The c-fos proto oncogene is the cellular homologue of
the transforming gene of FBJ osteosarcoma virus. It encodes a
nuclear protein that is most likely involved in normal cellular
growth and differentiation. Transcription of c-fos is transiently
and rapidly activated by growth factors and by inducers of other
cell surface proteins, including hormones, differentiation-
specific agents, stress, mitogens and other known inducers of
cell surface proteins. Activation is protein synthesis
independent. The c-fos regulatory elements include a TATA box
that is required for transcription initiation, two upstream
elements for basal transcription, and an enhancer, which includes
an element with dyad symmetry and which is required for induction
by TPA, serum, EGF, and PMA. The 20 bp transcriptional enhancer
element located between -317 and -298 bp upstream from the c-fos
mRNA cap site, which is essential for serum induction in serum
starved NIH 3T3 cells. One of the two upstream elements is
located at -63 to -57 and it resembles the consensus sequence for
cAMP regulation.

[0082] Transcriptional control elements, particularly as they
relate to a preferred embodiment of the present invention where
Type I and/or Type II interferon is the extracellular signal, are
preferably an interferon stimulatory response element (ISRE)
and/or a gamma activated sequence (GAS). There are a number of
ISREs characterized from different human genes responsive to Type
I interferon and a consensus sequence, ggraaagwGAAActg (SEQ ID
NO:1; capital letters denote core sequence; underlines denote
high conservation), to which the STAT1/STAT2/IRF9 complex binds,
was identified for ISRE (Levy et al., 1988). A preferred ISRE is

from the human ISG15 gene and is presented as SEQ ID NO:2 where
nucleotides 41-55 correspond to the consensus ISRE sequence.
ISRE is also highly conserved among species. For example, a
sequence present in the promoter region of the interferon
inducible chicken Mx gene (Schumacher et al., 1994) is similar to
that found in primates and conforms to the ISRE consensus
sequence for mammalian interferon responsive genes including
rodents and cows (see Fig. 2 of Perry et al., 1999).

[0083] Regarding GAS, to which the STAT1 homodimer binds in
genes responsive to Type II interferon, a consensus sequence,
nnnsanttccgGGAAntgnsn (SEQ ID NO:3; capital letters denote core
sequence; underlines denote high conservation), from many
selected binding sequences was identified (Horvath et al., 1995).

[0084] In the instance where Type I interferon or Type II
interferon is the extracellular ligand signal, a preferred
combination of transcriptional control elements is an interferon
responsive chimeric promoter in which an ISRE and/or GAS controls
a SV40 minimal promoter operatively linked to a nucleotide
sequence encoding a first reporter gene product as a first
measurable tag.

[0085] When the extracellular ligand is TNFα, a preferred
combination of transcriptional control elements is a TNF-α-
responsive chimeric promoter in which repeats (i.e., 5x tandem
repeats; SEQ ID NO:11) of the NFκB recognition site controls a
SV40 minimal promoter operatively linked to a nucleotide sequence
encoding a first reporter gene product.

[0086] When the extracellular ligand is erythropoietin (EPO),
a preferred combination of transcriptional control elements is an
EPO-responsive chimeric promoter in which repeats of the signal
transducer and activator of transcription #5 (STAT5) sequence(5x
tandem repeats is tcgagTTCGAAGAAaacTTCTTGGAAgaTTCCTGGAgcTTCTAG
GAAgaTTCCGGGAA (SEQ ID NO:4), where the sequence in capital

letters represent variants of the STAT5 consensus sequence),
through which EPO signals from its cell surface receptor to the
nucleus, controls a SV40 minimal promoter operatively linked to a
nucleotide sequence encoding a first reporter gene product.

[0087] The first reporter gene product (also known herein as a
first measurable tag), whose level is a measure of the presence
and/or the level of an extracellular ligand that activates the
signal transduction activity of a cell surface protein or pattern
recognition receptor, may be RNA or protein, as long as it is
readily detectable, although it is preferably a protein. For
instance, luciferases, such as firefly luciferase, Renilla
luciferase, Gaussia luciferase and Metridia luciferase, enhanced
green fluorescent protein (EGFP) and jellyfish aequorin are most
preferred embodiments of reporter gene products (measurable tags)
in the cell according to the present invention. In the case of
the enzyme firefly luciferase (deWet et al., 1987) and other
luciferases, and jellyfish aequorin (Rider et al., 2003), the
result of its enzymatic activity, light, is detected and measured
using a luminometer, whereas in the case of EGFP, a fluorescence
activated cell sorter or analyzer (FACS) can be used at an
appropriate wavelength to detect and quantify the amount of EGFP
expressed in a cell. The distribution curve of the amount of
luciferase, aequorin or EGFP expressed in a sample of cells will
be determined by the amount of ligand to which the cell is
exposed in the immediate external environment surrounding the
cell. Non-limiting examples of other suitable reporter gene
products include dsRED, chloramphenicol acetyl transferase (CAT)
(Alton et al., 1979) other enzyme detection systems, such as β-
galactosidase, bacterial luciferase (Engebrecht et al., 1984 and
Baldwin et al. 1984), alkaline phosphatase (Toh et al. 1989 and
Hall et al. 1983), and bacterial or humanized β-lactamase
(Zlokarnik et al., 1998).

[0088] The second reporter gene product (also known as the second measurable tag), whose level is a measure of the level of the ligand, expressed together with the second reporter gene product (and on a separate cistron from the same promoter) and secreted into the immediate external environment surrounding the cell, and which ligand is capable of activating the signal transduction activity of a cell surface protein/receptor or a pattern recognition receptor. The second reporter gene product can be any of those disclosed above with regard to the first reporter gene product except that the first and second reporter gene products must be different from each other such that one reporter gene product can be independently measured in the presence of the other reporter gene product, and vice versa. The term "cistron" is intended to have the meaning commonly understood in the art as a segment of DNA coding for a single polypeptide but expressed from the same set of one or more transcription control elements (i.e., promoter) as the second reporter gene product.

[0089] When the cell is to be used to assay for neutralizing antibodies to a ligand antagonist/antibody (e.g., TNFα antagonists such as Eubrel, Infliximab, Adalimumab, Etanercept, etc.), the cell would further carry a third construct which includes a segment encoding a third reporter gene product (third measurable tag) driven by a third set of one or more transcription control elements different from the first and second set of transcription control elements in the first and second constructs. This third reporter gene product is expressed together with a ligand antagonist (on a separate cistron driven from the same transcription control elements/promoter) and whose level is a measure of the level of expressed ligand antagonist. The ligand antagonist is the expressed and secreted into the immediate external environment surrounding the cell along with

the ligand expressed from the second construct. The level of measurable ligand activity is a measure of the amount of neutralizing antibodies to the ligand antagonist that block the ligand antagonist from inhibiting the signaling activity of the ligand. The third reporter gene product can be any of those disclosed above with regard to the first and second reporter gene products, including preferably a CBG68Luc reporter gene, except that the third reporter gene product and the first and second reporter gene products must each be different such that each reporter gene product can be independently measured in the presence of the other two.

[0090] In the case of extracellular ligands that inhibit cell proliferation, induce apoptosis, or induce receptor down-regulation, the expression of the ligand is controlled by a set of one or more transcription control elements that is inducible (i.e., no expression unless an inducer is present). This would prevent the undesirable inhibitory activity of the extracellular ligand while the cell is growing or before the cell is ready for use in a cell-based assay. Another instance in which inducible transcription control elements are desirable for expressing the ligand is when the cell is used in a cell-based assay where only the level of the extracellular ligand in a sample, without any such ligand being produced from the second set of one or more transcription control elements, is sought to be determined rather than the level of neutralizing antibodies to the extracellular ligand. In the case that a ligand antagonist is to be expressed in a ligand antagonist is to be expressed in a third construct, the expression of the ligand antagonist is preferably controlled by a set of one or more transcription control elements that is inducible.

[0091] Inducible promoters and other transcriptional control elements, some of which are disclosed above, are well-known in

the art. A preferred well known inducible transcriptional
control element for use in controlling the expression of the
ligand and a reporter gene product is a tetracycline-responsive
element from the Tet-On/Tet-Off gene expression system (such as
provided by Clontech Laboratories, Inc., Palo Alto, CA). This
element, to which a reverse tetracycline repressor (rTetR; a
mutated version of the tetracycline repressor) attaches, thereby
inhibiting transcription from the Tet-On construction, is placed
upstream of preferably a minimal promoter, such as the cytomegalo
virus (CMV) immediate early minimal promoter. In the presence of
an inducer, e.g., tetracycline or doxycycline, the mutated
version of the TetR (rTetR) becomes a reverse tetracycline-
controlled transactivator (rtTA) and binds to the TRE allowing
transcription to start.

[0092] In another embodiment, the inducible promoter is a Tet-
Off promoter in which the TetR binds to the TRE, silencing
transcription in the presence of tetracycline or doxycycline.
Following removal of tetracycline or doxycycline, the
tetracycline-controlled transactivator (tTA) binds to the TRE,
thereby activating transcription.

[0093] One embodiment of the cell according to the present
invention is derived from the human pro-monocytic cell line U937
transfected with the firefly luciferase reporter gene controlled
by an interferon responsive chimeric promoter containing a SV40
minimal promoter and the ISRE from the ISG 15 gene as described
previously in WO 2004/039990 and US 2004/023517, which are
incorporated herein by reference and shown in Fig. 3. These
cells were then transfected with the 5991 bp pIRES/IFNA2/hRL
vector (SEQ ID NO:5) which comprises the coding sequence of human
IFN α2a gene (nucleotides 1-586 of SEQ ID NO:5), the IRES
(Internal Ribosome Entry Site; SEQ ID NO:6) of cytomegalovirus
(CMV), together with the coding sequence of Renilla luciferase

reporter gene (nucleotides 1532-2484 of SEQ ID NO:5), under the control of a constitutive CMV promoter (nucleotides 5282-5991 of SEQ ID NO:1) as shown in Fig. 3. Thus, this construction allows the primary RNA transcript to be translated into two distinct native proteins (IFNα2a and Renilla luciferase) so as to preserve the tertiary structure of the human IFNα2a protein and hence its recognition by anti-IFNα antibodies.

[0094] In a preferred embodiment of the cell of the present invention, the Renilla luciferase reporter-gene was cloned upstream of the IRES and the human IFNα2a gene (Fig. 4) in order to increase the low levels of Renilla gene expression observed when the human IFNα2a gene, or other human type I IFN genes (i.e., IFNβ), was cloned upstream of the Renilla luciferase reporter gene.

[0095] In a second preferred embodiment of the cell of the present invention, the Renilla luciferase reporter-gene and the human IFNα2a gene were expressed under the control of an inducible promoter in order to prevent continued expression of human type I IFNs from inhibiting cell proliferation and hence preventing cultivation of the transfected cell line. Thus, the Renilla luciferase reporter gene and the human IFNα2a gene, were expressed under the control of a CMV promoter (Fig. 5), the expression of which is induced in the presence of doxycycline or tetracycline (Tet-On).

[0096] In a third preferred embodiment cell of the present of the invention, the Renilla luciferase reporter-gene and the human IFNβ1a gene, are expressed under the control of a Tet-On CMV promoter (Fig. 6).

[0097] In a further embodiment of the cell of the present invention, the Renilla luciferase reporter gene and the human TNFα gene, are expressed under the control of a tet-on CMV promoter (Fig. 7). The use of an inducible promoter is essential

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for the expression of TNFα, the uncontrolled production of which
would induce apoptosis in the TNFα-sensitive host cells. The
sequence of the 5x tandem repeats of the canonical NFκB
recognition site used in the other construct in Fig. 7 to drive
the expression of firefly luciferase in combination with a SV40
minimal promoter is SEQ ID NO:11.

[0098] In still a further embodiment of the cell of the
present invention, the Renilla luciferase reporter gene and the
human erythropoietin (EPO) gene, are expressed under the control
of a Tet-On CMV promoter (Fig. 9). The use of an inducible
promoter is essential for the expression of EPO, the continuous
production of which would cause down-regulation of EPO-specific
receptors on the EPO sensitive host cells.

[0099] In yet a further embodiment of the cell of the present
invention, the Renilla luciferase reporter gene is expressed
under the control of chimeric promoter consisting of a 5x tandem
repeat of the canonical NFκB recognition site/SV40 minimal
promoter. In addition, the TNFα and the CBRLuc reporter gene are
both expressed under the control of a Tet-responsive element
(TRE)/CMV immediate early minimal promoter(Tet-On) as a second
construct, and the TNFα antagonist of interest and the CBG68Luc
reporter gene are expressed in a third construct under the
control of a different inducible promoter such as the chimeric
mifepristone inducible promoter. In this system, the GAL4-UAS and
the TATA sequence are expressed from the Adenovirus E1b minimal
promoter that is transcriptionally silent in the absence of
activation. The Gal4 DNA binding domain, which binds the
regulatory protein to the GAL4-E1b promoter, and the truncated
human progesterone receptor ligand binding domain (hPR-LBD),
which undergoes conformational change when it binds the
progesterone antagonist mifepristone, are expressed from a
minimal TK promoter on the vector. Thus, upon addition of

mifepristone, the antagonist binds to the hPR-LBD region of the
vector, causing a conformational change in the regulatory protein
resulting in transcription of the TNFα antagonist and the
CBG68Luc reporter gene (Fig. 8).

[00100] In order to make the cell according to the present
invention a one time use cell that cannot be propagated for
further use, the cell (after having been transformed/transfected
with the first, second, and optionally, third DNA construct) is
treated with an anti-mitotic or pro-apoptotic agent so as to
acquire the property that it will maintain the ligand-specific
signal transduction activity for at least about 1 hour but no
more than about 30 days at a temperature above freezing before
losing the signal transduction activity and undergoing cellular
death.

[00101] One preferred embodiment of the present invention is
where the anti-mitotic or pro-apoptotic agent is γ-radiation and
the cell has been treated by irradiating with γ-radiation at an
intensity and for a sufficient time such the irradiated cell
maintains the signal transduction activity of a cell surface
protein/receptor or a pattern recognition receptor for a period
of at least about 1 hour, preferably 7 days but no more than 30
days at a temperature above freezing following irradiation, after
which period of time the irradiated cell immediately undergoes
cellular death (i.e., apoptosis).

