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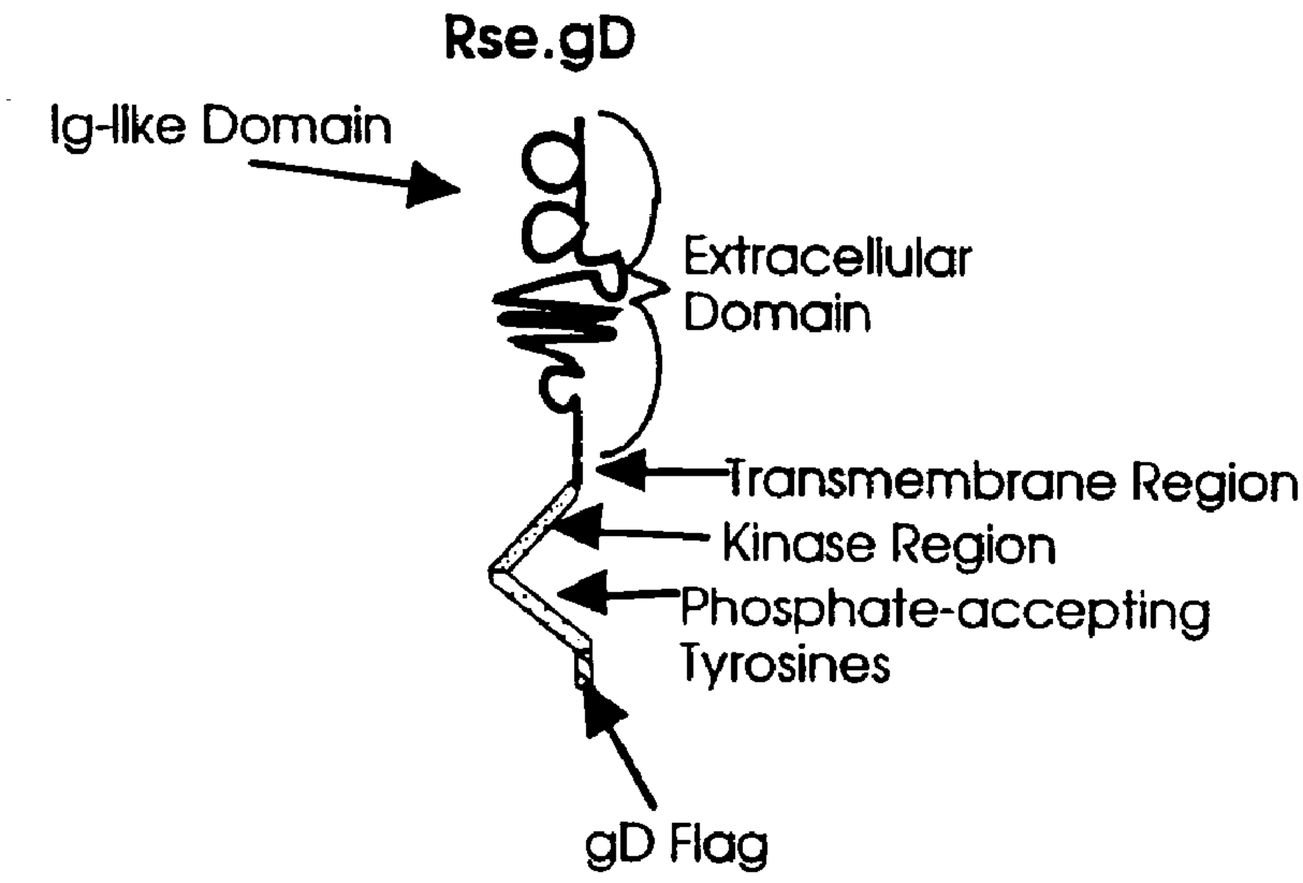
(54) **METHODE D'ACTIVATION DU RECEPTEUR DE KINASE**
(54) **KINASE RECEPTOR ACTIVATION ASSAY**



(57) L'invention se rapporte à une méthode de mesure de l'activation (c'est-à-dire l'autophosphorylation) d'un récepteur de tyrosine kinase d'intérêt. a) Une première phase solide est recouverte d'une population de cellules pratiquement homogène de sorte que ces dernières adhèrent à la première phase solide. Les cellules ont soit un récepteur de tyrosine kinase endogène, soit ont été transformées par l'ADN codant un récepteur ou "un produit de recombinaison du récepteur", et l'ADN a été exprimé de sorte que le récepteur ou le produit de recombinaison du récepteur soit présent dans les membranes des cellules. b) Un ligand est alors ajouté à la

(57) An assay for measuring activation (i.e., autophosphorylation) of a tyrosine kinase receptor of interest is disclosed. a) A first solid phase is coated with a substantially homogeneous population of cells so that the cells adhere to the first solid phase. The cells have either an endogenous tyrosine kinase receptor or have been transformed with DNA encoding a receptor or "receptor construct" and the DNA has been expressed so that the receptor or receptor construct is presented in the cell membranes of the cells. b) A ligand is then added to the solid phase having the adhering cells, such that the tyrosine kinase receptor is exposed to the ligand. c)

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phase solide comportant les cellules d'adhésion de sorte que le récepteur de tyrosine kinase soit mis en contact avec le ligand. c) Après la mise en contact du récepteur avec le ligand, les cellules d'adhésion sont solubilisées, libérant ainsi le lysat cellulaire. d) Une seconde phase solide est recouverte d'un agent de capture qui se fixe spécifiquement au récepteur de tyrosine kinase, ou, dans le cas d'un produit de recombinaison du récepteur, au polypeptide drapeau. e) Le lysat cellulaire obtenu à l'étape c) est ajouté aux puits contenant l'agent de capture d'adhésion de façon à capturer le récepteur ou le produit de recombinaison du récepteur dans les puits. f) Une étape de lavage est ensuite effectuée de façon à retirer le lysat cellulaire non lié, laissant le récepteur ou le produit de recombinaison du récepteur capturé. g) Le récepteur ou le produit de recombinaison du récepteur est mis en contact avec un anticorps anti-phosphotyrosine marqué qui identifie les restes phosphorylés du récepteur de tyrosine kinase. h) On mesure la liaison de l'anticorps anti-phosphotyrosine au récepteur ou au produit de recombinaison du récepteur capturé.

Following exposure to the ligand, the adherent cells are solubilized, thereby releasing cell lysate. d) A second solid phase is coated with a capture agent which binds specifically to the tyrosine kinase receptor, or, in the case of a receptor construc, to the flag polypeptide. e) The cell lysate obtained in step c) is added to the wells containing the adhering capture agent so as to capture the receptor or receptor construct to the wells. f) A washing step is then carried out, so as to remove unbound cell lysate, leaving the captured receptor or receptor construct. g) The captured receptor or receptor construct is exposed to a labelled anti-phosphotyrosine antibody which identifies phosphorylated residues in the tyrosine kinase receptor. h) Binding of the anti-phosphotyrosine antibody to the captured receptor or receptor construct is measured.

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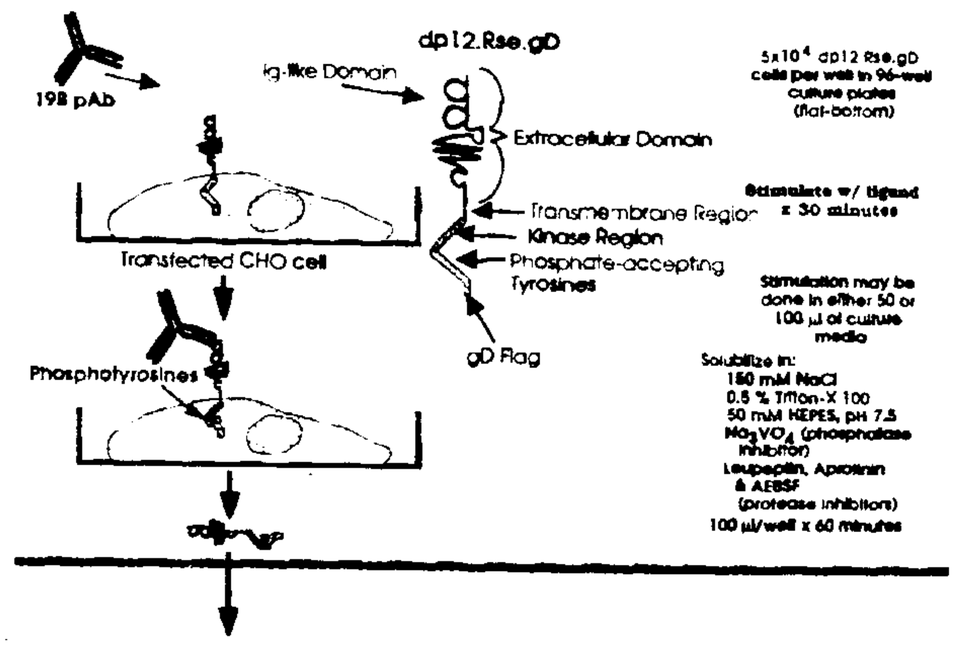
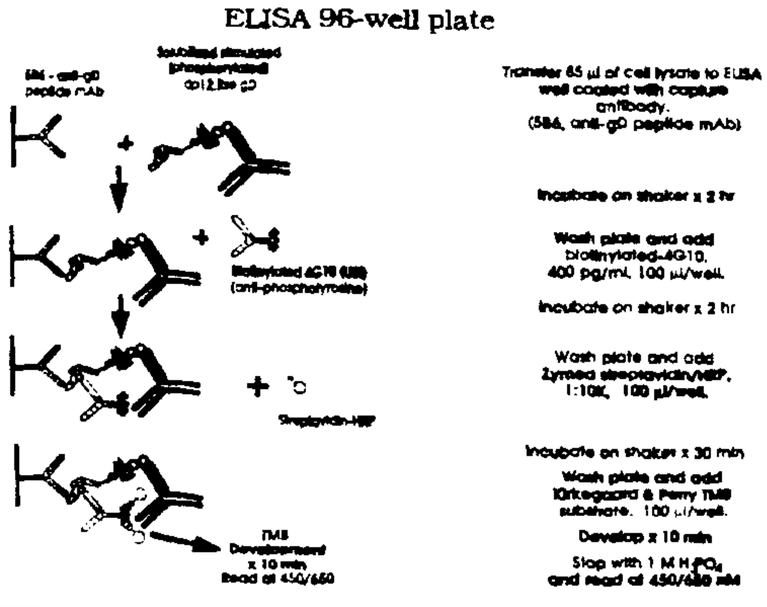
(54) Title: KINASE RECEPTOR ACTIVATION ASSAY