[00102] It is known that γ-irradiation at a high dose causes a
cell to lose its signal transduction activity. Irradiation at a
somewhat lower dose causes a cell to cease replication and
undergo cellular death. The present inventors previously
discovered that it is possible to determine a dose which inhibits
replication but still allows a cell to maintains its signal
transduction activity for a period of time before undergoing cell
death. For example, γ-irradiation at about 9 Grays allows U937

cells to retain signal transduction activity for 14 days, after which the cells undergo cell death. However, during those 14 days, the signal transduction activity in response to, for example, Type I interferon that is being assayed functions as well as in a non-irradiated control. Thus, by irradiating a cell with γ radiation, the treated cell has a 14-day shelf life, but which becomes inactive (undergoes cellular death) after a period of about 14 days so that it cannot be maintained and reproduced by an end user. The dose of γ-irradiation required will vary as a function of the particular cell line employed but this can be determined with only routine experimentation based on the guidance in WO 2004/039990 and US 2004/023517.

[00103] The dose (intensity and duration) of γ radiation to which the transformed cell is treated is preferably about 6 to 12 Grays (Gy). As the experiments in WO 2004/039990 and US 2004/023517 demonstrate, the temperature above freezing, at which the cell is kept or stored, affects the shelf-life of the cell. Preferably, this temperature is room temperature, which advantageously maintains maximum interferon sensitivity while providing for ease of storage and shipping of the commercial one time use cell.

[00104] A second preferred embodiment of the present invention is where the cell (after having been transformed/transfected with the first, second, and optionally, third DNA construct) is treated with an anti-mitotic or pro-apoptotic chemical agent such as vinblastin, 5-fluorouracil (5-Fu), cisplatin or an anti-tumor intercalating agent (i.e., mitomycin C) in a sufficient amount and for a sufficient time such that the treated cell maintains the signal transduction activity of the cell surface protein or pattern recognition receptor for a period of at least about 1 hour but no more than about 30 days at a temperature above freezing following treatment with the agent, after which period

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of time the treated cell immediately undergoes cellular death.
An anti-mitotic or pro-apoptotic agent will affect a treated cell
when it begins to replicate, such as for example by preventing
spindle formation, thereby inducing apoptosis and killing the
cell. Thus, cells which have been treated with an anti-mitotic
or pro-apoptotic agent, such as transformed human promonocytic
cells, will have a shelf life of about 24 hours during which the
signal transduction assay can be conducted and after which period
of time the cells will die. It will be appreciated that a cell
having only a 24 hour shelf life is not desirable from a
commercial standpoint. In order to extend the shelf life, the
treated cells may be immediately frozen, in which state they will
have a much longer shelf life, depending upon the manner of
freezing and thawing. Once thawed, however, they must be used
within 24 hours, after which they will undergo cellular death
(i.e., apoptosis).

[00105] It should be understood that conventional wisdom is
that cryopreservation of cells requires a special freezing and
thawing process (and equipment) in which the cells are frozen at
a rate of 1°C per minute until it reaches -80°C or liquid
nitrogen temperatures of about -200°C, where it may be stored
indefinitely, and after which it must be thawed very rapidly.
Often, dimethyl sulfoxide (DMSO) or another cryopreservative is
also used in order to help protect the cells. As most
laboratories do not have storage facilities at -200°C or even
-80°C, it would be useful to allow freezing of the cells to occur
at -20°C. However, it is known that cell viability is poor when
cells are frozen at -20°C and then thawed. It was previously
found by the present inventors that DMSO will protect the cells
even when frozen at -20°C without any special freezing or thawing
techniques or equipment. While glycerol, a known
cryopreservative compound, will protect cells at -20°C, there is

the possibility that it may prevent protein ligands from
interacting with surface receptors at the high percentage (50%)
of glycerol conventionally used for cryopresevation. However, a
low percentage of glycerol (much less than the 50% conventionally
used) can be used. DMSO does not have this disadvantage. DMSO
can thus protect cells frozen at -20°C without any special
freezing or thawing techniques or equipment being required and
without adversely affecting their sensitivity to IFN. After
treating with an anti-mitotic or pro-apoptotic agent, a cell may
achieve a long shelf life even at standard freezer temperatures
of -20°C if further treated with DMSO and that once thawed such a
cell will remain active, i.e., for signal transduction assays
used for determining the amount of ligand or neutralizing
antibodies to the ligand or to an anti-ligand antibody, for
approximately 24 hours until it undergoes apoptosis as a result
of being treated with an anti-mitotic and pro-apoptotic agent.
Any anti-mitotic or pro-apoptotic agent which kills cells during
the process of replication by inducing apoptosis, such as γ-
radiation and chemical agents such as vinbastin, 5-FU, cisplatin,
doxorubicin, or an anti-tumor intercalating agent (i.e.,
mitomycin C) can be used for this purpose as it would be expected
that the cells will remain biologically active during a quiescent
period and until such time the treated cells start to die.

[00106] The treated transformed cell (transformed with the
first, second, and optionally, third DNA construct) is frozen at
a temperature and under conditions such that it will resume
signal transduction after thawing. While the cell is preferably
frozen at a temperature between -20°C and -200°C, more preferably
at -80°C, cells may be subsequently stored at -20°C, a commonly
available freezer temperature in almost all laboratories, it is
intended that other suitable temperatures for cryopreservation of
cells, such as the liquid nitrogen temperature of about -200°C,

be encompassed. It is further preferred that the treated
transformed cell be resuspended in a solution containing a
cryopreservative before freezing the cell. Dimethyl sulfoxide
(DMSO) is the preferred cryopreservative although other suitable
cryopreservatives which have a high bonding affinity to water,
such as ethylene glycol, polyethylene glycol, propylene glycol,
glycerol, butane diol, propanediol, and formamide, may be used so
long as they do not interfere with the use of the cell after
thawing. When DMSO is used alone as the cryopreservative, the
solution containing DMSO preferably contains about 10% DMSO.
More preferably, 2.5% DMSO is used in combination with 10%
glycerol as the cryopreservative.

[00107] The cell according to the present invention is
preferably a mammalian or avian cell, more preferably a human
cell, and most preferably a human promonocytic cell. A preferred
human promonocytic cell carrying the ISRE-luc vector containing
the firefly luciferase gene reporter construct is a PIL5 cell.
Other preferred cell lines include, but are not limited, to human
myeloid (i.e., U266R), human T-cell lymphoma (i.e., Jurkatt),
human breast adenocarcinoma (i.e., MCF7) cell lines and mouse
lymphoma (i.e., L1210) and mouse erythroid leukemia cell lines.
The cell is treated to make a commercial cell line that has the
commercially desirable properties of a sufficient shelf life for
the purpose of the assay and of being a one time use cell that
cannot be propagated for possible further use. Preferably, the
cell is treated either 1) by irradiating with 6 to 12 Gy of γ
radiation, more preferably about 9 Gy, and storage at room
temperature for up to 14 days after irradiation or 2) by exposure
to an anti-mitotic or pro-apoptotic agent, such as vinblastin,
cisplatin, or 5-fluorouracil, most preferably vinblastin, for 10
minutes at 37°C prior to resuspending in a solution containing

40% fetal bovine serum (FBS) and 2.5% DMSO + 10% glycerol and
freezing at -80°C.

[00108] In order to optimize the method of obtaining a cell
with an indefinite shelf life during frozen storage, but which
will die approximately 24 hours after being thawed (once thawed,
however, the product has excellent sensitivity, and precision as
well as selectivity), the parameters which can be varied in the
course of such optimization include:

        1)   Concentration of FBS. Besides FBS, most any serum
could be used as it acts as a toxic sink to protect the cells
from toxins, such as while being thawed or while being treated
with an anti-mitotic or pro-apoptotic agent. The concentration
of FBS can cause the results to vary.

        2)   Time is a variable. The amount of time of exposure
to an anti-mitotic or pro-apoptotic chemical agent, such as
vinblastin, before the cells are centrifuged out and washed to
remove the agent (i.e., vinblastin).

        3)   Using vinblastin as a non-limiting example, the
formulation of the vinblastin makes a difference. Presently,
soluble vinblastin in a proprietary prebuffered formulation sold
by Eli Lilly under the name Velbe in France is preferably used.
A different formulation may require slightly different
combination of parameters.

        4)   The concentration of vinblastin.

        5)   Cell concentration during the vinblastin treatment.

        6)   The amount of cryopreservative or combination of
cryopreservatives.

[00109] All of these parameters can be varied empirically and
the results after freezing tested for sensitivity and precision,
assuming that the cells stay alive for approximately 24 hours
after being thawed. This can be readily determined by one of
ordinary skill in the art without undue experimentation,

particularly in view of the guidance provided in the experiments shown in Figs. 11-24 for PIL5 cells in WO 2004/039990 and US 2004/023517, in order to arrive at a product having substantially the same sensitivity as the untreated live cells for a period of at least one hour, preferably 8-24 hours, following thawing but having a viability of no more than 30 days, preferably no more than 14 days, more preferably no more than 5 days, most preferably no more than 3 days.

[00110] Exemplified below are protocols for preparation of microtiter assay plates and ampoules/vials of PIL5 cells (as model cells) treated with the anti-mitotic and pro-apoptotic agent 1 μg/ml vinblastin for 10 minutes at 37°C prior to frozen storage at -20°C and thawing at a later time for purposes of conducting the assay.

PREPARATION OF MICROTITER ASSAY PLATES

1.   PIL5 cells at a concentration of about 2×10^5 to 7×10^5 cells/ml in RMPI 1640 medium with 10% fetal bovine serum (FBS) are treated with a fresh solution of 1 μg/ml vinblastin (commercially available from Eli Lilly under the pre-buffered formulation VELBE), diluted from 1 mg/ml in H_2O, for 10 minutes at 37°C in an atmosphere of 5% CO_2 in air. A CO_2 incubator can be used for convenience.

2.   The PIL5 cells are centrifuged at 800 x g for 10 minutes at 4°C, and washed once with the same volume of RPMI 1640 medium with 10% FBS to remove the vinblastin.

3.   The PIL5 cells are re-suspended at a concentration of 2×10^7 cells/ml in RMPI 1640 medium with 40% fetal bovine serum (FBS) and 2.5% dimethylsulfoxide + 10% glycerol.

4.   The cell suspension is dispensed into the wells of a flat-bottom micro-plate to give 300,000 cells per well (equivalent to 25 μl of cell suspension per well).

5.   The micro-plate is frozen at -80°C in an aluminum bag sealed under vacuum with the cover uppermost.

6.   The micro-plates can be subsequently stored for limited periods at -20°C until use.

[00111] Alternatively, PIL5 cells at a concentration of 2x107 cells/ml in RMPI 1640 medium with 40% fetal bovine serum (FBS) and 2.5% dimethylsulfoxide + 10% glycerol can be frozen at -80°C or -200°C in a single or multiple cryopreservation vials. Immediately prior to use the viale is thawed rapidly and the cells distributed into one or more microtiter plates. Vials may also be prepared containing sufficient cells for half or a quarter of a microtiter plate as required.

PREPARATION OF CRYOPRESERVATION AMPOULES/VIALS

1.   PIL5 cells at a concentration of about $2x10^5$ to $7x10^5$ cells/ml in RMPI 1640 medium with 10% fetal bovine serum (FBS) are treated with a fresh solution of 1 μg/ml vinblastin (commercially available from Eli Lilly under the prebuffered formulation VELBE), diluted from 1 mg/ml in H_2O for 10 minutes at 37°C in an atmosphere of 5% CO_2 in air. A CO_2 incubator can be used for convenience.

2.   The PIL5 cells are centrifuged at 80 x g for 10 minutes at 4°C, and washed once with the same volume of RPMI 1640 medium with 10% FBS to remove the vinblastin.

3.   The PIL5 cells are re-suspended at a concentration of $2x10^7$ cells/ml in RMPI 1640 medium with 40% fetal bovine serum (FBS) and 2.5% dimethylsulfoxide + 10% glycerol.

4.   The cell suspension (1 ml) is dispensed into a cryopreservation vial and frozen at -80%.

5.   The cryopreservation vial can be subsequently stored at -20°C for limited periods until use.

[00112] The present invention also provides a method of using
the cell according to the present invention for determining the
level in a sample of an extracellular ligand that initiates a
ligand-specific signal at the nucleus (i.e., by signal
transduction from a cell surface receptor or from a pattern
recognition receptor) or the level of neutralizing antibodies
either against the extracellular ligand or an antagonist against
the extracellular ligand, or the level of a soluble form of the
ligand receptor. This method involves incubating the cell of the
present invention in a mixture with a sample in which the level
of the extracellular ligand or the neutralizing antibody is
sought to be determined. The level of the first measurable tag
(first reporter gene product, such as firefly luciferase in the
embodiments shown in the drawings) in the mixture is determined
relative to the level of the first measurable tag in the absence
of the sample to calculate the level in the sample of the
extracellular ligand or neutralizing antibody.

[00113] The present invention also provides a means of
detecting the presence of residual drug in the sample to be
tested for the presence of anti-drug NAbs. The presence of
residual drug can render the results of neutralization assays
uninterpretable. In the case of patients treated with a
biopharmaceutical drug which is, for instance, a recombinant form
of a cytokine such as IFNβ or a growth factor such as EPO, the
presence of the drug (IFNβ or a growth factor) in the sample
(serum or other biological fluid) to be assayed for the presence
of anti-cytokine or anti-growth factor NAbs can be determined
using the method of the present invention prior to carrying out
the neutralization assay according to the present invention by
simply first incubating the sample with the assay cell of the
present invention for an appropriate time (3 to 6 hours).
Activation of firefly luciferase, in the absence of addition of

tetracycline or doxycycline, will indicate the presence of residual drug. The degree of activation of the firefly luciferase will allow the level of residual drug to be quantified. Residual drug can then be removed by the use of an appropriate procedure. For example in the case of IFNβ, the drug can be separated from any anti-IFN antibodies present in the sample by the use of a molecular sieve with a 20 to 30 kDa cut-off or an anti-IFNβ affinity column. Alternatively, anti-IFN NAbs can be removed from the sample using a protein-A or protein G affinity column.

[00114] The sample can then be assayed for the presence of anti-IFN NAbs following activation of the ligand-Renilla luciferase construct in the presence of tetracycline or doxycycline.

[00115] In the case of TNFα antagonists such as Infliximab, a chimeric antibody, Adalimumab, a fully human antibody, or Etanercept, an IgG1FC-TNFp75 receptor fusion protein, the presence of residual drug can again interfere with the detection of NAbs against the TNFα antagonist. The presence of residual drug in the sample can be detected using the one-step assay method according to the present invention simply by incubating the serum sample with the assay cells in the presence of tetracycline or doxycycline prior to activation of the inducible Infliximab construct. In this construct, the nucleic acid encoding Infliximab is under the control of an inducible promoter different from the Tet-On or Tet-Off promoter. For example, a mifepristone-regulated promoter can be employed such as that commercialized by Invitrogen (Carlsbad, CA). In this system, a chimeric promoter, consisting of the GAL4-UAS and the TATA sequence from the Adenovirus E1b minimal promoter, is transcriptionally silent in the absence of activation. The Gal4 DNA binding domain, which binds the regulatory protein to the GAL4-E1b promoter, and the truncated human progesterone receptor

ligand binding domain (hPR-LBD), which undergoes conformational change when it binds the progesterone antagonist mifepristone, are expressed from a minimal TK promoter on the vector. Thus, upon addition of mifepristone, the antagonist binds to the hPR-LBD region of the vector, causing a conformational change in the regulatory protein resulting in transcription from the GAL4-E1b promoter.

[00116] Thus, a reduction in the Tet-On regulated TNFα-induced Renilla luciferase signal, due to the production of endogenously expressed TNFα, following addition of a sample to the cells of the present invention, will indicate the presence of the TNFα antagonist in the sample to be assayed for the presence of anti-TNFα antagonist NAbs. The degree of reduction of the Renilla signal produced by endogenously expressed TNFα, will allow the concentration of the TNFα antagonist in the sample to be quantified. The TNFα antagonist can then be removed from the sample by an appropriate means. For example in the case of Infliximab, which is composed solely of kappa light chains, the drug can be removed from the sample to be assayed for anti-Infliximab NAbs using an anti-kappa affinity column. Alternatively, anti-Infliximab NAbs can be removed from the sample using a protein-A or protein G affinity column and then quantified using the "One-Step" assay method according to the present invention.

[00117] The sample which is assayed in the method according to the present invention is a biological fluid of a mammalian subject, preferably a human subject, in which the extracellular ligand or neutralizing antibodies are expected to be present, such as blood. Most preferably the sample is serum, saliva, bronchoaveolar lavage, or cerebrospinal fluid.

[00118] A preferred embodiment of the method according to the present invention is where the cell used in the assay is a cell

treated with an anti-mitotic or pro-apoptotic agent frozen as described above, and which is then thawed, prior to use, within a period of time that the thawed cell maintains the ligand-specific signal transduction activity.

[00119] When the method according to the present invention is used for determining the level in a sample of an extracellular ligand that initiates a ligand-specific signal at the nucleus, the cell according to the present invention would need to have any endogenous production of the ligand by the cell itself to be negligible or absent. Since the ligand is expressed from a second set of one or more transcription control elements in the second DNA construct present in the cell, the expression of the ligand in this situation is controlled from an inducible set of one or more transcription control elements which is turned off so as to not interfere with the determination of the level of extracellular ligand in the sample itself.

[00120] When the method according to the present invention is used to determine the level in a sample of neutralizing antibodies either against the extracellular ligand or against an antagonist to the ligand, the ligand can be expressed from a constitutive promoter or from an inducible set of one or more transcription control elements. However, for the case of determining the level of neutralizing antibodies against a ligand antagonist (i.e., against Enbrel, Infliximab, etc., for TNFα as the extracellular ligand), the cell according to the present invention preferably includes an additional construct (i.e., third DNA construct) from which the ligand antagonist and a third different measurable tag are expressed. In this way, the cell according to the present invention would have all the necessary components of ligand, ligand antagonist and ligand-responsive expression of a reporter gene present in a single cell to assay for neutralizing antibodies in a sample. Such a cell would only

require addition of an undiluted sample to initiate the assay (and the presence of substrate for the reporter-gene product).

[00121] When the method of the present invention is assaying for the level of a Type I interferon to determine either the titer of the Type I interferon as extracellular ligand or of neutralizing antibodies for Type I interferon, the first reporter gene product is preferably firefly luciferase, jellyfish aequorin, or enhanced green fluorescent protein (EGFP) and is preferably under the control of an interferon-sensitive chimeric promoter containing the ISRE from ISG15 and a minimal SV40 promoter. Examples of such reporter gene constructs are presented in Figures 9 and 10. Figure 9 is a schematic representation of a luciferase gene reporter construct in an ISRE-luc vector (SEQ ID NO:7), where the ISRE from ISG15 (SEQ ID NO:2) is positioned at nucleotides 38-97 of SEQ ID NO:7, the SV40 minimal promoter is positioned at nucleotides 103-288 of SEQ ID NO:7, and the coding sequence of the luciferase reporter gene having the amino acid sequence of SEQ ID NO:8 is positioned at nucleotides 328-1980 of SEQ ID NO:7. Similarly, Figure 10 is a schematic representation of a EGFP gene reporter construct in an ISRE-EGFP vector (SEQ ID NO:9), where the ISRE from ISG15 is positioned at nucleotides 30-89 of SEQ ID NO:9, the SV40 minimal promoter is positioned at nucleotides 95-290 of SEQ ID NO:9, and the coding sequence of the EGFP reporter gene having the amino acid sequence of SEQ ID NO:10 is positioned at nucleotides 358-1077 of SEQ ID NO:9.

[00122] The sample of serum (10 μl) to be tested for the presence of anti-ligand antibodies is added to the well of a 96-well plate containing 50,000 One-Step cells (cells according to the present invention) cells in 50 ml of RPMI 1640 medium containing 2% BFS and a suitable concentration of doxycycline in the range 1.0 ng/ml to 10.0 μg/ml, most preferably 1.0 μg/ml. The

sample is incubated with the cells at 37°C in an atmosphere of 5% CO_2 in air for a period ranging from 4 to 18 hours, most preferably 5 to 6 hours. The activities of the firefly luciferase and Renilla luciferase can then be determined sequentially following addition of 50 µl of the DUAL-GLOW luciferase assay reagent (Promega, Madison, WI). Thus, expression of the firefly luciferase can first be quantified using the Luciferase Assay Reagent II (Promega, Madison, WI). This reaction is then quenched and the Renilla luciferase reaction is initiated by addition of 50 µl of the STOP & GLO reagent (Promega, Madison, WI) to the same sample and expression of Renilla luciferase is quantified. Neutralization titer is determined from the level of expression of the firefly luciferase gene expressed in relative luminescence units (RLU) following addition of the anti-ligand antibody (FL2) relative to the level of expression in RLU of the Renilla luciferase (RL2) divided by level of expression of the firefly luciferase gene without addition of the anti-ligand antibody (FL1) relative to that of the Renilla luciferase (RL1). Thus, neutralization titre = FL2/RL2 divided by FL1/RL1. This titer can then be quantified relative to an anti-ligand NAb reference preparation of known titer. A titration curve of anti-INFα neutralizing antibodies using the present method is presented in Fig. 11.

[00123] An assay for the quantification of anti-IFN alpha NAbs is described herein that overcomes many of the limitations of conventional cell-based neutralization assays or other reporter-gene assays noted in the "Description of the Related Art" section. The assay is based on a cell that has been engineered to express and secrete IFNα2 and to express the Renilla luciferase reporter-gene transcribed from the same inducible promoter. The cell also contains the firefly luciferase reporter gene controlled by a chimeric IFN-responsive promoter. Expression of

the Renilla reporter gene is strictly proportional to expression
of IFNα2 and therefore allows IFN *expression* to be quantified
with precision while expression of firefly luciferase allows IFN
activity to be quantified. The presence of anti-IFNα NAbs in the
immediate environment of the cell will neutralize a quantity of
IFNα secreted from the cell proportional to the neutralizing
capacity of the antibody, and thus prevent IFN from interacting
with its specific cell-surface receptor. This will result in a
corresponding reduction in the activity of IFNα and hence
expression of the IFN-responsive firefly luciferase reporter
gene, the activity of which can be quantified. The degree of
reduction in the expression of the IFNα-responsive reporter gene
in the presence or absence of the NAb sample will allow the
relative neutralizing titer of the sample to be quantified,
relative to a given level of expression of the renilla reporter
gene transcribed from the same promoter as IFNα.

[00124] The one-step assay according to the present invention
is applicable to a wide range of biopharmaceuticals and allows
residual drug levels to be quantification in a sample from the
expression the drug-responsive reporter gene prior to induction
of autocrine drug synthesis. Drug synthesis is then induced and
NAb activity is quantified in the same sample from the change in
expression of the drug-responsive reporter gene in the presence
or absence of anti-drug antibodies, without the need for serial
dilution of the sample, or addition of exogenous drug. Although
the one-step NAb assay is based on the same principal as a
conventional constant antibody neutralization assay, results are
normalized relative to the expression of an internal standard and
are consequently independent of cell number affording a high
degree of assay precision. The one-step assay is thus ideally
suited to high through-put analysis of anti-drug NAbs.

[00125] In another embodiment of the invention, the cell line used as the basis for the method of the present invention, the so called NanoLite One-Step neutralization assay, was derived from the human pro-monocytic cell line U937 transfected with the Renilla luciferase reporter gene under the control of an interferon-responsive chimeric promoter comprising a SV40 minimal promoter, and the ISRE from the ISG 15 gene. The cell line is also transfected with a second construct consisting of a vector expressing the ligand of interest and a firefly luciferase reporter gene product under the control of an inducible promoter such as the Tet-On CMV promoter.

[00126] The ligand reporter activity of the Renilla luciferase reporter gene is determined at various time points following addition of a luciferase reagent such as EnduRen, or ViviRen (Promega, Madison, WI) that allows Renilla luciferase activity to be determined continuously within live-cells. Alternatively, non-limiting examples of luciferases that can be used instead of Renilla luciferase include luciferases such as Gaussia Luciferase or Metridia luciferase together with the appropriate luciferase substrate. Thus, 10 µl of serum to be tested for the presence of anti-ligand antibodies is added to the well of a 96-well plate containing 50,000 One-Step cells (cells according to the present invention) in 50 µl of RPMI 1640 medium containing 2% BFS and 1.0 µg/ml of doxycycline and the appropriate luciferase substrate. The sample is incubated with the cells at 37°C in the luminometer for a period ranging from 4 to 18 hours, most preferably 5 to 6 hours and RLU readings are taken at regular intervals. The neutralizing titer of the sample is calculated from the time taken, T2, in the presence of the anti-ligand antibody, to reach a relative level of Renilla luciferase expression obtained at a time, T1, in the absence of anti-ligand antibody.

[00127] Another aspect of the present invention is directed to
a kit for determining the level in a sample of an extracellular
ligand that initiates a ligand-specific signal at the nucleus of
a cell. This kit includes a reagent containing a plurality of
the cell of the present invention and either a testing device
having a plurality of wells or a container for storing the
reagent prior to use. The testing device is preferably a multi-
well microtiter plate (e.g., 96 well microtiter plate), but can
also be any type of receptacle such as petri dishes or plates
with a plurality of wells in which an assay can be conducted.
The reagent containing the cells may be disposed in the wells of
the testing device, although it will be appreciated that such
cells can instead be dispensed in the wells of the testing device
by the end user just prior to conducting the assay. The kit may
further include a set of instructions for using the kit in an
assay. Preferably, the reagent in the kit is supplied frozen
and, most preferably, the frozen cells according to the present
invention as contained in the reagent have been treated with an
anti-miotic or pro-apoptotic agent, as discussed above, prior to
being frozen in a cryopreservative.

[00128] Having now generally described the invention, the same
will be more readily understood through reference to the
following example which is provided by way of illustration and is
not intended to be limiting of the present invention.

                              EXAMPLE

[00129] A unique one-step cell-based assay for interferon alpha
(IFNα) has been developed that allows both drug concentration and
anti-drug neutralizing antibodies (NAbs) to be quantified in a
single serum sample without the need for sample dilution,
addition of exogenous drug, or other manipulation. IFN activity
is quantified with a high degree of precision and within a few

hours using cells, transfected with the firefly luciferase
reporter-gene controlled by an IFN-responsive chimeric promoter.
The assay cells have also been engineered to express and secrete
IFNα, the production of which is normalized relative to the
expression of the Renilla luciferase reporter gene transcribed
from a common doxycycline inducible promoter. Thus, following
quantification of residual IFN levels in a serum sample from an
IFNα treated patient, autocrine IFN synthesis is induced and NAb
activity can then be quantified instantaneously from the
difference in expression of the IFN-responsive reporter gene, in
the presence or absence of the sample. Assay results are
normalized relative to the expression of an internal standard
that renders results independent of cell number or differences in
cell viability thus affording a high degree of assay precision.
This unique assay platform is ideally suited for high throughput
analysis of samples and is applicable to the quantification of
both the activity and NAb levels for a number of
biopharmaceuticals allowing comparison of immunogenicity data
between assays and compounds.

MATERIALS AND METHODS

[00130] *PIL5 Reporter-Gene Assay.* The synthetic double-stranded
oligonucleotide CTCGGGAAAGGGAAACCGAAACTGAAGCC (SEQ ID NO:12),
corresponding to the IRSE from the ISG-15 gene, controlling a
SV40 minimal promoter was cloned upstream of the luciferase
reporter-gene by insertion into the Xho/BglII site of the pGL2-
promoter vector (Promega, Madison, WI) as described previously
(Lallemand et al., 2008). Human promonocytic U937 cells were
transfected with the IFN regulated gene reporter construct and
stable transfectants were isolated and cloned. A human cell line,
PIL5, carrying the luciferase reporter gene under the control of
an IFN responsive chimeric promoter was thus established. Assay-

ready vinblastin-treated, division-arrested PIL5 cells (i*Lite*
Alpha-Beta assay) were obtained from Biomonitor Ltd, Galway,
Ireland, and stored frozen at -80°C until use, according to the
manufacturer's instructions. Briefly, frozen cells were thawed
rapidly and incubated overnight in a 96-well micro-titer plate
(50,000 cells/well), in duplicate with serial dilutions of IFN in
a total volume of 100 µl/well. Cells were then lysed by the
addition of 100 µl/well of the luciferase substrate containing
reagent, and luciferase activity was determined in a luminometer
(LumiCount™, Packard Instriuments Inc, Downers, Grove IL).
Interferon activity was determined from the dose-response curve
of relative luminescence units (RLU) against dilutions of the
appropriate international IFN reference preparation using Excel™
software. Results are expressed in IU/ml.