(57) Abstract

An assay for measuring activation (i.e., autophosphorylation) of a tyrosine kinase receptor of interest is disclosed. a) A first solid phase is coated with a substantially homogeneous population of cells so that the cells adhere to the first solid phase. The cells have either an endogenous tyrosine kinase receptor or have been transformed with DNA encoding a receptor or "receptor construct" and the DNA has been expressed so that the receptor or receptor construct is presented in the cell membranes of the cells. b) A ligand is then added to the solid phase having the adhering cells, such that the tyrosine kinase receptor is exposed to the ligand. c) Following exposure to the ligand, the adherent cells are solubilized, thereby releasing cell lysate. d) A second solid phase is coated with a capture agent which binds specifically to the tyrosine kinase receptor, or, in the case of a receptor construc, to the flag polypeptide. e) The cell lysate obtained in step c) is added to the wells containing the adhering capture agent so as to capture the receptor or receptor construct to the wells. f) A washing step is then carried out, so as to remove unbound cell lysate, leaving the captured receptor or *receptor construct. g) The captured receptor* or receptor construct is exposed to a labelled anti-phosphotyrosine antibody which identifies phosphorylated residues in the tyrosine kinase receptor. h) Binding of the anti-phosphotyrosine antibody to the captured receptor or receptor construct is measured.





KINASE RECEPTOR ACTIVATION ASSAY

BACKGROUND OF THE INVENTION

Field of the Invention

This invention relates to a kinase receptor activation (KIRA) assay. In particular, the invention relates to an assay for measuring autophosphorylation of the kinase domain of a receptor protein tyrosine kinase (rPTK) using a kinase receptor activation, enzyme-linked immunosorbent assay (KIRA ELISA).

Description of Related Art

One mechanism for signal transduction in animals involves protein phosphorylation. Protein phosphorylation involves the action of protein kinase, an enzyme that transfers a phosphate group from a phosphate donor onto an acceptor amino acid in a substrate protein. Protein phosphatases provide a means for reversing the signal when the stimulus is removed.

Protein kinases have multiple substrates, and classification of the protein kinases is based on the acceptor amino acid specificity. The two most well characterized protein kinases are the protein kinases with a protein alcohol group as acceptor called protein serine/threonine kinases and the protein kinases with a protein phenolic group as acceptor called protein tyrosine kinases (Hunter, Methods in Enzymology **200**:3-9 [1991]).

The most well known type of signal-transducing protein kinases are growth factor receptor protein tyrosine kinases (rPTKs). rPTKs usually comprise a large, glycosylated, extracellular ligand binding domain (ECD) and an intracellular domain (ICD) which contains a tyrosine kinase catalytic domain. A single hydrophobic transmembrane (TM) domain connects the ECD and ICD. Examples of rPTKs include the insulin receptor, epidermal growth factor receptor (EGF-R), platelet-derived growth factor receptor (PDGF-R), insulin-like growth factor 1 receptor (IGF-1-R), and the HER2 receptor, to name a few. See, for example, Ullrich and Schlessinger Cell **61**:203-212 (1990) and Fantl *et al.*, Annu. Rev. Biochem. **62**:453-481 (1993). rPTKs can phosphorylate exogenous protein substrates and intrinsic tyrosine residues via their catalytic tyrosine kinase domain. The intrinsic tyrosine residues normally reside in the ICD of the rPTK (see Figure 1 herein). Activation of the intracellular kinase domain of rPTKs appears to be mediated by receptor oligomerization which results from the conformational alteration of the ECD upon ligand binding thereto. See

Ullrich and Schlessinger, *supra*.

Serine-threonine kinases have also been disclosed in the literature. While most of the known protein serine-threonine kinases are cytoplasmic proteins, a family of mammalian transmembrane receptors with serine-threonine kinase domains has recently been found. Members of this receptor family have been described as binding TGF-β's and activin. For reviews of serine-threonine kinases, see Sale, G., Biochem. Soc. Transactions 20: 664-670 (1992); ten Dijke *et al.*, Prog. in Growth Factor Res. 5: 55-72 (1994); and Mathews, L., Endoc. Rev. 15(3): 310-325 (1994).

Various assays have been developed which measure tyrosine kinase activity. Some of these assays measure the ability of a tyrosine kinase enzyme to phosphorylate a synthetic substrate polypeptide. For example, an assay has been developed which measures growth factor-stimulated tyrosine kinase activity by measuring the ability of the kinase to catalyze the transfer of the γ-phosphate of ATP to a suitable acceptor substrate. See Pike, L., Methods of Enzymology **146**:353-362 (1987) and Hunter, Journal of Biological Chemistry **257(9)**:4843-4848 (1982), for example. In this assay, the use of [γ-^{32}P]ATP permits the radioactive labeling of the phosphorylated substrate, which is a synthetic tyrosine-containing peptide. Others have described protein kinase assays wherein incorporation of ^{32}P into a tyrosine kinase receptor, such as the EGF receptor (see Donato *et al.*, Cell Growth Differ. 3:259-268 [1992]), insulin receptor (see Kasuga *et al.*, Journal of Biological Chemistry **257(17)**:9891-9884 [1982] and Kasuga *et al.*, Methods in Enzymology **109**:609-621 [1985]), and liver growth hormone receptor (see Wang *et al.*, Journal of Biological Chemistry **267(24)**:17390-17396 [1992]), is measured.

The discovery of anti-phosphotyrosine antibodies has provided a non-radioactive, alternative means for measuring phosphorylation of tyrosine residues. For example, White and Backer (Methods in Enzymology **201**:65-67 [1991]) mention polyclonal antibodies which selectively bind to phosphotyrosine and are considered to be useful for studying rPTKs. An anti-phosphotyrosine monoclonal antibody was used in one of the assays referred to in Madden *et al.* (Anal Biochem **199**:210-215 [1991]), which measured phosphatase activity toward the insulin receptor. Anti-phosphotyrosine antibodies were also used by Cleaveland *et al.*, in their protein tyrosine kinase ELISA assay. See Cleaveland *et al.*, Analytical Biochemistry **190**:249-253 (1990). The method of Cleaveland *et al.* utilizes purified high-activity oncogene tyrosine kinases, v-src and v-fps, and

measures the ability of these tyrosine kinases to phosphorylate synthetic polymeric substrates which are coated on an ELISA microtiter plate. The phosphotyrosine produced by src-induced phosphorylation of the polymeric substrate is then quantitated by addition of an anti-phosphotyrosine antibody, the presence of which is detected using a second rabbit anti-mouse antibody which is linked to a reporter enzyme, horseradish peroxidase (HRPO). A similar ELISA assay has been developed by Lazaro *et al.*, which is used for detection of a protein tyrosine kinase. See Lazaro *et al.*, Analytical Biochemistry **192**:257-261 (1991). Like the assay of Cleaveland *et al.*, this assay also measures the ability of a protein tyrosine kinase to phosphorylate a synthetic substrate which is bound to microELISA wells.

A direct way to assess specific activation of rPTKs is by analysis of receptor autophosphorylation. See, *e.g.*, Hunter and Cooper Ann Rev Biochem **54**:897-930 (1985) and Ullrich and Schlessinger, Cell **61**:203-212 (1990). Using this direct approach, Knutson and Buck disclose assays for measuring autophosphorylation of the insulin receptor under *in situ* or *in vitro* conditions (Archives of Biochemistry and Biophysics **285(2)**:197-204 [1991]). In the *in situ* assay, monolayer cultures of embryonic mouse 3T3-C2 fibroblasts (having the endogenous insulin receptor) are incubated with insulin in large cell culture dishes. Following incubation, the insulin receptor is extracted from the membranes. To achieve extraction of the insulin receptor, the cell monolayers are scraped into a buffer containing protease inhibitors and the cells are then disrupted in a homogenizer. The cellular homogenate is subsequently subjected to centrifugation for 60 min., and the pellet which forms is extracted into buffer containing detergent. Following a further centrifugation step, the supernatant (containing the insulin receptor) is incubated with an anti-insulin receptor antibody. Then, the receptor-antibody complex is incubated with protein A-agarose and unoccupied protein A sites are blocked with normal rabbit IgG. The agarose beads are then centrifuged, the supernatants aspirated and the pellets are re-suspended in buffer containing the radiolabelled anti-phosphotyrosine antibody. The amount of bound iodinated anti-phosphotyrosine antibody is consequently measured.

Klein and his colleagues discuss an assay for measuring insulin activation of the insulin receptor (Klein *et al.*, Diabetes **42**:883-890 [1993]). In this assay, aliquots of a heterogeneous population of mononuclear blood cells (including T cells, B cells, macrophages etc) having the insulin receptor are exposed to insulin in centrifuge tubes.