[00131] ***Construction of the pTRE/IFNa2/hRL Vector.*** The
TRE/IFNalpha2/hRL vector was constructed as follows: The coding
region of the human IFNalpha2a gene was amplified by PCR from a
human genomic extract using the following primers, which contain
respectively EcoRI and BamHI restriction sites at their 5'
extremities:

IFNalpha2 Sense: 5' ACGTGAATTCGCAACATCTACAATGGCCTTGACCTTT 3'
(SEQ ID NO:13)

FNalpha2 Anti-sense: 5' GATCGGATCCAGTTTTCATTCCTTACTTCTTAAAC 3'
(SEQ ID NO:14)

The humanized version of the Renilla luciferase gene (hRenilla)
was amplified by PCR from the psiCHEK-2 vector (Promega,
Catalog ref C8011) using the following primers which contained
respectively SmaI and XbaI restriction sites at their 5'
extremities:

hRenilla Sense : 5' TCGTCCCGGGATGGCTTCCAAGGTGTACGACCCC 3'
(SEQ ID NO:15)

hRenilla Anti-sense : 5′ CTAGTCTAGATTACTGCTCGTTCTTCAGCACG 3′
(SEQ ID NO:16)

The IFNalpha2 and the hRenilla amplification products were
cloned in the EcoRI/BamHI and the SmaI /XbaI sites respectively
of the pIRES2Neo plasmid (Clontech, Palo Alto, CA, Catalog ref
6938-1). The EcoRI/XbaI restriction fragment of this construct,
containing the coding region of the human IFNalpha2 gene, the
IRES and the hRenilla gene, were cloned in the EcoRI/XbaI
restrictions site of the pTRE-Tight vector (Clontech, Catalog
ref. 631059). The integrity of each construct was verified by
sequencing.

[00132] *Patient Sera.* Sera from patients treated with
recombinant IFN α, or IFN β and monitored for the presence of
neutralizing anti-IFN antibodies were randomly selected for
evaluation in the present study.

[00133] *Recombinant IFNα2a* (Roferon-A™) was purchased from
Hoffmann-La Roche, Neuilly-sur-Seine, France. The preparation
used in this study had a titer of 9.0×10^6 IU/ml on human HuH7
cells challenged with vesicular stomatitis virus (VSV). The
preparation was standardized against the human IFNα international
reference preparation (G023-901-527). The specific activity of
the interferon preparation was 2×10^8 IU/mg protein.

[00134] *Recombinant IFNα2b* (Intron-A™) was purchased from
Schering-Plough, Levallois-Perret, France. The preparation used
in this study had a titer of 1.0×10^7 IU/ml on human HuH7 cells
challenged with VSV. The preparation was standardized against the
human IFNα international reference preparation (G023-901-527).
The specific activity of the interferon preparation was 2×10^8
IU/mg protein.

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[00135] *IFN Bioassay.* IFN activity was assayed by the inhibition of the cytopathic effect (CPE) of VSV on human HuH7 cells as described previously (Lallemand et al., 2008).

[00136] *Neutralization Assays.* Briefly, serial dilutions of human serum were incubated in duplicate for 1 hour at 37°C followed by 2 hours at 4°C with a constant quantity (10 LU/ml) of a particular IFN preparation diluted in RPMI 1640 medium + 2% fetal bovine serum (FBS) in a 96-well micro-titer plate (*constant IFN method*), or a constant dilution of serum was incubated under the same conditions with serial dilutions of IFN (*constant antibody method*). Residual IFN activity was then assayed using either the IFN viral cytopathic effect (CPE) bioassay or the PIL5 gene-reporter assay. The IFN preparation used in each neutralization test was also assayed simultaneously to determine its precise IFN activity in that day's assay. The lowest dilution of serum tested was also assayed alone for the presence of IFN activity or toxicity.

[00137] Neutralizing titer was determined using the Kawade methodology (Grossberg et al., 2001a and 2001b) which determines the reciprocal of the antibody dilution that reduces IFN activity from 10 to 1.0 LU/ml according to the formula; t = f (n-1)/9, where f = the reciprocal of the antibody dilution, and n = IFN concentration in LU/ml (Grossberg et al., 2001b; and Lallemand et al., 2008). Thus, when n = 10 LU/ml, t = f. Neutralizing titers are expressed as **Ten-fold Reduction Units/ml**, or TRU/ml (Grossberg et al., 2001a and 2001b). Neutralization titers were corrected for the actual number of LU/ml of IFN used in the neutralization assay from the value obtained in the simultaneous IFN titration.

[00138] *One-Step Neutralization Assay.* PIL5C2.2 were incubated overnight in a 96-well micro-titer plate (37,500 cells/well), in duplicate with a single dilution of the serum sample to be tested

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in a total volume of 75 µl/well and 25ng/ml Doxycycline (Clontech
Catalog ref. 631311). The activities of both Firefly and Renilla
luciferase were determined sequentially in the same well using
the Dual-Glo luciferase assay system (Promega, Catalog ref.
E2940) according to the manufacturer's instructions. The cells
were first lysed by the addition of 75 µl/well of the Firefly
luciferase substrate containing reagent, and FireFly luciferase
activity was determined in a luminometer (LumiCount™, Packard
Instruments Inc, Downers Grove IL). Renilla luciferase activity
was then determined following addition in the same well of 50µl
the Renilla luciferase substrate. The neutralizing activity of
the NAb sample was determined from the ratio of Firefly
luciferase activity in the presence of the NAb containing sample
(FL2) normalized relative to Renilla luciferase expression (RL2)
and Firefly luciferase activity of the control sample (FL1)
normalized relative to Renilla luciferase expression of the
control sample (RL1): (FL2/RL2)/(FL1/RL1).

RESULTS

*Establishment of the IFN Secreting IFN Responsive Reporter-Gene
Cell Line*

[00139] The human pro-monocytic cell line U937 was transfected
with the firefly luciferase reporter gene controlled by an
interferon responsive chimeric promoter containing a SV40 minimal
promoter and the interferon sensitive response element (ISRE)
from the ISG 15 gene as described previously (Lallemand et al.,
2008). These cells were then co-transfected firstly with the
5,000 bp pTRE/IRES/IFNA2/hRL vector (SEQ ID NO:17) comprising the
coding sequence of human IFNα2a gene including its natural signal
peptide (nucleotides 323-913), the internal ribosome entry site
(IRSE) of cytomegalovirus (CMV), nucleotides 914-1,859, together
with the coding sequence of Renilla luciferase (nucleotides

1,860-2,801), under the control of a composite Tet-responsive element (TRE)-CMV promoter (nucleotides 1-323). This construction allows the primary RNA transcript to be translated into two distinct native proteins (IFNα2a and Renilla luciferase) so as to preserve the tertiary structure of the human IFNα2a protein and hence its recognition by anti-IFNα antibodies. Secondly, with a vector encoding the reverse Tet-controlled transactivation protein that confers tetracycline/doxycycline (Tet)-induced gene expression, as shown in Figure 14.

[00140] The Renilla luciferase reporter-gene and the human IFNα2a gene were expressed under the control of a doxycycline inducible (Tet-On) CMV promoter in order to prevent continued expression of human type I IFNs from inhibiting cell proliferation and hence preventing cultivation of the transfected cell line.

[00141] Stable clones were isolated and tested for both strict inducibility of IFN expression and IFN responsiveness, following induction with doxycycline. Clone C2.2 (PIL5C2.2) was then characterized further.

[00142] Treatment of PILC2.2 cells with increasing concentrations of doxycycline (0.1 to 100 ng/ml) resulted in a dose-dependent increase in the expression of Renilla luciferase (Figure 15A). Increased expression of Renilla luciferase (RL-hatched bars) was accompanied by a corresponding increase in IFNα2a expression as demonstrated by increased production of IFNα2a in the culture supernatent (data not shown). Increased expression of IFNα2a was also accompanied by a corresponding increase in the expression of IFN-responsive firefly luciferase (FL) expression (Figure 15A). Addition of a constant concentration of a polyclonal anti-IFNα neutralizing antibody resulted in a marked decrease in the expression of firefly luciferase (solid white bars-designated Luc in Figs. 15A-15B),

relative to the value observed in the presence of an equivalent dilution of control serum, without affecting the expression of Renilla luciferase expression significantly over a wide range of doxycycline concentrations (Figure 15B).

[00143] The ratio of firefly luciferase expression (abbreviated "FL") to Renilla luciferase (abbreviated "RL") expression in the absence of antibody relative to that in the presence of anti-IFNα antibody (FL1/RL1)/(FL2/RL2) remained relatively constant at doxycycline concentrations ranging from 0.1 to 10 ng/ml even though IFNα2 expression increased some 8 fold (Figure 16A). The ratio (FL1/RL1)/(FL2/RL2) also remained relatively constant at either a 1:10 or 1:100 dilutions of individual sera from patients with chronic hepatitis C treated with IFNα2a or IFNα2b and containing neutralizing anti-IFNα antibodies (Figure 16B).

[00144] Expression of firefly luciferase was found to follow a typical sigmoidal dose-response curve characteristic of a classic IFN dose-response curve following treatment of cells PILC2.3 cells with increasing concentrations of doxycycline (Figure 17). In contrast, expression of Renilla luciferase did not saturate at the concentrations of doxycycline tested (Figure 17).

[00145] Treatment of PILC2.2 cells with different concentrations of doxycycline (25 or 250 ng/ml) did not change significantly the anti-IFNα neutralizing activity of the human sera tested. Thus a series of sera from patients with chronic hepatitis C containing varying anti-IFNα neutralizing activities were quantified using the one-step assay following treatment with either 25 or 250 ng/ml of doxycycline (Figure 18).

[00146] Two principal approaches are used to quantify anti-IFN NAbs: the constant IFN method and the constant antibody method. In the former, a constant quantity of IFN is mixed with increasing dilutions of serum while in the later a fixed dilution of serum in mixed with varying concentrations of IFN. Although

both methods give similar results (Figures 19A and 19B) the
constant antibody method has been reported to be the more
sensitive of the two approaches and to be able to detect weakly
neutralizing sera not detected by the constant IFN method (Lam et
al., 2008). As the one-step NAb assay is based on the same
principal as the constant antibody method, sera from patients
with chronic hepatitis C treated with IFNα2a or IFNα2b were
tested for the presence of neutralizing antibodies to IFNα using
the constant antibody method and either a cytopathic inhibition
(CPE) assay or luciferase reporter-gene assay to quantify IFNα
activity. In keeping with a previous report (Lallemand et al.,
2008), similar results were obtained for the neutralizing titers,
expressed as TRU/ml, for individual sera using the constant IFN
NAb assay, when tested using either the CPE or luciferase
reporter-gene assays to measure IFN activity (R^2 = 0.85, Figure
20A). The results were then compared with those obtained using
the one-step method. Not surprisingly a somewhat lower degree of
correlation (R2 = 0.63) was observed between the results obtained
using the CPE assay to quantify anti-IFNα NAb levels using the
constant IFN method and those obtained using the one-step assay
(equivalent to the constant antibody method) to quantify the
neutralizing titer in TRU/ml of the same samples (Figure 20B). In
contrast, a high degree of correlation (R^2 = 0.85), was observed
for the anti-IFNα neutralizing titers of individual human sera,
expressed as TRU/ml, using the constant IFN NAb assay and the
luciferase reporter-gene assay to measure IFN activity compared
with the results obtained for the same sera using the one-step
method (Figure 20C).

[00147] Although the one-step NAb assay is based on the same
principal as a conventional constant antibody neutralization
assay, the results are normalized relative to the expression of

an internal standard and consequently are not influenced by variations in cell number or errors in sample dilution.

[00148] Having now fully described this invention, it will be appreciated by those skilled in the art that the same can be performed within a wide range of equivalent parameters, concentrations, and conditions without departing from the spirit and scope of the invention and without undue experimentation.

[00149] While this invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications. This application is intended to cover any variations, uses, or adaptations of the inventions following, in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth as follows in the scope of the appended claims.

[00150] All references cited herein, including journal articles or abstracts, published or corresponding U.S. or foreign patent applications, issued U.S. or foreign patents, or any other references, are entirely incorporated by reference herein, including all data, tables, figures, and text presented in the cited references. Additionally, the entire contents of the references cited within the references cited herein are also entirely incorporated by reference.

[00151] Reference to known method steps, conventional methods steps, known methods or conventional methods is not in any way an admission that any aspect, description or embodiment of the present invention is disclosed, taught or suggested in the relevant art.

[00152] The foregoing description of the specific embodiments will so fully reveal the general nature of the invention that

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others can, by applying knowledge within the skill of the art
(including the contents of the references cited herein), readily
modify and/or adapt for various applications such specific
embodiments, without undue experimentation, without departing
from the general concept of the present invention. Therefore,
such adaptations and modifications are intended to be within the
meaning and range of equivalents of the disclosed embodiments,
based on the teaching and guidance presented herein. It is to be
understood that the phraseology or terminology herein is for the
purpose of description and not of limitation, such that the
terminology or phraseology of the present specification is to be
interpreted by the skilled artisan in light of the teachings and
guidance presented herein, in combination with the knowledge of
one of ordinary skill in the art.

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WHAT IS CLAIMED IS:

1.   A cell for use in assaying antibodies to an extracellular ligand that initiates a ligand-specific signal at the nucleus of the cell, said cell comprising:

(a)   a first DNA construct having a sequence comprising a first set of one or more transcription control elements, said first set of one or more transcription control elements being inducible by the ligand, and a portion encoding a first measurable tag, driven by said first set of one or more transcription control elements, which tag can be detected when the first set of one or more transcription control elements is induced by the ligand; and

(b)   a second DNA construct having a sequence comprising:

(i)   a second set of one or more transcription control elements different from said first set;

(ii) a segment driven by said second set of one or more transcription control elements encoding a second measurable tag, which second tag can be independently measured in the presence of said first tag, and vice versa; and,

(iii) on a separate cistron, a segment encoding said ligand, also driven by said second set of one or more transcription control elements.

2.   The cell of claim 1, wherein said second set of one or more transcription control elements comprises an inducible transcription control element.

3.   The cell of claim 2, wherein said inducible transcription control element is a tetracycline (Tet)-responsive element (TRE) and wherein a reverse tetracycline repressor (rTetR), which is expressed constitutively in the cell, turns off/represses the action of said inducible TRE in the absence of

tetracycline or doxycycline as an inducer in a Tet-On expression
system.

4.   The cell of claim 2, wherein said inducible
transcription control element is a tetracycline (Tet)-responsive
element (TRE) and wherein a tetracycline-controlled
transactivator (tTA) binds to said inducible TRE in the absence
of tetracycline or doxycycline to activate transcription from TRE
as a Tet-Off expression system.