The cells are then lysed in detergent using a motordriven homogenizer and the lysates are concentrated two- to fourfold using vacuum centrifugation. Sometimes, the insulin receptor is also partially purified using wheat germ agglutin agarose. The supernatants which form following centrifugation, are then transferred to anti-insulin receptor-coated microtiter plates. Insulin (8.7nM) as well as kinase and phosphatase inhibitors are present during receptor immobilization in order to optimize the percentage of receptors captured to the microtiter plates. Activation of the insulin receptor is then measured by transphosphorylation of the substrate Poly-Glu,Tyr with ^{32}P labeled ATP. The supernatants are then spotted onto absorbent paper and the paper is washed with cold TCA to remove unbound ^{32}P-ATP. Remaining ^{32}P-labeled Poly-Glu,Tyr on the washed absorbent paper is subsequently counted by scintillation counting.

Hagino *et al.* were also interested in studying the insulin receptor in patients (Hagino *et al.*, Diabetes **43**:274-280 [1994]). As a first step in the assay, Hagino *et al.* stimulate a primary cell suspension, which is not particularly homogeneous with respect to cell type. In particular, heparinized blood (1ml washed twice with medium and resuspended in 1 ml of medium containing bovine serum albumin, BSA) is exposed to varying concentrations of insulin. The autophosphorylation reaction is stopped, the cells centrifuged for 30 min, the supernatant is discarded and the erythrocyte ghosts thus obtained are resuspended in buffer and centrifuged again. The pellet thereby obtained is adjusted to 500μl and solubilized in detergent. The solubilized materials are then centrifuged and the resulting supernatant is subjected to sandwich ELISA (using anti-insulin receptor antibodies to capture the insulin receptor) to determine the extent of insulin receptor autophosphorylation.

King *et al.* in Life Sciences 53: 1465-1472 (1993) describe a colorimetric assay for examining inhibitors of the epidermal growth factor (EGF) receptor-associated tyrosine kinase in human intact epidermal A431 cells.

Several others have used an enzyme-conjugated form of the anti-phosphotyrosine antibody in Western blot analyses which measure receptor autophosphorylation. Briefly, Western blotting generally involves electrophoresing activated rPTK on polyacrylamide gel. The rPTK is then transferred to nitrocellulose and immunoblotted with the anti-phosphotyrosine antibody which is labelled to enable detection. See, for example, Wang, Molecular and Cellular Biology **5(12)**:3640-3643 (1985);

Glenney *et al.*, Journal of Immunological Methods **109**:277-285 (1988); Kamps, Methods in Enzymology **201**:101-110 (1991); Kozma *et al.*, Methods in Enzymology **201**:28-43 (1991); Holmes *et al.*, Science **256**:1205-10 (1992); and Corfas *et al.*, PNAS, USA **90**:1624-1628 (1993). However, with Western blot analysis, accurate quantitation can be very tedious. Furthermore, this technique tends to be time- consuming and generally does not allow high sample throughput.

It is an object of the instant invention to provide a sensitive, reliable assay that measures receptor protein tyrosine kinase (rPTK) autophosphorylation. The assay is desirably useful for qualitatively and quantitatively measuring kinase activation as well as facilitating identification and characterization of potential agonists and antagonists for a selected rPTK. It is a further object of the invention to provide an assay which enables ligand-receptor ınteractions to be studied for any selected rPTK.

This assay must have a capacity for high throughput, that is, the ability to reliably evaluate large numbers of samples in a relatively short period of time (*e.g.*, in one day). The assay ideally does not use radioactive materials and is also amenable to automation.

It is a further object, in at least one embodiment of the invention, to provide a generic assay which enables a rPTK of interest to be studied, regardless of whether or not a receptor-specific capture agent having the desired characteristics is available. Furthermore, it is an object of the invention to provide an assay which substantially represents the activity of the tyrosine kinase receptor *in situ*. This is desirable insofar as it reduces the possibility that altered interactions between the receptor and the ligand may occur as a consequence of the receptor not being membrane-bound. Furthermore, if the receptor is a multimeric complex, this assay enables the correctly assembled receptor to be studied. It is an additional object to provide a method for measuring serine-threonine kinase phosphorylation, phosphorylation of intracellular kinases and phosphatase activity.

These and other objects will be apparent to the ordinary artisan upon consideration of the specification as a whole.

SUMMARY OF THE INVENTION

Accordingly, the invention provides an assay for measuring activation (*i.e.*, autophosphorylation) of a tyrosine kinase receptor of interest.

The assay can be divided into two major stages, each of which is generally performed in separate assay plates. The first stage of the assay involves activating the receptor and is termed the kinase receptor activation (KIRA) stage of the assay. The second stage of the assay involves measuring receptor activation. Conveniently, this is achieved using an enzyme-linked immunosorbent assay (ELISA) to measure receptor activation.

The KIRA stage of the assay involves activating a tyrosine kinase receptor which is located in the cell membrane of an eukaryotic cell such that the extracellular domain of the receptor faces the external milieu of the cell, the transmembrane domain is located in the cell membrane and the kinase domain is located intracellularly. This stage of the overall assay involves steps (a) to (c) below:

(a) The first solid phase (*e.g.*, a well of a first assay plate) is coated with a substantially homogeneous population of cells (usually a mammalian cell line) so that the cells adhere to the solid phase. Often, the cells are adherent and thereby adhere naturally to the first solid phase. In one embodiment of the invention, the cells have an endogenous tyrosine kinase receptor presented in the cell membrane as discussed above. In an alternative embodiment, the cells have been transformed with DNA encoding a tyrosine kinase receptor or a "receptor construct" defined further below, which DNA is expressed by the cells such that the receptor or receptor construct is suitably positioned in the cell membranes thereof.

The receptor construct comprises a fusion of a kinase receptor and a flag polypeptide. The flag polypeptide is recognized by the capture agent, often a capture antibody, in the ELISA part of the assay. Use of a receptor construct as disclosed herein is particularly advantageous since it provides a "generic" assay wherein autophosphorylation of any tyrosine kinase receptor can be measured, regardless of whether or not a receptor-specific capture agent having the required characteristics is available. Often, the tyrosine kinase receptor is a fusion protein comprising the ECD of a selected tyrosine kinase and the catalytic ICD (and possibly the transmembrane domain) of another well characterized tyrosine kinase (*e.g.*, the Rse receptor).

(b) An analyte is then added to the wells having the adhering cells, such that the tyrosine kinase receptor is exposed to (or contacted with) the analyte. This assay enables identification of agonist and antagonist ligands for the tyrosine kinase receptor of interest. In order to detect

the presence of an antagonist ligand which blocks binding and/or activation of the receptor by an agonist ligand, the adhering cells are exposed to the suspected antagonist ligand first and then to the agonist ligand (or to a mixture of the agonist and antagonist) so that competitive inhibition of receptor binding and activation can be measured. Also, the assay can identify an antagonist which binds to the agonist ligand and thereby reduces or eliminates its ability to bind to, and activate, the rPTK. To detect such an antagonist, the suspected antagonist and the agonist for the rPTK are incubated together and the adhering cells are then exposed to this mixture of ligands.

(c) Following exposure to the analyte, the adhering cells are solubilized using a lysis buffer (which has a solubilizing detergent therein) and gentle agitation, thereby releasing cell lysate which can be subjected to the ELISA part of the assay directly, without the need for concentration or clarification of the cell lysate. Thus, this assay provides a significant improvement over assays described by Knutson and Buck, *supra*, Klein *et al.*, *supra*, and Hagino *et al.*, *supra*, insofar as it is surprisingly unnecessary to concentrate the cell lysate prior to the ELISA. Furthermore, unlike the other assays, in the instant assay the cells can be lysed in lysis buffer using gentle agitation without the need for homogenizing, centrifuging or clarifying the cells. The cell lysate thus prepared is then ready to be subjected to the ELISA stage of the assay. It has been discovered that, surprisingly, the first assay plate can be stored at freezing temperatures (*i.e.*, at about -20° to -70°C) for significant periods of time (at least 6 months) before the ELISA stage of the assay. This is a significant finding insofar as the KIRA and ELISA stages of the assay can be performed on separate days.

The ELISA component of the assay comprises steps (d) to (h), described below.

(d) As a first step, the second solid phase (usually a well of an ELISA microtiter plate) is coated with a capture agent (often a capture antibody) which binds specifically to the tyrosine kinase receptor, or, in the case of a receptor construct, to the flag polypeptide. Coating of the second solid phase is carried out so that the capture agent adheres to the second solid phase. The capture agent is generally a monoclonal antibody, but, as is described in the examples herein, polyclonal antibodies may also be used.

(e) The cell lysate obtained in step (c) of the above-mentioned KIRA stage of the assay is exposed to, or contacted with, the adhering capture agent so that the receptor or receptor construct adheres to (or is captured in) the second solid phase. Unlike the assay of Klein *et al.*, the instant assay does not require the ligand for the receptor as well as kinase inhibitors to be present to achieve suitable immobilization of the receptor or receptor construct to the second solid phase.

(f) A washing step is then carried out, so as to remove unbound cell lysate, leaving the captured receptor or receptor construct.

(g) The adhering or captured receptor or receptor construct is then exposed to, or contacted with, an anti-phosphotyrosine antibody which identifies phosphorylated tyrosine residues in the tyrosine kinase receptor. In the preferred embodiment, the anti-phosphotyrosine antibody is conjugated (directly or indirectly) to an enzyme which catalyses a color change of a non-radioactive color reagent. Accordingly, phosphorylation of the receptor can be measured by a subsequent color change of the reagent. The enzyme can be bound to the anti-phosphotyrosine antibody directly, or a conjugating molecule (*e.g.*, biotin) can be conjugated to the anti-phosphotyrosine antibody and the enzyme can be subsequently bound to the anti-phosphotyrosine antibody via the conjugating molecule.

(h) Finally, binding of the anti-phosphotyrosine antibody to the captured receptor or receptor construct is measured, *e.g.*, by a color change in the color reagent.

The invention also pertains to a Rse.flag reagent which is particularly useful for use in the KIRA ELISA assay. The Rse.flag reagent is a polypeptide comprising a fusion of a flag polypeptide (usually the gD flag described herein) to the carboxyl terminus of the intracellular domain of the Rse rPTK. Generally, the transmembrane domain of Rse and the extracellular domain of another rPTK of interest are also present in the fusion polypeptide reagent. The nucleic acid encoding this reagent and a cell transformed therewith are also claimed.

In yet a further aspect, the invention relates to a kit which can be used in the KIRA ELISA disclosed above which comprises an anti-flag polypeptide capture agent (*e.g.* a capture antibody) which is usually bound to the second solid phase as described herein. Thus, the kit generally provides an ELISA microtiter plate having an anti-flag polypeptide capture antibody adhering to a well thereof. Optionally, the kit also provides an anti-phosphotyrosine antibody which is often labelled, or reagents for

labelling the anti-phosphotyrosine antibody are supplied with the kit. Sometimes, a homogeneous population of cells which have been transformed with a receptor construct as described herein are also provided with the kit. The kit also suitably includes instructions for carrying out the KIRA ELISA.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A-1C are diagrammatic representations of Rse.gD (Figure 1A), Receptor ECD/Rse.gD chimera (Figure 1B) and a CHO cell transformed with the Receptor ECD/Rse.gD chimera (Figure 1C).

Figures 2A-2C depict an alignment of the amino acid sequence (SEQ ID NO: 1) and nucleotide sequence (SEQ ID NO: 2) of Rse.gD. The residues of the signal sequence are indicated with an (*), the transmembrane domain of Rse is boxed and the ECD and ICD of Rse are also delineated. The residues of the gD flag sequence are underlined.

Figure 3 is a flow diagram of an exemplary strategy for selecting a suitable capture agent for use in the assay.

Figure 4 is a flow diagram of an exemplary strategy for selecting a transformed cell suitable for use in the assay, where the cell has a receptor construct with an amino-terminal flag polypeptide located in the cell membrane thereof.

Figure 5 is a flow diagram of an exemplary strategy for selecting a transformed cell suitable for use in the assay, where the cell has a receptor construct with a carboxyl-terminal flag polypeptide located in the cell membrane thereof.

Figures 6A-6B is a flow chart and cartoon illustrating the KIRA ELISA assay for the HER2 receptor described in Example 1.

Figure 7 depicts a $p185^{HER2}/HRG\beta1_{177-244}$ KIRA ELISA standard curve obtained using the assay described in Example 1. To obtain the standard curve, MCF-7 cells ($2x10^5$) were stimulated with 3000, 1000, 333, 111, 37, 12, 4, or 0 pM $HRG\beta1_{177-244}$, as determined by quantitative amino acid analysis (q.a.a.a.). Each calibrator concentration was run in triplicate. The values derived from 10 such standard curves were averaged (total n = 30) and are presented as mean $ABS_{450/650}$ ± sd vs. $HRG\beta1_{177-244}$ concentration.

Figure 8 depicts heregulin specificity of $p185^{HER2}/HRG$ KIRA ELISA of Example 1. In the assay, MCF-7 cells ($2x10^5$) were stimulated with either $HRG\beta1_{177-244}$ (■) at 3000, 1000, 333, 111, 37, 12, 4 or 0 pM or IGF-1(▲), EGF (□), VEGF (●) or insulin (♦) at 30000, 10000, 3333, 1111, 370, 120, 40 or

0 pM. For all concentrations of ligands, n = 3 and data are presented as average $ABS_{450/650}$ ± sd vs. ligand concentration.

Figures 9A-9B is a flow chart and cartoon illustrating the KIRA ELISA assay for the Rse receptor described in Example 2.

Figure 10 depicts a Rse KIRA ELISA standard curve obtained using the assay described in Example 2. To obtain the standard curve, CHO cells transformed with the Rse.gD construct were stimulated with 1:100, 1:200, 1:400, 1:800, 1:1600, 1:3200 or 0 diluted, anti-Rse agonist antibody. Each calibrator concentration was run in triplicate. The values are presented as mean $ABS_{450/650}$ ± sd vs. 1/dilution agonist antibody.

Figures 11A-11B is a flow chart and cartoon illustrating the KIRA ELISA assay for the trk receptors (*i.e.*, trk A, trk B, and trk C) described in Example 3.

Figures 12A-12G depict an alignment of the amino acid sequence (SEQ ID NO: 3) and nucleotide sequence (SEQ ID NO: 4) of gD.trk A used in the assay described in Example 3. The residues of the signal sequence are indicated with an (*), the residues of the gD flag sequence are underlined, the residues of the transmembrane domain of trk A are in bold and the ECD and ICD thereof are also delineated.

Figures 13A-13G depict an alignment of the amino acid sequence (SEQ ID NO: 5) and nucleotide sequence (SEQ ID NO: 6) of gD.trk B used in the assay described in Example 3. The residues of the signal sequence are indicated with an (*), the residues of the gD flag sequence are underlined, the residues of the transmembrane domain of trk B are in bold and the ECD and ICD thereof are also delineated.

Figures 14A-14G depict an alignment of the amino acid sequence(SEQ ID NO: 7) and nucleotide sequence (SEQ ID NO: 8) of gD.trk C used in the assay described in Example 3. The residues of the signal sequence are indicated with an (*), the residues of the gD flag sequence are underlined, the residues of the transmembrane domain of trk C are in bold and the ECD and ICD thereof are also delineated.

Figures 15A-15C depict standard curves for trk A, B and C, respectively, which were obtained using the assay described in Example 3. To obtain the standard curves, CHO cells transformed with the gD.trk constructs were stimulated with 3000, 1000, 333, 111, 37, 12, 4 or 0 pM of ligand, *i.e.* nerve growth factor (NGF, ■), neurotrophin 3 (NT3, ●) or neurotrophin 5 (NT5, ▲). The values are presented as mean $ABS_{450/650}$ ± sd vs. ligand concentration.

Figures 16A-16Z-5 depict the nucleotide sequence (SEQ ID NO 9) of the pSVI17.ID.LL expression vector used for expression of Rse.gD in Example 2.

Figure 17 is a diagrammatic representation of the *MPL*/Rse.gD chimeric receptor described in Example 4.

Figures 18A-B is a flow chart and cartoon illustrating the KIRA ELISA for the *MPL*/Rse.gD chimeric receptor described in Example 4.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

I. Abbreviations and Definitions

"rPTK" means a receptor protein tyrosine kinase.

"ECD", "TM domain" and "ICD" refer to the extracellular domain, transmembrane domain and intracellular domain of a rPTK, respectively.

"Kinase Receptor Activation" or "KIRA" when used throughout this application refers to the first stage of the instantly claimed assay wherein a cell-bound rPTK is exposed to a potential agonist/antagonist ligand which may (or may not) induce phosphorylation of tyrosine residues in the intracellular domain of the rPTK. The KIRA is generally carried out in the "first assay plate" as defined herein.

"Enzyme-Linked Immunosorbent Assay" or "ELISA" refers to the second stage of the instantly claimed assay and involves measuring tyrosine phosphorylation of the rPTK. The ELISA is normally carried out in the "second assay plate" as disclosed in this application. The ELISA is a "sandwich ELISA" insofar as it involves capturing the rPTK or receptor construct to the second solid phase (usually the well of an ELISA microtiter plate). ELISA assays generally involve the preparation of enzyme-antibody conjugates. The conjugated enzyme cleaves a substrate to generate a colored reaction product that can be detected spectrophotometrically. In this assay, the absorbance of the colored solution in individual microtiter wells is proportional to the amount of phosphotyrosines. A review of ELISA is found in Current Protocols in Molecular Biology, Vol. 2, chapter 11 (1991). While the term "ELISA" is used to describe the second stage of the instant assay, it is only a preferred embodiment of the invention, since, as disclosed herein, techniques other than enzymatic detection are available for measuring binding of the anti-phosphotyrosine antibody to the activated receptor.

The terms "receptor", "kinase receptor", "tyrosine kinase", "tyrosine kinase receptor", "receptor protein tyrosine kinase" and "rPTK" are used

interchangeably herein and refer to a protein having at least one phosphate accepting phenolic group. The protein is usually a receptor insofar as it has a ligand-binding ECD, TM domain and ICD. The ICD usually comprises a catalytic kinase domain and has one or more phosphate accepting tyrosine residues. See Figures 1A and 1B, for example. Examples of tyrosine kinase receptors include the insulin receptor, insulin related receptor, epidermal growth factor receptor (EGF-R), platelet-derived growth factor receptors A and B (PDGF-R-A and PDGF-R-B), insulin-like growth factor 1 receptor (IGF-1-R), macrophage colony-stimulating factor receptor (M-CSF-R), HER2/*neu*/c-*erb*B-2 receptor, HER3/c-*erb*B-3 receptor, *Xmrk* receptor, IRR receptor, fibroblast growth factor (FGF) receptors *bek* and *flg*, c-*kit* receptor, Flk/kDR receptor, Rse receptor, the *Eph, Elk, Eck, Eek, Erk, Cek4/Mek4/HEK* and *Cek5* receptors, Axl receptor, hepatocyte growth factor receptor (HGF-R), Flt1 VEGF receptor, SAL-S1 receptor, HpTK 5 receptor, trkA receptor, trkB receptor, and trkC receptor. See, for example, Ullrich and Schlessinger Cell 81:203-212 (1990); Fantl *et al.*, Annu. Rev. Biochem. 62:453-481 (1993); Mark *et al.*, Journal of Biological Chemistry 269(14):10720-10728 (1994); and WO 93/15201.