5.   The cell of claim 1 for use in assaying antibodies
against a ligand antagonist, further comprising a third DNA
construct having a sequence comprising:

(i) a third set of one or more transcription control
elements different from the first and second set;

(ii) a segment driven by said third set of one or more
transcription control elements encoding a third measurable tag,
which third tag can be independently measured in the presence of
said first and second tags; and,

(iii) on a separate cistron, a segment encoding said
ligand antagonist, also driven by said third set of one or more
transcription control elements.

6.   The cell of claim 1, wherein said ligand is
selected from the group consisting of a cytokine, chemokine
growth factor, integrin, immunoglobulin and a protein that
interacts with a cell surface molecule to transmit the ligand-
specific signal from the cell surface to the nucleus.

7.   The cell of claim 1, wherein said ligand is a
cytokine.

8.   The cell of claim 1, wherein said ligand is an
interferon.

9.   The cell of claim 8, wherein said first set of one
or more transcription control elements comprises an interferon
stimulatory response element (ISRE).

10.   The cell of claim 1, wherein said ligand is tumor necrosis factor α (TNFα).

11.   The cell of claim 10, wherein said first set of one or more transcription control elements comprises an NFκB binding site.

12.   The cell of claim 1, wherein said ligand is erythropoietin (EPO).

13.   The cell of claim 12, wherein said first set of one or more transcription control elements comprises a signal transducer and activator of transcription #5 element (SAT5).

14.   The cell of claim 1, wherein said first or second measurable tag is a luciferase.

15.   The cell of claim 14, wherein said luciferase is Renilla luciferase.

16.   The cell of claim 14, wherein said luciferase is firefly luciferase.

17.   The cell of claim 1, wherein said first and second measurable tags are different luciferases.

18.   The cell of claim 1, which has the property that it will maintain the activity of ligand-specific signal transduction to the nucleus for at least about one hour but will lose said ligand-specific signal transduction activity and undergo cellular death in no more than 30 days at a temperature above freezing.

19.   The cell of claim 18, which has the property that it will maintain the activity of ligand-specific signal transduction to the nucleus for at least about eighteen hours but will lose said ligand-specific signal transduction activity and undergo cellular death in no more than 14 days at a temperature above freezing.

20.   The cell of claim 18, which has the property that it will maintain the activity of ligand-specific signal

transduction to the nucleus for at least about eight hours but will lose said ligand-specific signal transduction activity and undergo cellular death in no more than 24 hours at a temperature above freezing.

21.   The cell of claim 18, wherein the cell has been treated with an anti-mitotic or pro-apoptotic agent so as to acquire said property.

22.   The cell of claim 21, wherein the treated cell, substantially immediately after being treated, has been resuspended in a solution containing a cryopreservative and frozen at about -80°C.

23.   The cell of claim 22, wherein said cryopreservative is dimethylsulfoxide (DMSO) and said solution contains 10% DMSO.

24.   The cell of claim 22, wherein said cryopreservative is a combination of 2.5% dimethylsulfoxide (DMSO) and 10% glycerol.

25.   The cell of claim 21, wherein said anti-mitotic or pro-apoptotic agent is vinblastin.

26.   The cell of claim 21, wherein said anti-mitotic or pro-apoptotic agent is 5-fluorouracil.

27.   The cell of claim 21, wherein said anti-mitotic or pro-apoptotic agent is mitomycin C, an anti-tumor intercalating agent.

28.   The cell of claim 21, wherein said anti-mitotic or pro-apoptotic agent is γ-irradiation and wherein the cell has been irradiated with γ radiation at an intensity and for a sufficient amount of time such that the cell line maintains said ligand-specific signal transduction activity for at least about one hour but loses said ligand-specific signal transduction activity and undergoes cellular death in no more than about 30 days at a temperature above freezing.

29.   The cell of claim 1, which is a mammalian or avian cell.

30.   The cell of claim 1, which is a human cell.

31.   The cell of claim 1, which is a human promonocytic cell.

32.   The cell of claim 31, wherein the human promonocytic cell is a U937 cell.

33.   A kit for determining the level in a sample of an extracellular ligand that initiates a ligand-specific signal at the nucleus of a cell, comprising:

a reagent containing a plurality of the cell of claim 1; and

either a testing device having a plurality of wells or a container.

34.   The kit of claim 33, wherein said reagent is disposed in the wells of said testing device or in said container.

35.   The kit of claim 33, wherein said reagent is frozen.

36.   The kit of claim 33, wherein the level of the extracellular ligand determined in the sample is to be used to determine the level of neutralizing antibodies either against the extracellular ligand or against an antagonist to the extracellular ligand.

37.   A kit for determining the level in a sample of an extracellular ligand that initiates a ligand-specific at the nucleus of a cell, comprising:

a reagent containing a plurality of the cell of claim 22 in a frozen state; and

either a testing device having a plurality of wells or a container.

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38.   The kit of claim 37, wherein said reagent is disposed in the wells of said testing device or in said container.

39.   The kit of claim 37, wherein the level of the extracellular ligand determined in the sample is used to determine the level of neutralizing antibodies either against the extracellular ligand or against an antagonist to the extracellular ligand.

40.   A method for determining the level in a sample of an extracellular ligand that initiates a ligand-specific signal at the nucleus of a cell or of a neutralizing antibody either against the extracellular ligand or against an antagonist of the extracellular ligand, comprising:

incubating the cell of claim 1 in a mixture with a sample in which the level of the extracellular ligand or the neutralizing antibody is sought to be determined; and

determining the level of the first measurable tag in the mixture relative to the level of the first measurable tag in the absence of the sample to thereby determine the level in the sample of the extracellular ligand or neutralizing antibody.

41.   A method for determining the level in a sample of an extracellular ligand that initiates a ligand-specific signal at the nucleus of a cell or of a neutralizing antibody either against the extracellular ligand or against an antagonist of the extracellular ligand, comprising:

thawing the cell of claim 22 from its frozen state within a period of time that the thawed cell maintains the ligand-specific signal transduction activity;

incubating the thawed cell in a mixture with a sample in which the level of the extracellular ligand or the neutralizing antibody is sought to be determined; and