The terms mentioned above encompass chimeric "receptor" molecules which comprise at least the extracellular domain of a selected tyrosine kinase and the intracellular domain, and optionally, the transmembrane domain of another tyrosine kinase. Of course, the tyrosine kinase of interest can provide the transmembrane domain and/or intracellular domain. The terms also encompass amino acid sequence variants and covalent derivatives of the various rPTKs provided they still display tyrosine kinase phosphorylation activity in the KIRA ELISA. Therefore, the variants will general have conservative amino acid alterations. The individual domains of the tyrosine kinases can be delineated based on sequence homology to known tyrosine kinases and hydrophobicity plots. For example, the hydrophobic transmembrane domain can be readily determined and the ECD and ICD are usually amino-terminal and carboxy terminal to the transmembrane domain, respectively. Conveniently, the transmembrane domain and the ICD of the Rse receptor can be fused to the ECD of a tyrosine kinase of interest, thereby forming a chimeric receptor which is encompassed by the terms denoting a receptor as mentioned above.

In the preferred embodiment, the rPTK is selected from the group consisting of HER2 receptor (Ullrich and Sclessinger, supra), Rse receptor

(Mark *et al.*, *supra* and SEQ ID NO: 1), trk A receptor (SEQ ID NO: 3), trk B receptor (SEQ ID NO: 5) and trk C receptor (SEQ ID NO: 7).

By "autophosphorylation" is meant activation of the catalytic kinase domain of the rPTK, whereby at least one intrinsic tyrosine residue is phosphorylated. Generally, autophosphorylation will result when an agonist molecule binds to the extracellular domain of the kinase receptor. Without being limited to any particular mechanism of action, it is thought that binding of the agonist molecule may result in oligomerization of the kinase receptor which causes activation of the catalytic kinase domain.

By "solid phase" is meant a non-aqueous matrix to which the cells (in the KIRA stage of the assay) or the capture agent (in the ELISA stage of the assay) can adhere. Usually, the solid phase comprises the well of an assay plate but the invention is by no means limited to this embodiment. For example, the solid phase can comprise a discontinuous solid phase of discrete particles. The particles can be porous and formed from a number of different materials, *e.g.*, polysaccharides (*e.g.* agarose), polyacrylamides, polystyrene, polyvinyl alcohol, silicones and glasses. For examples of suitable particulate solid phases, see U.S. Patent No. 4,275,149.

By "well" is meant a recess or holding space in which an aqueous sample can be placed. The well is provided in an "assay plate". The invention usually employs a "first assay plate" which is formed from a material (*e.g.* polystyrene) which optimizes adherence of cells (having the receptor or receptor construct) thereto. Generally, the individual wells of the first assay plate will have a high surface area to volume ratio and therefore a suitable shape is a flat bottom well (where the cells are adherent). The "second assay plate" is generally formed from a material (*e.g.* polystyrene) which optimizes adherence of the capture agent thereto. The second assay plate may have the same general construction and/or characteristics as the first assay plate. However, separate plates are used for the KIRA stage of the assay and the ELISA stage of the assay.

In the preferred embodiment of the invention, both the first assay plate and the second assay plate are "microtiter" plates. The term "microtiter" plate when used herein refers to an assay plate having between about 30 to 200 individual wells, usually 96 wells. Often, the individual wells of the microtiter plate will hold a maximum volume of about 250μl. Conveniently, the first assay plate is a 96 well polystyrene or plastic, cell culture microtiter plate (such as that

sold by Becton Dickinson Labware, Lincoln Park, NJ), which allows for automation. Often, about 50μl to 300μl, more preferably 100μl to 200μl, of an aqueous sample comprising cell culture media with the cells suspended therein will be added to each well of the first assay plate in the KIRA stage of the assay. It is desirable to seed between about 1×10^4 to 3×10^5 cells per well. More preferably, 5×10^4 to 1×10^5 cells per well are seeded. Usually, the second assay plate will comprise a polystyrene microtiter ELISA plate such as that sold by Nunc Maxisorp, Inter Med, Denmark.

The term "homogeneous population of cells" refers to a substantially homogeneous population of cells wherein at least about 80%, and preferably about 90%, of the cells in the population are of the same cell type. Therefore, it is convenient to use a cell line. The cell line is a eukaryotic cell line, normally an animal cell line and desirably a mammalian cell line.

The cells have, or are transformed to produce, the selected receptor or a receptor construct. For example, where the kinase receptor is known to be present in a certain cell line (*e.g.*, the HER2 receptor in the MCF-7 cell line) no transformation step is required. Conversely, it may be necessary to transform a cell with a nucleic acid encoding the receptor, where the cell does not make the receptor, or does not have suitable numbers of the receptor in the cell membrane thereof. Accordingly, the cell is transformed with a nucleic acid encoding the receptor (or receptor construct) and the nucleic acid is expressed so that the ECD of the receptor faces the external milieu of the cell, the transmembrane domain is located in the cell membrane and the kinase domain is located intracellularly.

Where the assay relies on activating the endogenous rPTK, a cell line is selected which is known to produce the rPTK of interest, provided sufficient levels of the rPTK are present in the cell membrane thereof to enable detection. As a general proposition, a minimum number of about 1×10^4 receptors/cell is required. For example, the MCF-7 cell line (ATCC-HTB 22) which produces the HER2 receptor was shown to be useful in the assay. There are 5×10^4 HER2 receptors/MCF-7 cell. Examples of other cell lines and their respective rPTKs include, embryonic mouse 3T3-C2 fibroblast cell line and the insulin receptor, and Hep 3B (ATCC # HB 8064) cell line and the Rse receptor. However, the degree of expression of the rPTK nucleic acid in the cell line is not so high that it results in constitutive

phosphorylation of the rPTK. For example, the SK-BR-3 cell line (ATCC HTB30), which has 3 x 10^6 HER2 receptors/cell, was found to be unsuitable for use in the assay disclosed herein. Therefore, it may be useful to use a cell line which has less than about 3 x 10^6 receptors/cell, depending on the type of receptor. The number of receptors/cell can be measured using Scatchard analysis, for example (Scatchard, Ann. NY Acad. Sci. 51:660-672 [1949]; and Goodwin *et al.*, Cell 73:447-456 [1993]). However, selection of a cell line having a suitable number of receptors/cell is possible using the techniques described herein.

The term "adherent" when used herein to describe the cell, refers to a cell which naturally adheres to the first solid phase (often the well of the first assay plate), thereby forming a fairly uniform coating of the cells on the inside surface of the well. The uniform coating of cells generally forms following incubation of the cells in the wells of the first assay plate for about 8-16 hours. After incubation, non-adhering cells and cell culture medium are decanted off the first assay plate. Incubation is usually carried out at a temperature which is optimal for cell growth, *i.e*, about 37°C. Examples of adherent cell lines include CHO cells (Urlaub and Chasin, Proc. Natl. Acad. Sci. USA 77:4216 [1980]), MCF-7 cells (ATCC HB 22), 293 cells (Graham *et al.*, J. Gen Virol. 36:59 [1977]), Swiss albino 3T3 fibroblast cell line (ATCC No. CCL 92) and U937 macrophage cell line (ATCC No. CRL 1593).

A "flag polypeptide" comprises a short polypeptide which has enough residues to provide an epitope (preferably a linear epitope) against which a "capture agent" thereagainst can be made, yet is short enough such that it does not interfere with activity of the rPTK. The flag polypeptide is also sufficiently unique so that the capture agent thereagainst does not bind to other reagents in the assay. Selection of a "unique" flag polypeptide sequence can be accomplished by comparing the sequence of a proposed flag polypeptide against other known sequences in Genbank or EMBL, for example. Suitable flag polypeptides generally have at least 6 amino acid residues and usually between about 8-80 amino acid residues (*preferably between* about 9-30 amino acid residues).

By "receptor construct" is meant a polypeptide which comprises a fusion of a kinase receptor and a flag polypeptide as defined above. The flag polypeptide is provided at a location in the receptor construct such that: a) the flag polypeptide does not interfere with ligand binding to the receptor; b) the flag polypeptide does not interfere with

autophosphorylation of the receptor and c) the flag polypeptide is presented in a suitable configuration so that it can bind to the capture agent in the ELISA stage of the assay. Often, the polypeptide flag will be present at the N-terminus of the receptor construct. See, for example, Example 3 which refers to the gD.trk constructs. Alternatively, the flag polypeptide may be present at the C-terminus of the receptor construct. See, for example, Example 2 which refers to the Rse.gD construct. See also Figures 1A-1C. The Rse construct disclosed herein is particularly useful, since the ICD (and optionally the transmembrane domain) thereof can be fused to the ECD of a kinase receptor of interest, thereby obviating the need to establish where the flag polypeptide should be located with respect to the kinase receptor of interest.

"Rse.gD" refers to a receptor construct which is the Rse receptor protein tyrosine kinase with the Herpes Simplex virus glycoprotein D (gD) flag polypeptide fused to the COOH-terminus thereof.

"Rse.flag reagent" refers to a polypeptide which comprises the ICD of the Rse receptor fused at its COOH-terminus to a flag polypeptide (normally the gD flag polypeptide). Sometimes, the TM domain of Rse and the ECD of a rPTK of interest will also be present in the Rse.gD. reagent.

"Receptor ECD/Rse.gD Chimera" refers to a fusion of the ECD of a rPTK of interest to the TM and ICD domains of Rse which are fused COOH-terminally to the gD flag polypeptide.

"gD.trkA", "gD.trkB" and "gD.trkC" refer to each of the trk receptors (A-C) having the gD flag polypeptide fused to the amino-termini thereof.

By "capture agent" is meant a compound or agent which is able to adhere to the second solid phase, as herein defined, and which is selective for a rPTK or receptor construct. Thus, the capture agent captures the receptor or receptor construct to the wells of the second assay plate. Usually, the capture agent binds selectively to the flag polypeptide which has been fused to the receptor of interest. Binding of the capture agent is not affected by the presence or absence of ligand bound to the receptor and does not induce receptor activation upon capture. Furthermore, the capture agent does not sterically block access to the phosphorylated tyrosine(s) by the anti-phosphotyrosine antibody. Means for selecting suitable capture agents are described herein. Generally, the capture agent will comprise an antibody (*e.g.*, an affinity purified polyclonal antibody or a monoclonal antibody), but other selective agents, such as streptavidin which binds selectively to the "*strep*-tag" polypeptide can also be used

(see Schmidt *et al.*, Protein Engineering 6(1):109-122 [1993]). Streptavidin can be purchased commercially from Zymed Laboratories, S. San Francisco, CA, for example. Alternatively, the capture agent can comprise protein A (which binds specifically to immunoglobulins). In this embodiment of the invention, the activated receptor or receptor-construct present in the cell lysate is incubated with an antibody which binds specifically thereto, thereby forming a receptor-antibody complex. This complex can be captured by protein A by virtue of its specific binding to the antibody present in the complex. Protein A can be purchased commercially from Pharmacia Biotech, Inc., Piscataway, New Jersey, for example. A strategy for selecting a suitable capture agent is depicted in Figure 3 and will be described in more detail later herein.

In the most preferred embodiment, the capture agent is a monoclonal antibody which binds specifically to a flag polypeptide (which is present in the receptor construct). Examples of suitable flag polypeptides and their respective capture antibodies include the flu HA flag and its antibody 12CA5, (Field *et al.*, Mol. Cell. Biol. 8:2159-2165 [1988]); the c-myc flag and the 8F9, 3C7, 6E10, G4, B7 and 9E10 antibodies thereto (Evan *et al.*, Molecular and Cellular Biology 5(12):3610-3616 [1985]); as well as the Herpes Simplex virus glycoprotein D (gD) flag and the 5B6 antibody thereto (Paborsky *et al.*, Protein Engineering 3(6):547-553 [1990] and Mark *et al.*, Journal of Biological Chemistry 269(14):10720-10728 [1994]). Other flag polypeptides have been disclosed. Examples include the Flag-peptide (Hopp *et al.*, BioTechnology 6:1204-1210 [1988]); the KT3 epitope peptide (Martin *et al.*, Science 255:192-194 [1992]); an α-tubulin epitope peptide (Skinner *et al.*, J. Biol. Chem 266(22):14163-16166 [1991]); and the T7 gene 10 protein peptide tag (Lutz-Freyermuth *et al.*, Proc. Natl. Acad. Sci. USA 87:6393-6397 [1990]). Once the flag polypeptide has been selected as discussed above, a capture antibody thereto can be generated using the techniques disclosed herein.

The term "analyte" refers to a compound or composition to be studied, usually to investigate its ability to activate (or prevent activation of) the tyrosine kinase receptor of interest. The analyte can comprise a bodily fluid (such as plasma or amniotic fluid) or a composition known to contain, or suspected of containing, a ligand for the tyrosine kinase receptor. The analyte can also comprise a cell which has a ligand to the rPTK of interest.

"Ligand" when used herein refers to a molecule which is able to bind to the ECD of the tyrosine kinase of interest or to a known agonist for the tyrosine kinase of interest. The ligand will usually be an agonist or antagonist for the tyrosine kinase.

By "agonist" is meant a molecule which is able activate the intracellular kinase domain of the tyrosine kinase upon binding to the ECD. Often, the agonist will comprise a growth factor (*i.e.*, a polypeptide that is able to stimulate cell division). Exemplary growth factors include heregulin (HRG), insulin, insulin-like growth factors I and II (IGF-I and IGF-II), epidermal growth factor (EGF), interleukins (*e.g.*, IL-8), macrophage colony-stimulating factor (M-CSF), erythropoietin (EPO), platelet-derived growth factor (PDGF), fibroblast factor growth (FGF), transforming growth factors alpha and beta (TGF-α and TGF-ß), hepatocyte growth factor (HGF), and the nerve growth factor (NGF). Alternatively, the agonist can be an antibody against the rPTK (*see, e.g.*, Yarden, Proc. Natl. Acad. Sci., USA 87:2569-2573 [1990]). However, other non-protein agonists such as small organic molecules are also encompassed by the invention.

By "antagonist" is meant a molecule which blocks agonist action. Usually, the antagonist will either: (a) bind to the rPTK and thereby block binding and/or activation of the rPTK by an antagonist thereto (the antagonist may bind to the ECD of the rPTK, but this is not necessarily the case) or (b) bind to the agonist and this precent activation of the rPTK by the agonist. This assay facilitates the detection of both types of antagonist. The antagonist may, for example, comprise a peptide fragment comprising the receptor binding domain of the endogenous agonist ligand for the receptor. The antagonist may also be an antibody which is directed against the ECD of the rPTK, or against a known agonist for the rPTK. However, other non-protein molecules are also encompassed by this term.

The term "antibody" is used in the broadest sense and specifically covers monoclonal antibodies and antibody compositions with polyepitopic specificity (*i.e.* polyclonal antibodies). The polyclonal antibodies are preferably "affinity purified" antibodies. The term "affinity purified" means that the antibodies have been purified using the antigen (*e.g.* the rPTK or fragment thereof or the flag polypeptide) to selectively purify the polyclonal antibodies. Affinity purification can be achieved by immobilizing the antigen on an affinity column (*e.g.* an agarose column) and passing the polyclonal antibodies through the column. The affinity purified antibodies can be subsequently eluted from the column by changing

the elution conditions or by adding a chaotropic agent, for example. For a review of affinity purification techniques with respect to antibodies, see Current Protocols in Immunology, Ed. Coligen *et al.*, Wiley publishers, Vols. 1 and 2, for example.

The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, *i.e.*, the individual antibodies comprising the population are identical except for possible naturally-occurring mutations that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigenic site. Furthermore, in contrast to conventional (polyclonal) antibody preparations which typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen.

The monoclonal antibodies herein include hybrid and recombinant antibodies produced by splicing a variable (including hypervariable) domain of a selected antibody with a constant domain (*e.g.* "humanized" antibodies), or a light chain with a heavy chain, or a chain from one species with a chain from another species, or fusions with heterologous proteins, regardless of species of origin or immunoglobulin class or subclass designation, as well as antibody fragments (*e.g.*, Fab, $F(ab')_2$, and Fv), so long as they exhibit the desired biological activity. [See, *e.g.* U.S. Patent No. 4,816,567 and Mage & Lamoyi, in Monoclonal Antibody Production Techniques and Applications, pp.79-97 (Marcel Dekker, Inc., New York (1987)].

Thus, the modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present invention may be made by the hybridoma method first described by Kohler & Milstein, Nature, **256**: 495 (1975), or may be made by recombinant DNA methods (U.S. Patent No. 4,816,567). The "monoclonal antibodies" may can also be isolated from phage antibody libraries using the techniques described in Clackson *et al.*, Nature, **352**: 624-628 (1991) and Marks *et al.*, J. Mol. Biol., **222**: 581-597 (1991), for example.

The term "anti-phosphotyrosine antibody" refers to a molecule, usually an antibody, which binds selectively to phosphorylated tyrosine

residues in the kinase domain of a rPTK. The antibody can be polyclonal, but is desirably a monoclonal antibody. Anti-phosphotyrosine polyclonal antibodies can be made using the techniques disclosed in White and Backer, Methods in Enzymology **201**:65-67 [1991] and monoclonal anti-phosphotyrosine antibodies can be obtained commercially from Upstate Biologicals, Inc. (UBI, Lake Placid, NY), for example.

The word "label" when used herein refers to a detectable compound or composition which is conjugated directly or indirectly with a molecule (such as the anti-phosphotyrosine antibody). The label may be detectable by itself (*e.g.* radioisotope labels or fluorescent labels) or, in the case of an enzymatic label, may catalyze a chemical alteration of a substrate compound or composition which is detectable. The preferred label is an enzymatic one which catalyzes a color change of a non-radioactive color reagent.

By "washing" is meant exposing the solid phase to an aqueous solution (usually a buffer or cell culture media) in such a way that unbound material (*e.g.*, non-adhering cells, non-adhering capture agent, unbound ligand, receptor, receptor construct, cell lysate, or anti-phosphotyrosine antibody) is removed therefrom. To reduce background noise, it is convenient to include a detergent (*e.g.* Triton X) in the washing solution. Usually, the aqueous washing solution is decanted from the wells of the assay plate following washing. Conveniently, washing can be achieved using an automated washing device. Sometimes, several washing steps (*e.g.*, between about 1 to 10 washing steps) may be required.