determining the level of the first measurable tag in the mixture relative to the level of the first measurable tag in the absence of the sample to thereby determine the level in the sample of the extracellular ligand or neutralizing antibody.
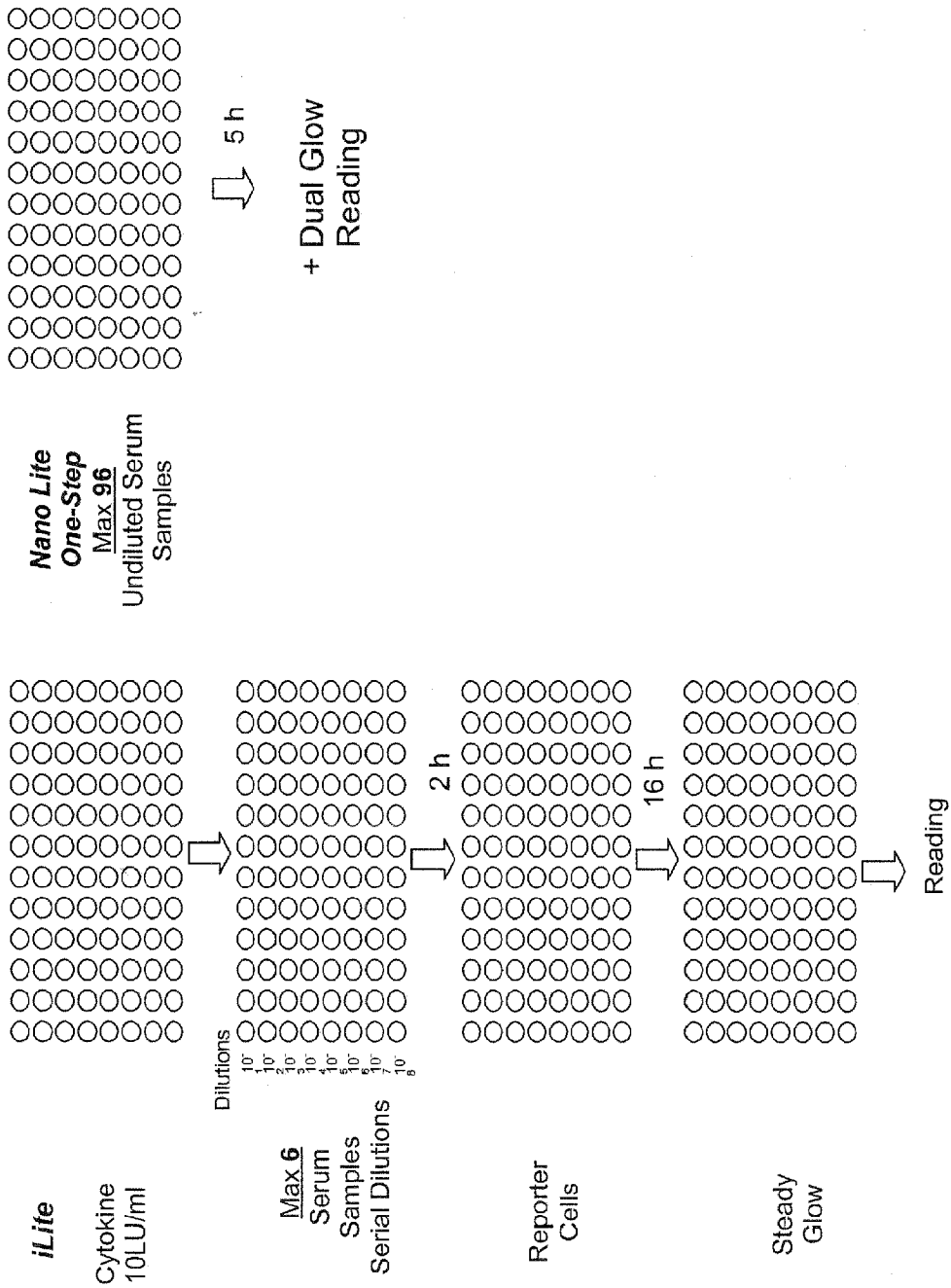
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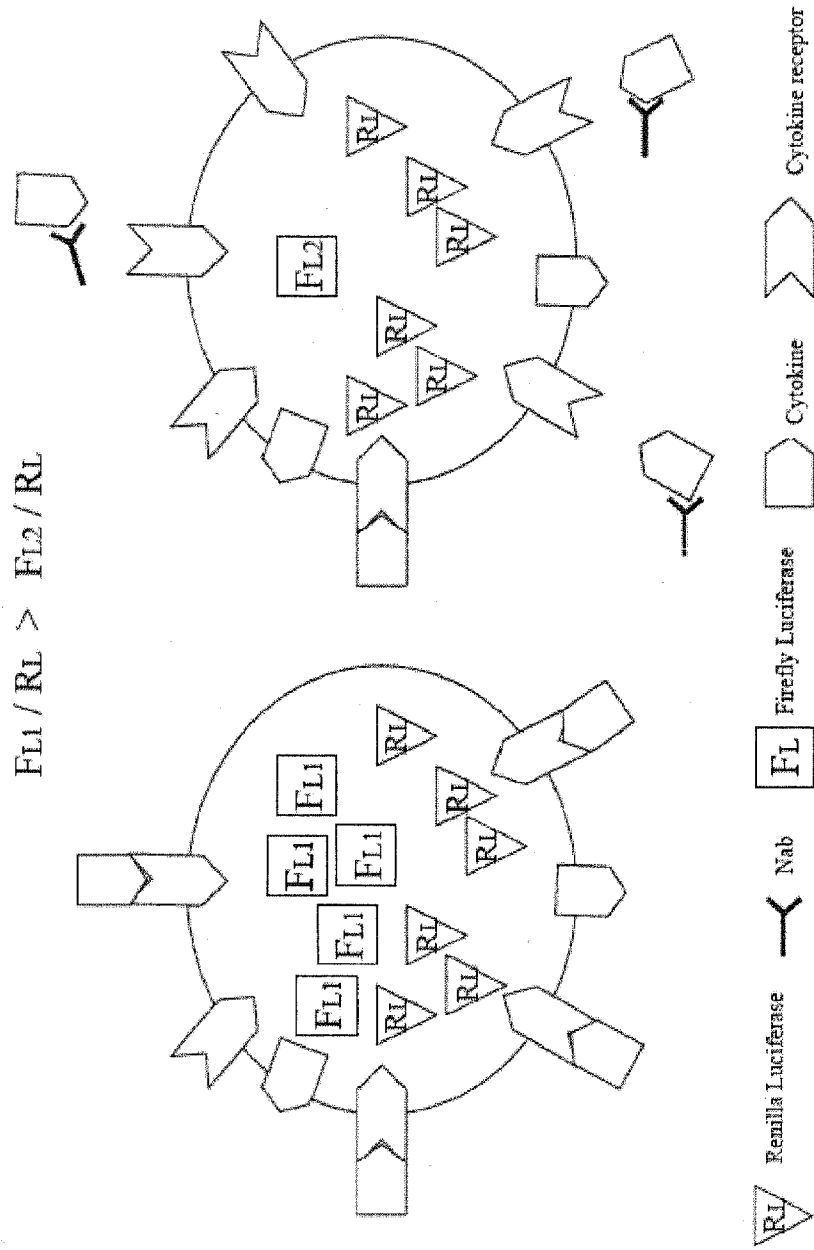
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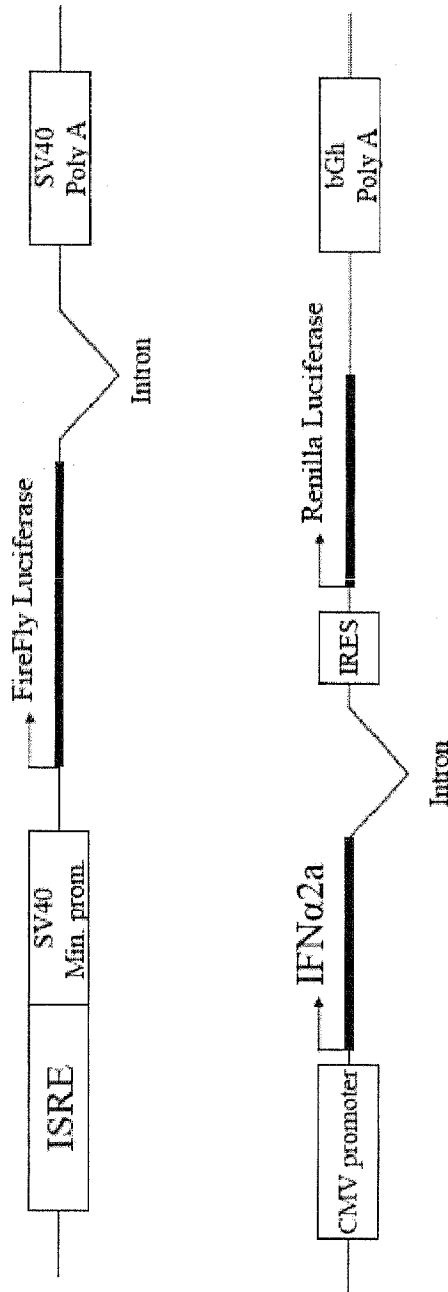
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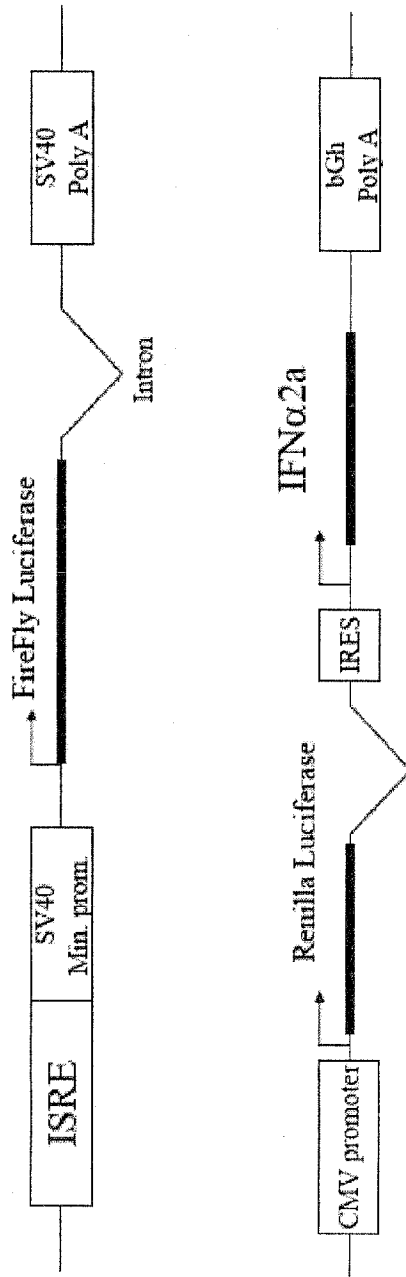
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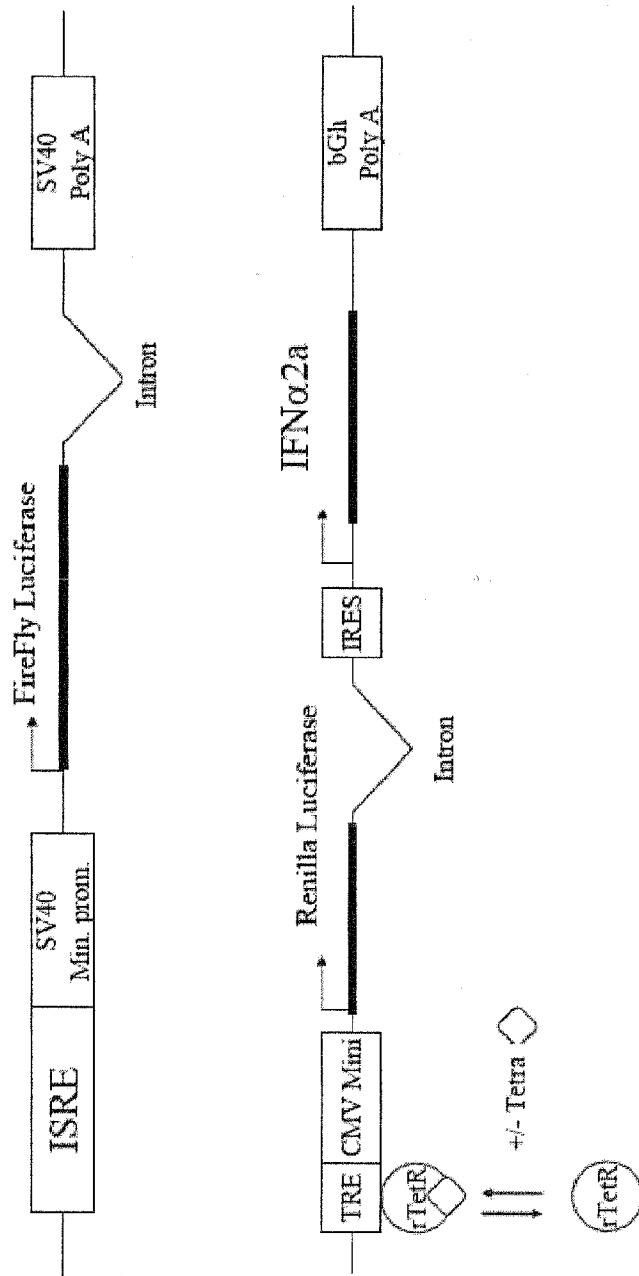
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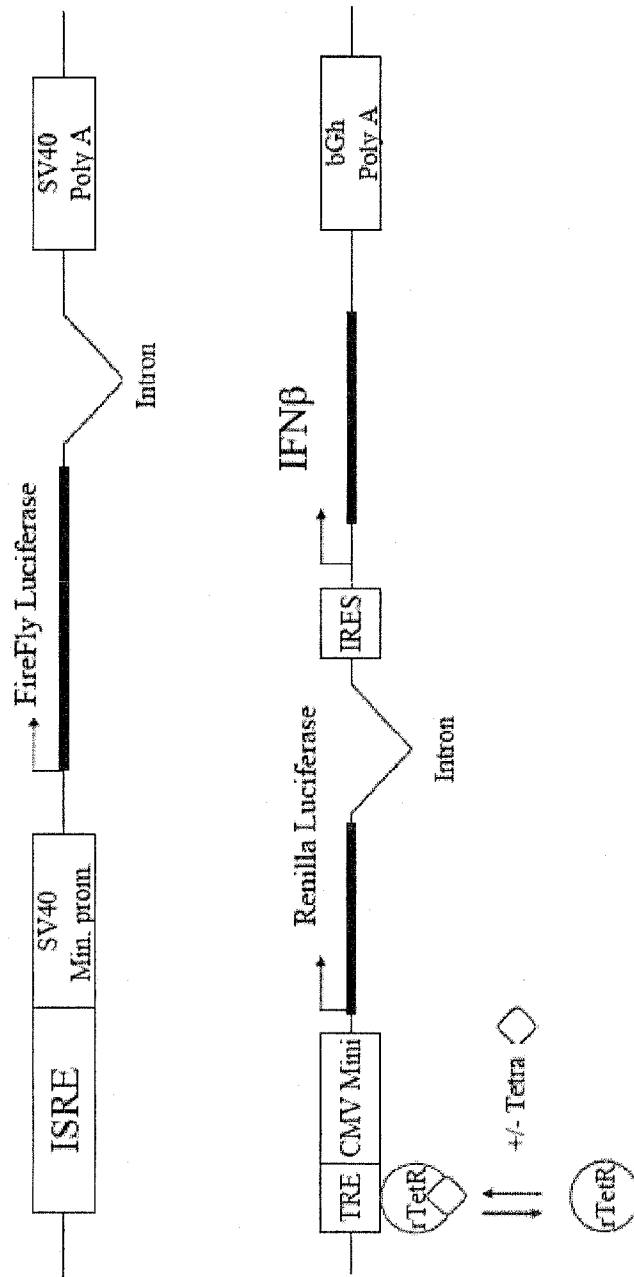