By "block buffer" is meant an aqueous, pH buffered solution containing at least one blocking compound which is able to bind to exposed surfaces of the second solid phase which are not coated with capture agent. The blocking compound is normally a protein such as bovine serum albumin (BSA), gelatin, casein or milk powder and does not cross-react with any of the reagents in the assay (*e.g.*, the anti-phosphotyrosine antibodies and detection reagents). The block buffer is generally provided at a pH between about 7 to 7.5 and suitable buffering agents include phosphate and *TRIS*.

By "lysis buffer" is meant an aqueous, pH buffered solution comprising a solubilizing detergent, one or more protease inhibitors and at least one phosphatase inhibitor (such as sodium orthovanadate). The term "solubilizing detergent" refers to a water miscible, non-ionic detergent which lyses cell membranes of eukaryotic cells but does not

denature or activate the receptor or receptor construct. Examples of suitable non-ionic detergents include Triton-X 100, Tween 20, CHAPS and Nonidet P-40 (NP40) available from Calbiochem, La Jolla, California, for example. Many other non-ionic detergents are available in the art. Examples of suitable protease inhibitors include phenylmethylsulfonyl fluoride (PMSF), leupeptin, pepstatin, aprotinin, 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride-bestatin, chymostatin and benzamidine. Preservatives (*e.g.*, thimerosal) and one or more compounds which maintain the isotonicity of the solution (*e.g.*, sodium chloride [NaCl] or sucrose) and a buffer (*e.g.*, Tris or PBS) are usually also present. Generally, the pH of the lysis buffer is in the range about 7 to 7.5.

Usually, following addition of the lysis buffer to the first assay plate, the first assay plate is "gently agitated" and this expression refers to the act of physically shaking the first assay plate (normally using a circular motion) at a substantially low velocity. Gentle agitation does not involve mechanically disrupting the cells (*e.g.* by homogenizing or centrifuging the cells). Exemplary shaking velocities are in the order of 200 to 500 rpm, preferably 300 to 400 rpm in a Bellco orbital shaker, for example.

II. Modes for Practicing the Invention

1. Kinase Receptor Activation - KIRA

The first stage of the assay involves phosphorylation of the kinase domain of a kinase receptor, wherein the receptor is present in the cell membrane of a eukaryotic cell. The receptor may be an endogenous receptor or nucleic acid encoding the receptor may be transformed into the cell. In one embodiment of the invention, nucleic acid encoding a receptor construct is transformed into the cell. Exemplary techniques for transforming the cell with either the receptor or the receptor construct nucleic acid follow.

A. Transformation of the cells

The instant invention provides a substantial improvement over soluble kinase receptor assays insofar as it is considered to more accurately reflect the activity of the receptor *in situ*. It has been discovered that it is possible to transform eukaryotic cells with a receptor construct (comprising the kinase receptor and either an amino- or carboxyl-terminal flag polypeptide) so that the receptor construct assembles itself appropriately in the cell membrane and still retains tyrosine kinase

activity which can be detected in the ELISA stage of the assay. This provides a generic assay for measuring tyrosine kinase activity of any tyrosine kinase of interest.

If a suitable capture agent as described herein is available for a selected rPTK, cells can be transformed with the nucleic acid encoding the receptor alone, without the flag polypeptide. Alternatively, if cells are available which produce the receptor (*e.g.*, MCF-7 cells which produce the HER2 receptor), it is not necessary to transform the cells for use in the assay.

In order to transform the cells with the nucleic acid encoding the rPTK or receptor construct, nucleic acid encoding the rPTK and, optionally, the flag polypeptide, is isolated. This can be achieved by screening a cDNA or genomic library known to contain the DNA encoding the rPTK or flag polypeptide of interest with a selected labelled probe (*e.g.*, an antibody or oligonucleotide- probe) for the rPTK or flag polypeptide, using standard procedures as described in chapters 10-12 of Sambrook *et al.*, Molecular Cloning: A Laboratory Manual (New York: Cold Spring Harbor Laboratory Press, 1989), for example. Alternatively, the nucleic acid encoding the flag polypeptide can be made synthetically using an oligo-synthesizing machine (Applied Biosystems, CA). An alternative means to isolate the nucleic acid encoding the rPTK or flag polypeptide is to use PCR methodology as described in section 14 of Sambrook *et al.*, *supra*. Isolation of only the ECD of the rPTK of interest is required, since this nucleic acid can be fused to the nucleic acid encoding the TM and ICD of the Rse-flag polypeptide construct disclosed herein. See Figures 1A - 1C and SEQ ID NOS: 1 and 2. If necessary however, conventional primer extension procedures as described in section 7.79 of Sambrook *et al.*, *supra*, can be used to detect precursors and processing intermediates of mRNA that may not have been reverse-transcribed into cDNA.

A preferred method of practicing this invention is to use carefully selected oligonucleotide sequences to screen cDNA libraries from various tissues, preferably mammalian cell lines having the rPTK of interest. The oligonucleotide sequences selected as probes should be of sufficient length and sufficiently unambiguous that false positives are minimized.

The oligonucleotide must be labeled such that it can be detected upon hybridization to DNA in the library being screened. The preferred method of labeling is to use ^{32}P- labeled ATP with polynucleotide kinase, as is well known in the art, to radiolabel the oligonucleotide. However, other

methods may be used to label the oligonucleotide, including, but not limited to, biotinylation or enzyme labeling.

In order to provide nucleic acid encoding a receptor construct, nucleic acid encoding the rPTK is fused at its 3' end to nucleic acid encoding the N-terminus of the flag polypeptide. Alternatively, the nucleic acid encoding the rPTK will be fused at its 5' end to nucleic acid encoding the carboxyl terminus of the flag polypeptide. Thus, the flag polypeptide is provided at either the carboxyl- or amino- terminus of the receptor construct. Examples of suitable flag polypeptides are provided above. Selection of other suitable flag polypeptides is possible using the techniques described herein.

In order to generate fusions between the Rse.flag reagent and a rPTK of interest, the nucleic acid encoding the ECD of the rPTK of interest is fused at its 3' end to the nucleic acid encoding the amino terminus of the Rse.flag reagent.

The nucleic acid (*e.g.*, cDNA or genomic DNA) encoding the rPTK or receptor construct is then inserted into a replicable vector for further cloning (amplification of the DNA) or for expression. Many vectors are available to the skilled practitioner but must be compatible with the cell which is to be used in the assay. The vector will have vector components the presence of which will depend on various factors. Such components include, for example, a signal sequence, an origin of replication, one or more marker genes, an enhancer element, a promoter, and a transcription termination sequence. Selection of these vector components shall be described below.

Incorporation of a signal sequence into the expression vector is required since the rPTK or receptor construct must be transported to the cell membrane where it is positioned such that the ECD faces the external milieu of the cell. Therefore, a signal sequence suitable for positioning the rPTK or receptor construct in such a manner is used. The signal sequence is generally a component of the vector, or it may be a part of the rPTK or receptor construct DNA that is inserted into the vector. If a *heterologous signal sequence* is used, it is from those that are recognized and processed (*i.e.*, cleaved by a signal peptidase) by the host cell.

For yeast secretion the native signal sequence may be substituted by, *e.g.*, the yeast invertase leader, alpha factor leader (including *Saccharomyces* and *Kluyveromyces* α-factor leaders, the latter described in U.S. Patent No. 5,010,182 issued 23 April 1991), or acid phosphatase

leader, the *C. albicans* glucoamylase leader (EP 362,179 published 4 April 1990), or the signal described in WO 90/13646 published 15 November 1990. In mammalian cells expression of the DNA encoding the native signal sequence (*e.g.*, the rPTK pre-sequence that normally directs secretion of rPTK from mammalian cells *in vivo*) is satisfactory, although other mammalian signal sequences may be suitable, such as signal sequences from other animal rPTKs, and signal sequences from secreted polypeptides of the same or related species, as well as viral secretory leaders, for example, the herpes simplex gD signal.

The DNA for such precursor region is ligated in reading frame to DNA encoding the rPTK or receptor construct.

Both expression and cloning vectors contain a nucleic acid sequence that enables the vector to replicate in one or more selected host cells. Generally, in cloning vectors this sequence is one that enables the vector to replicate independently of the host chromosomal DNA, and includes origins of replication or autonomously replicating sequences. The 2μ plasmid origin is suitable for yeast, and various viral origins (SV40, polyoma, adenovirus, VSV or BPV) are useful for cloning vectors in mammalian cells. Generally, the origin of replication component is not needed for mammalian expression vectors (the SV40 origin may typically be used only because it contains the early promoter).

Most expression vectors are "shuttle" vectors, *i.e.*, they are capable of replication in at least one class of organisms but can be transformed into another organism for expression. For example, a vector is cloned in *E. coli* and then the same vector is transformed into yeast or mammalian cells for expression even though it is not capable of replicating independently of the host cell chromosome.

DNA may also be amplified by insertion into the host genome. This is readily accomplished using *Bacillus* species as hosts, for example, by including in the vector a DNA sequence that is complementary to a sequence found in *Bacillus* genomic DNA. Transformation of *Bacillus* with this vector results in homologous recombination with the genome and insertion of rPTK or receptor construct DNA. However, the recovery of genomic DNA encoding the rPTK or receptor construct is more complex than that of an exogenously replicated vector because restriction enzyme digestion is required to excise the rPTK or receptor construct DNA.

Expression and cloning vectors usually contain a selection gene, also termed a selectable marker. This gene encodes a protein necessary for the

survival or growth of transformed host cells grown in a selective culture medium. Host cells not transformed with the vector containing the selection gene will not survive in the culture medium. Typical selection genes encode proteins that (a) confer resistance to antibiotics or other toxins, *e.g.*, ampicillin, neomycin, methotrexate, or tetracycline, (b) complement auxotrophic deficiencies, or (c) supply critical nutrients not available from complex media.