Fig. 6
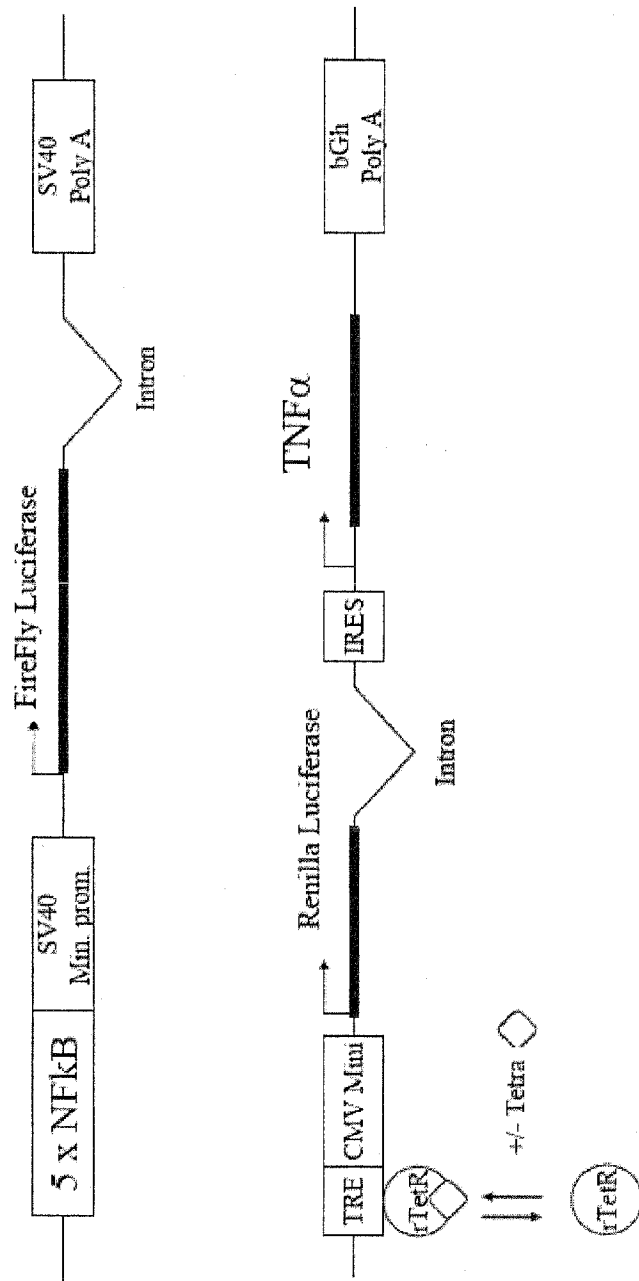
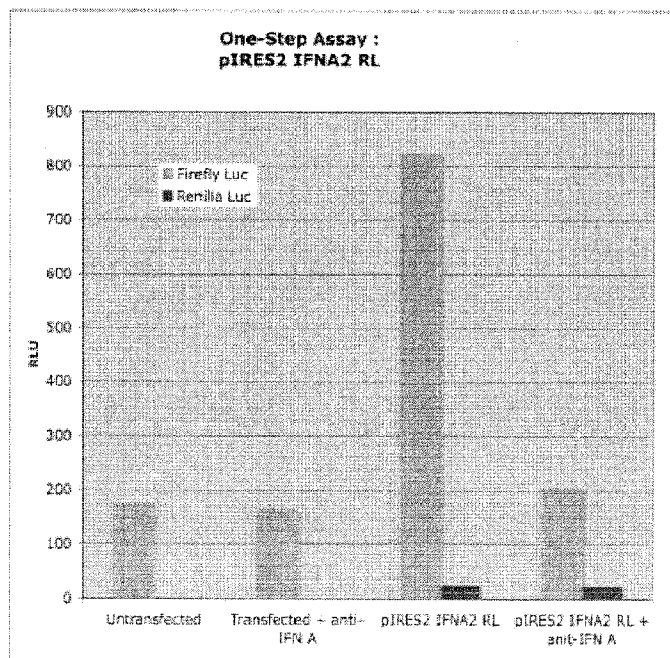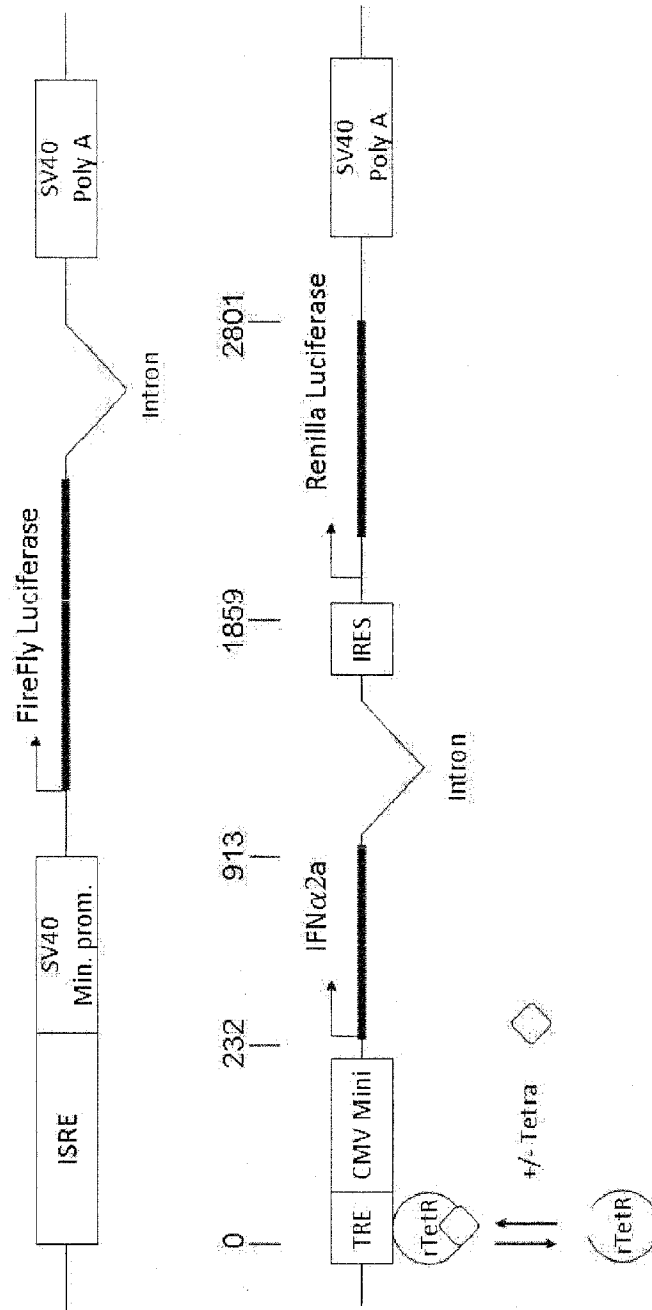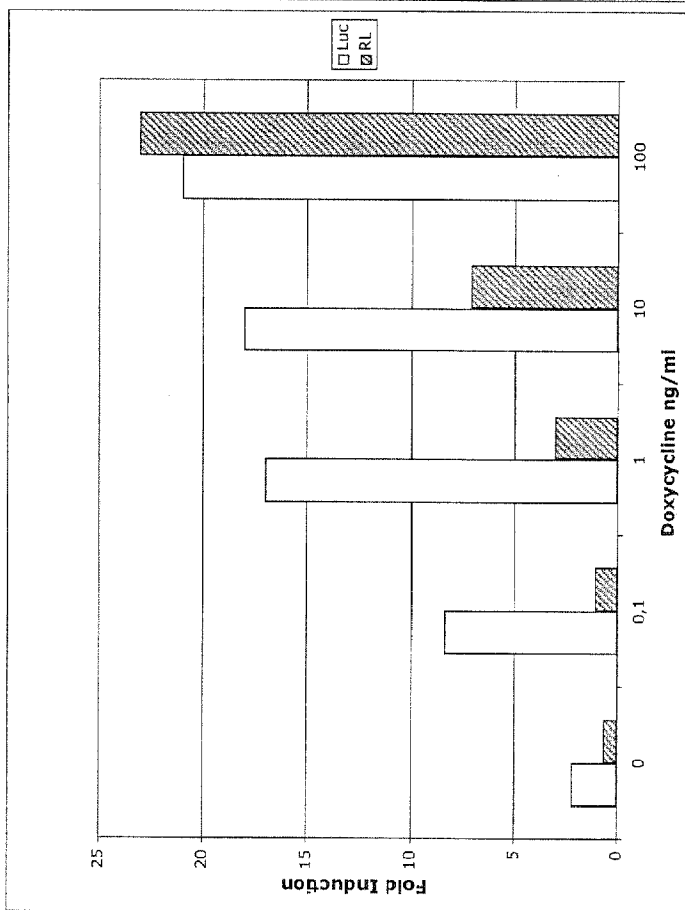**Fig. 7**
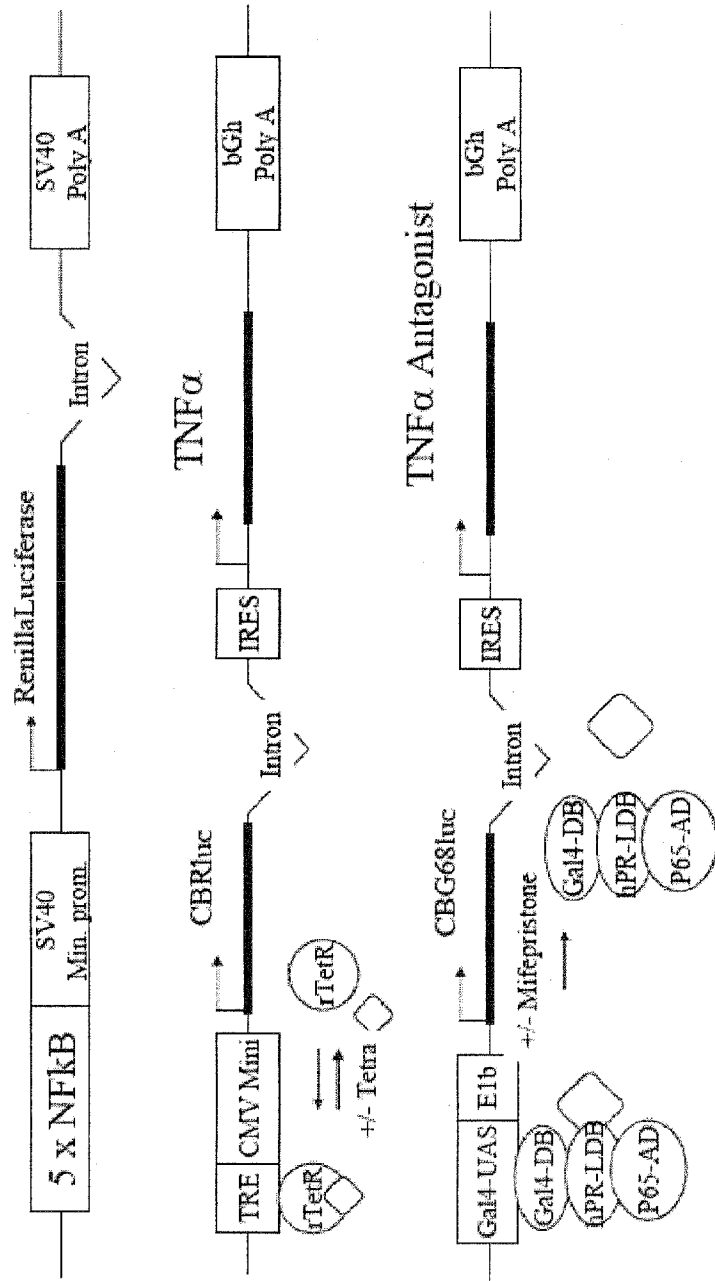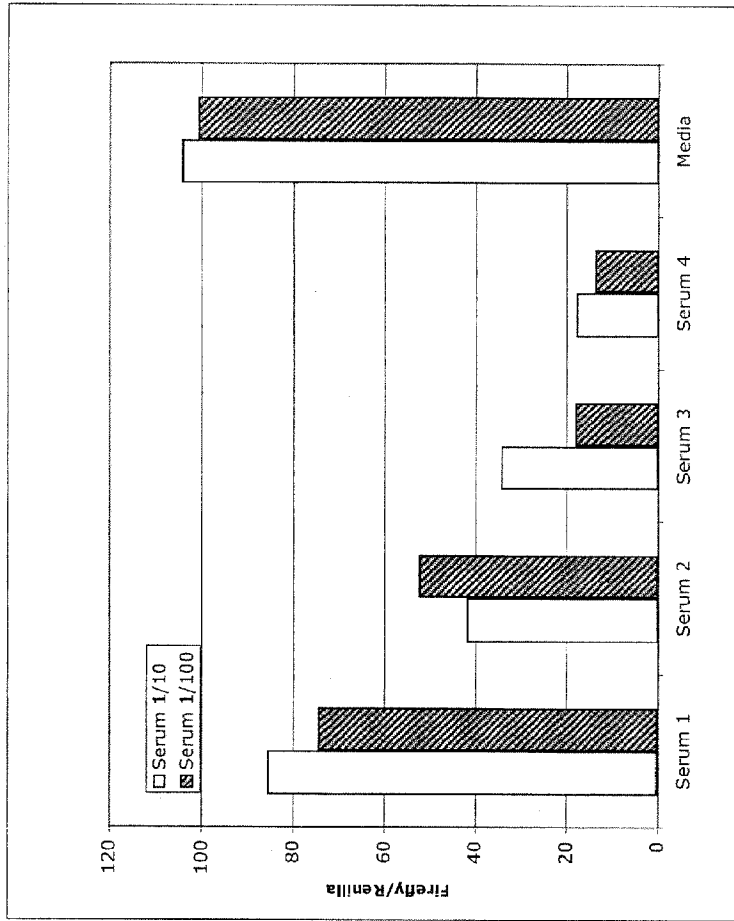
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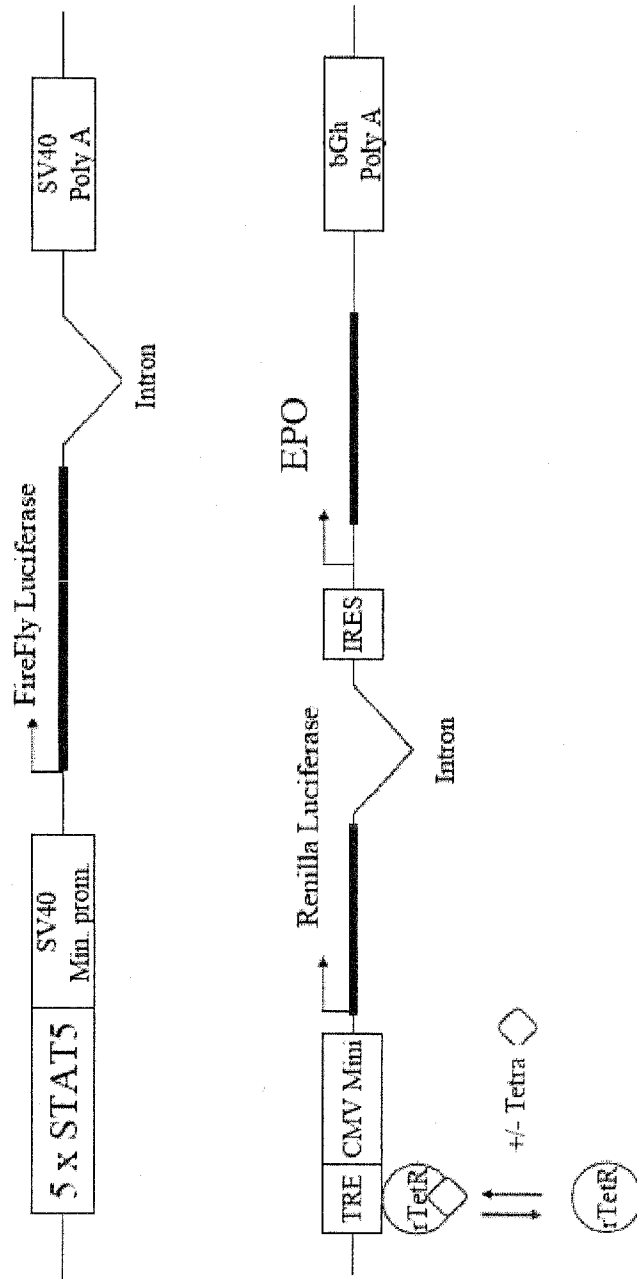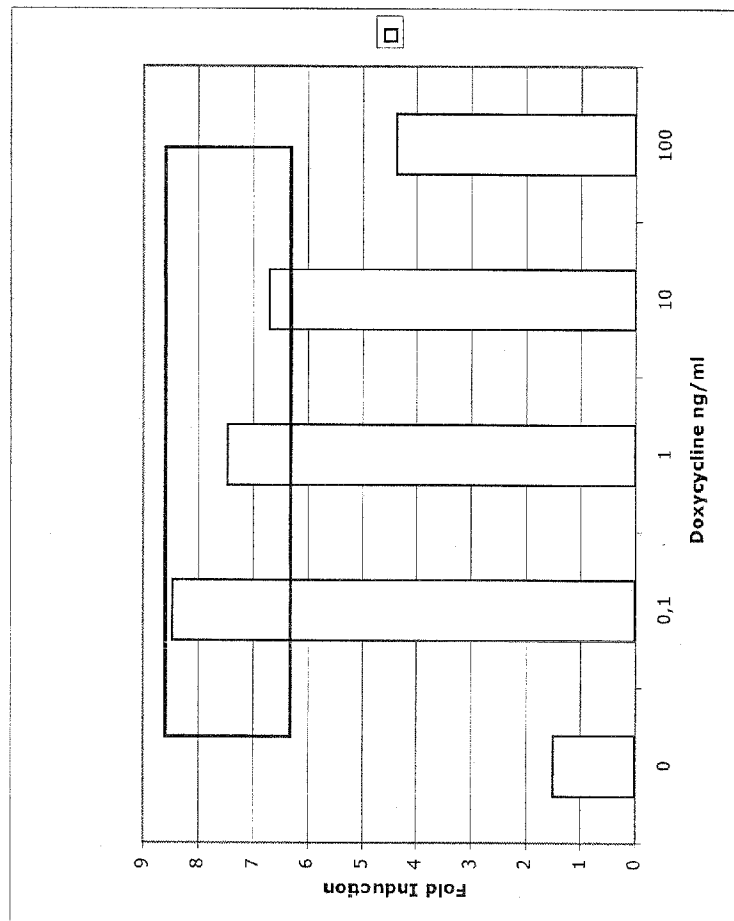
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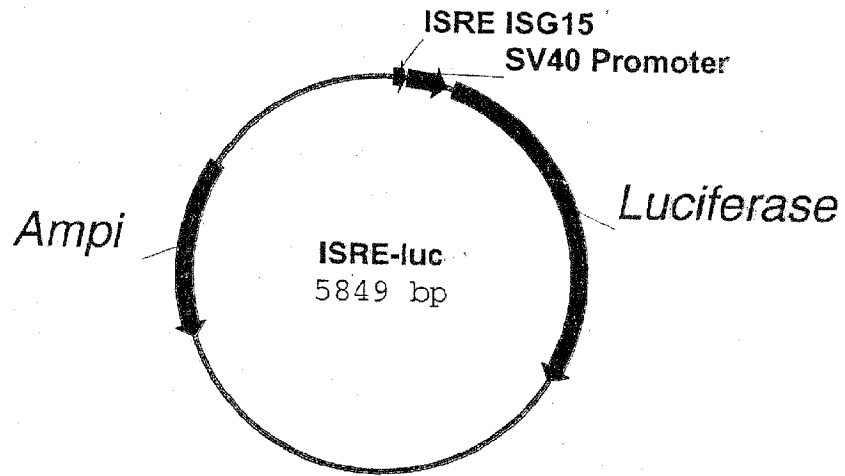
Fig. 10
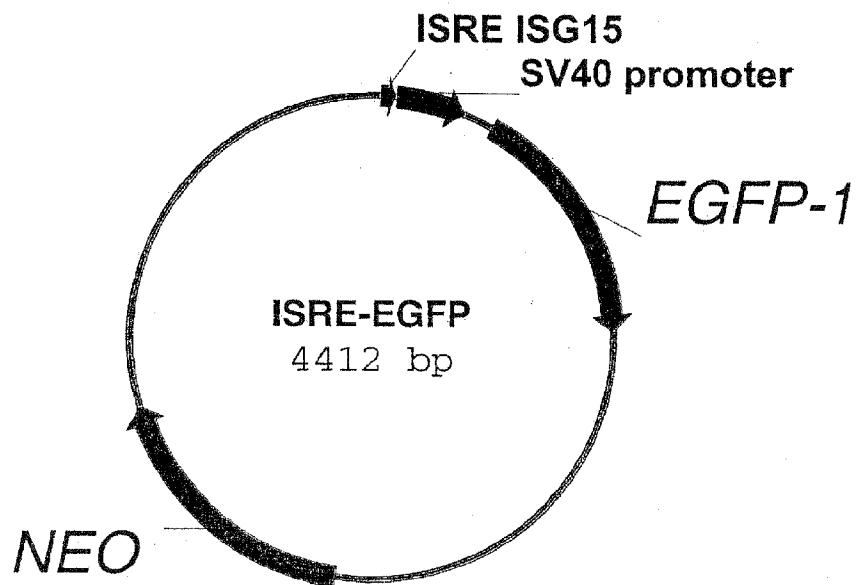
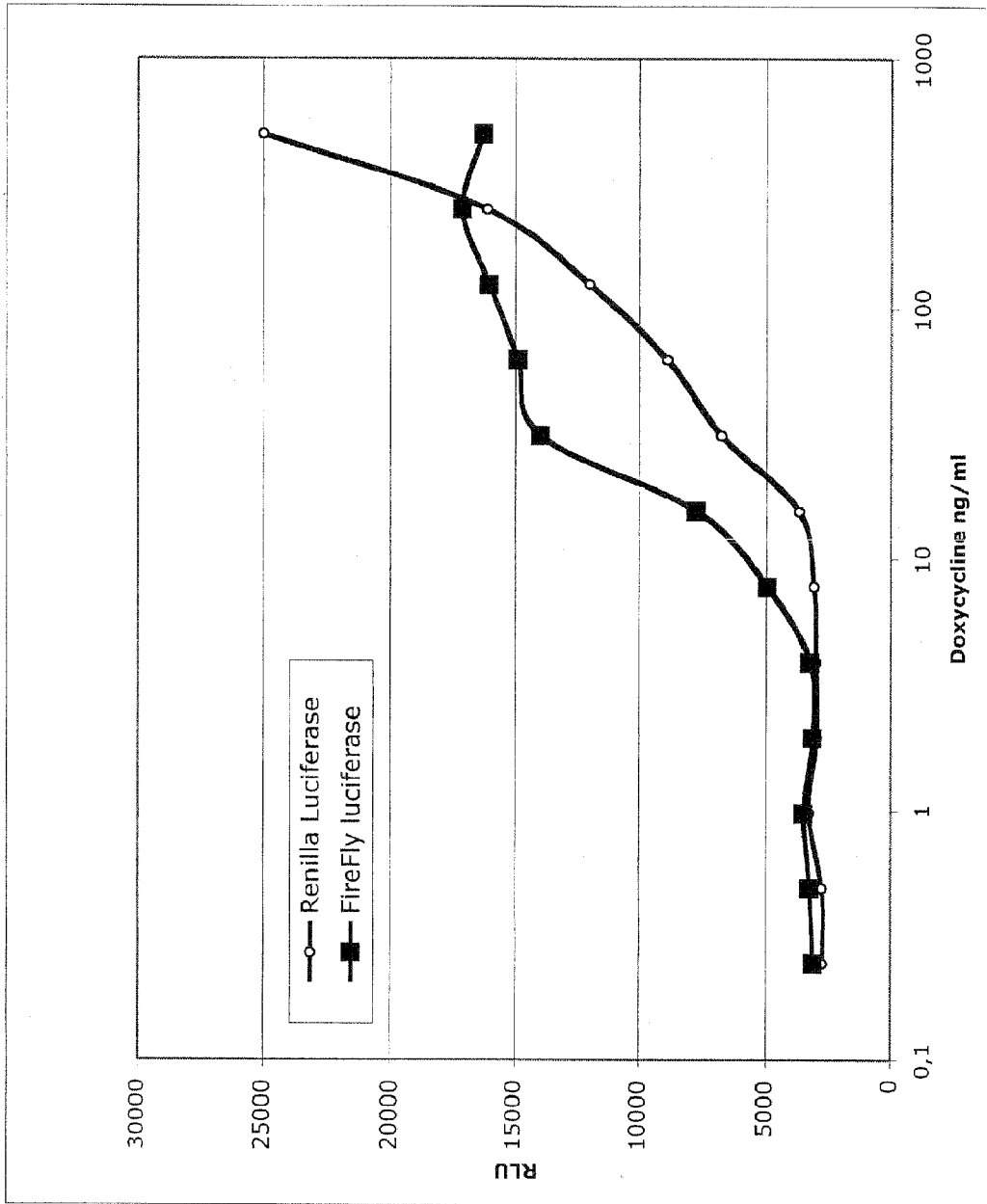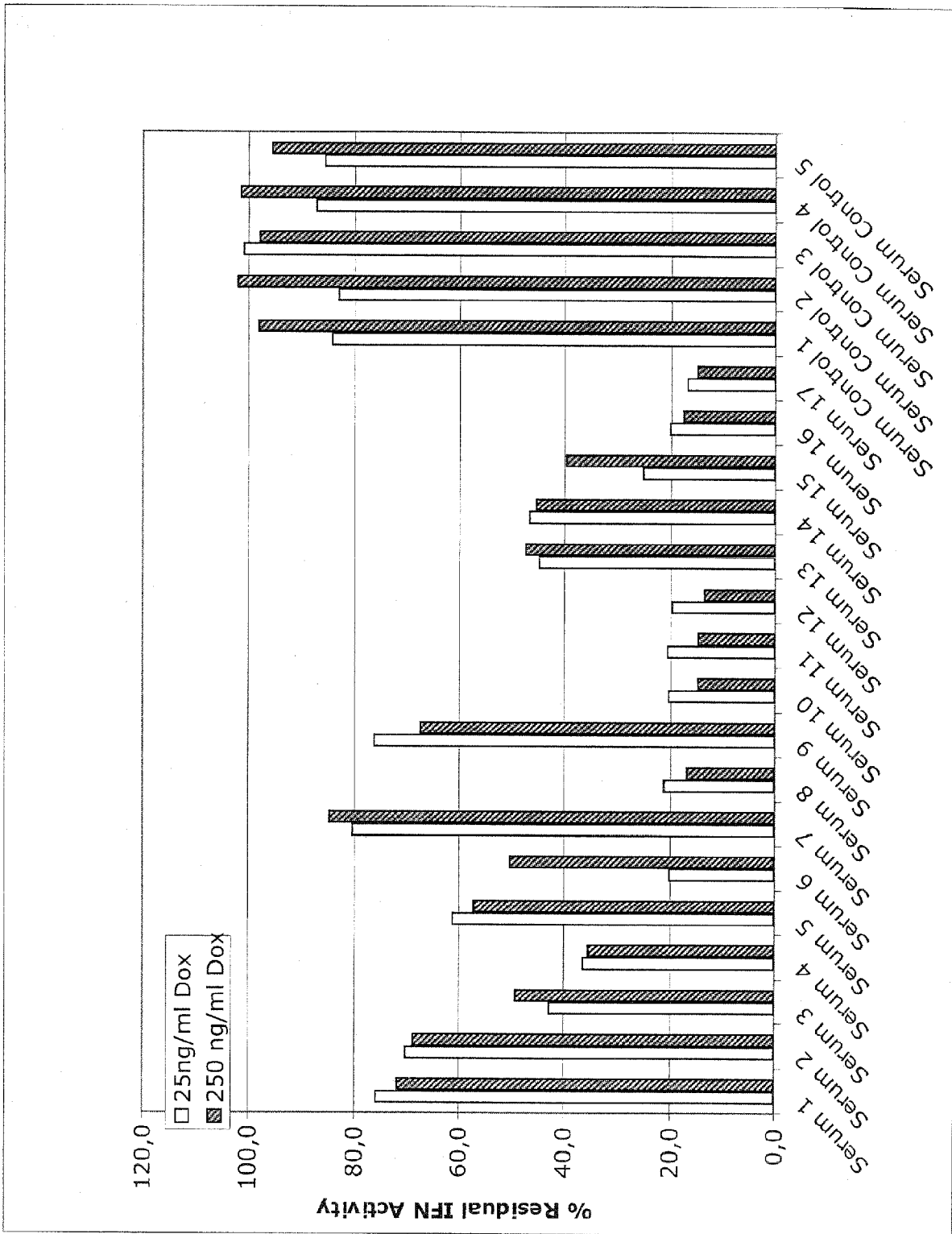Fig. 11
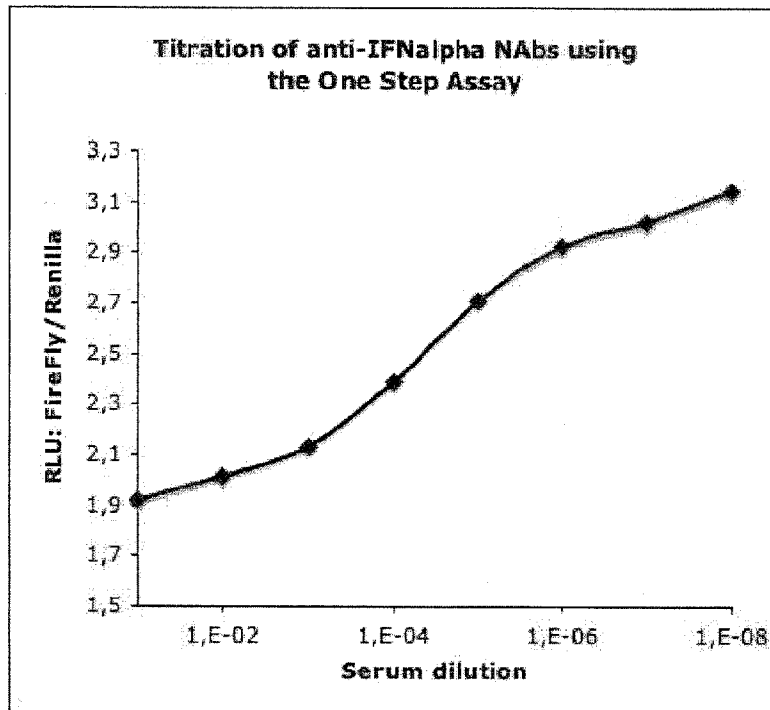
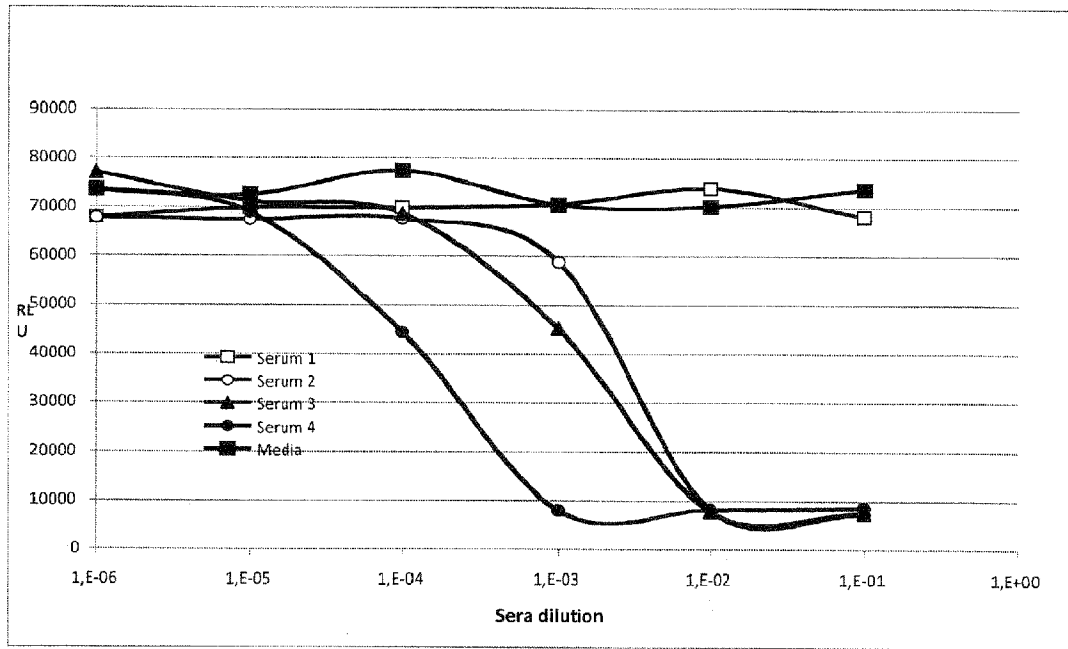**Fig. 12**

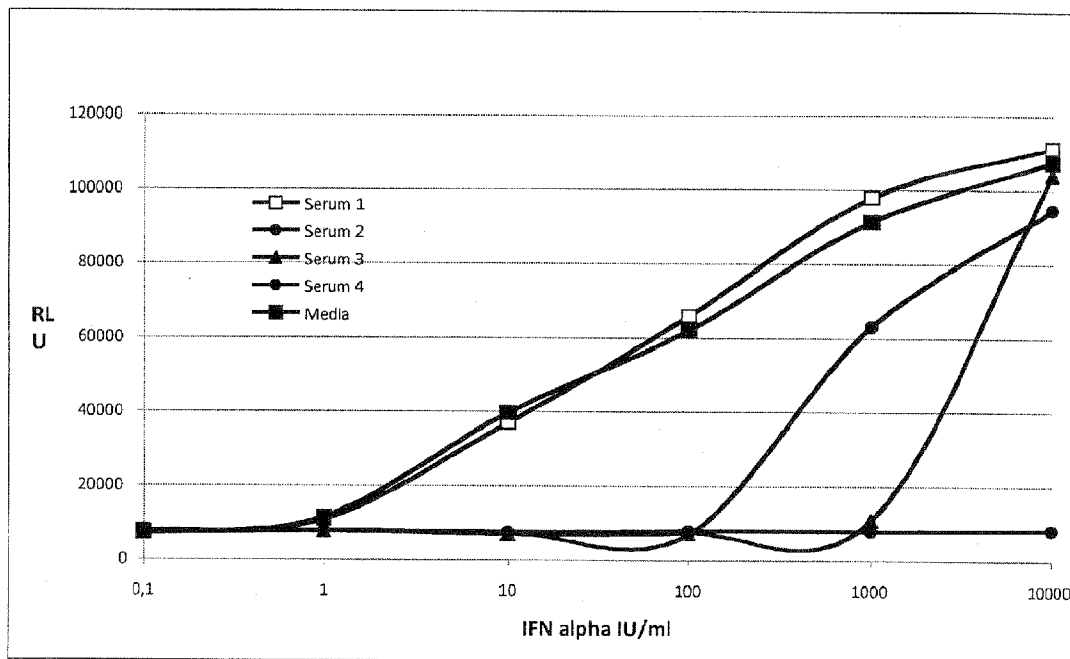

Fig. 13

Fig. 14

Fig. 15B

Fig. 15A

Fig. 16B

Fig. 16A

Fig. 17

Fig. 18

Fig. 19A

Fig. 19B

Fig. 20A

Fig. 20B

Fig. 20C