One example of a selection scheme utilizes a drug to arrest growth of a host cell. Those cells that are successfully transformed with a heterologous gene express the DNA encoding a protein conferring drug resistance and thus survive the selection regimen. Examples of such dominant selection use the drugs neomycin (Southern *et al.*, J. Molec. Appl. Genet. **1**:327 [1982]), mycophenolic acid (Mulligan *et al.*, Science **209**:1422 [1980]) or hygromycin (Sugden *et al.*, Mol. Cell. Biol. **5**:410-413 [1985]). The three examples given above employ bacterial genes under eukaryotic control to convey resistance to the appropriate drug G418 or neomycin (geneticin), xgpt (mycophenolic acid), or hygromycin, respectively.

Another example of suitable selectable markers for mammalian cells are those that enable the identification of cells competent to take up the rPTK or receptor construct nucleic acid, such as DHFR or thymidine kinase. The mammalian cell transformants are placed under selection pressure that only the transformants are uniquely adapted to survive by virtue of having taken up the marker. Selection pressure is imposed by culturing the transformants under conditions in which the concentration of selection agent in the medium is successively changed, thereby leading to amplification of both the selection gene and the DNA that encodes the rPTK or receptor construct. Amplification is the process by which genes in greater demand for the production of a protein critical for growth are reiterated in tandem within the chromosomes of successive generations of recombinant cells. Increased quantities of rPTK or receptor construct are synthesized from the amplified DNA. Other examples of amplifiable genes include metallothionein-I and -II, preferably primate metallothionein genes, adenosine deaminase, ornithine decarboxylase, *etc.*

For example, cells transformed with the DHFR selection gene are first identified by culturing all of the transformants in a culture medium that contains methotrexate (Mtx), a competitive antagonist of DHFR. An appropriate host cell when wild-type DHFR is employed is the Chinese hamster ovary (CHO) cell line deficient in DHFR activity, prepared and

propagated as described by Urlaub and Chasın, Proc. Natl. Acad. Sci. USA 77:4216 (1980). The transformed cells are then exposed to increased levels of methotrexate. This leads to the synthesis of multiple copies of the DHFR gene, and, concomitantly, multiple copies of other DNA comprising the expression vectors, such as the DNA encoding the rPTK or receptor construct. This amplification technique can be used with any otherwise suitable host, e.g., ATCC No. CCL61 CHO-K1, notwithstanding the presence of endogenous DHFR if, for example, a mutant DHFR gene that is highly resistant to Mtx is employed (EP 117,060).

Alternatively, host cells (particularly wild-type hosts that contain endogenous DHFR) transformed or co-transformed with DNA sequences encoding the rPTK or receptor construct, wild-type DHFR protein, and another selectable marker such as aminoglycoside 3' phosphotransferase (APH) can be selected by cell growth in medium containing a selection agent for the selectable marker such as an aminoglycosidic antibiotic, e.g., kanamycin, neomycin, or G418. See U.S. Patent No. 4,965,199.

A suitable selection gene for use in yeast is the *trp1* gene present in the yeast plasmid YRp7 (Stinchcomb *et al.*, Nature **282**:39 [1979]; Kingsman *et al.*, Gene 7:141 [1979]; or Tschemper *et al.*, Gene **10**:157 [1980]). The *trp1* gene provides a selection marker for a mutant strain of yeast lacking the ability to grow in tryptophan, for example, ATCC No. 44076 or PEP4-1 (Jones, Genetics **85**:12 [1977]). The presence of the trp1 lesion in the yeast host cell genome then provides an effective environment for detecting transformation by growth in the absence of tryptophan. Similarly, *Leu2*-deficient yeast strains (ATCC 20,622 or 38,626) are complemented by known plasmids bearing the *Leu2* gene.

In addition, vectors derived from the 1.6 μm circular plasmid pKD1 can be used for transformation of *Kluyveromyces* yeasts. Bianchi *et al.*, Curr. Genet. **12**:185 (1987). More recently, an expression system for large-scale production of recombinant calf chymosin was reported for *K. lactis*. Van den Berg, Bio/Technology **8**:135 (1990). Stable multi-copy expression vectors for secretion of mature recombinant human serum albumin by industrial strains of *Kluyveromyces* have also been disclosed. Fleer *et al.*, Bio/Technology **9**:968-975 (1991).

Expression and cloning vectors usually contain a promoter that is recognized by the host organism and is operably linked to the rPTK or receptor construct nucleic acid. Promoters are untranslated sequences located upstream (5') to the start codon of a structural gene (generally

within about 100 to 1000 bp) that control the transcription and translation of particular nucleic acid sequence, such as the rPTK nucleic acid sequence, to which they are operably linked. Such promoters typically fall into two classes, inducible and constitutive. Inducible promoters are promoters that initiate increased levels of transcription from DNA under their control in response to some change in culture conditions, *e.g.*, the presence or absence of a nutrient or a change in temperature. At this time a large number of promoters recognized by a variety of potential host cells are well known. These promoters are operably linked to rPTK or receptor construct-encoding DNA by removing the promoter from the source DNA by restriction enzyme digestion and inserting the isolated promoter sequence into the vector. Both the native rPTK promoter sequence and many heterologous promoters may be used to direct amplification and/or expression of the rPTK or receptor construct DNA. The promoter will be one which results in the accumulation of suitable numbers of receptor or receptor construct in the cell membrane of the transformed cell (*i.e.* so that autophosphorylation of the receptor is detectable in the ELISA but constitutive phosphorylation does not occur). Selection of a suitable promoter to achieve this is possible following the guidelines herein for selecting cells for use in the KIRA ELISA.

Virtually all eukaryotic genes have an AT-rich region located approximately 25 to 30 bases upstream from the site where transcription is initiated. Another sequence found 70 to 80 bases upstream from the start of transcription of many genes is a CXCAAT region where X may be any nucleotide. At the 3' end of most eukaryotic genes is an AATAAA sequence that may be the signal for addition of the poly A tail to the 3' end of the coding sequence. All of these sequences are suitably inserted into eukaryotic expression vectors.

Examples of suitable promoting sequences for use with yeast hosts include the promoters for 3-phosphoglycerate kinase (Hitzeman *et al.*, J. Biol. Chem. **255**:2073 [1980]) or other glycolytic enzymes (Hess *et al.*, J. Adv. Enzyme Reg., 7:149 [1968]; and Holland, Biochemistry **17**:4900 [1978]), such as enolase, glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase.

Other yeast promoters, which are inducible promoters having the additional advantage of transcription controlled by growth conditions, are

the promoter regions for alcohol dehydrogenase 2, isocytochrome C, acid phosphatase, degradative enzymes associated with nitrogen metabolism, metallothionein, glyceraldehyde-3-phosphate dehydrogenase, and enzymes responsible for maltose and galactose utilization. Suitable vectors and promoters for use in yeast expression are further described in Hitzeman *et al.*, EP 73,657A. Yeast enhancers also are advantageously used with yeast promoters.

rPTK or receptor construct transcription from vectors in mammalian host cells is controlled, for example, by promoters obtained from the genomes of viruses such as polyoma virus, fowlpox virus (UK 2,211,504 published 5 July 1989), adenovirus (such as Adenovirus 2), bovine papilloma virus, avian sarcoma virus, cytomegalovirus, a retrovirus, hepatitis-B virus and most preferably Simian Virus 40 (SV40), from heterologous mammalian promoters, *e.g.*, the actin promoter or an immunoglobulin promoter, from heat-shock promoters, and from the promoter normally associated with the rPTK or receptor construct sequence, provided such promoters are compatible with the host cell systems.

The early and late promoters of the SV40 virus are conveniently obtained as an SV40 restriction fragment that also contains the SV40 viral origin of replication. Fiers *et al.*, Nature **273**:113 (1978); Mulligan and Berg, Science **209**:1422-1427 (1980); Pavlakis *et al.*, Proc. Natl. Acad. Sci. USA **78**:7398-7402 (1981). The immediate early promoter of the human cytomegalovirus is conveniently obtained as a HindIII E restriction fragment. Greenaway *et al.*, Gene **18**:355-360 (1982). A system for expressing DNA in mammalian hosts using the bovine papilloma virus as a vector is disclosed in U.S. 4,419,446. A modification of this system is described in U.S. 4,601,978. See also Gray *et al.*, Nature **295**:503-508 (1982) on expressing cDNA encoding immune interferon in monkey cells; Reyes *et al.*, Nature **297**:598-601 (1982) on expression of human β-interferon cDNA in mouse cells under the control of a thymidine kinase promoter from herpes simplex virus; Canaani and Berg, Proc. Natl. Acad. Sci. USA **79**:5166-5170 (1982) on expression of the human interferon β1 gene in cultured mouse and rabbit cells; and Gorman *et al.*, Proc. Natl. Acad. Sci. USA **79**:6777-6781 (1982) on expression of bacterial CAT sequences in CV-1 monkey kidney cells, chicken embryo fibroblasts, Chinese hamster ovary cells, HeLa cells, and mouse NIH-3T3 cells using the Rous sarcoma virus long terminal repeat as a promoter.

Transcription of DNA encoding the rPTK or receptor construct by higher eukaryotes may be increased, if increased numbers of the rPTK or receptor construct per cell are required to facilitate detection in the ELISA stage of the assay. This may be achieved by inserting an enhancer sequence into the vector. Enhancers are cis-acting elements of DNA, usually about from 10 to 300 bp, that act on a promoter to increase its transcription. Enhancers are relatively orientation and position independent, having been found 5' (Laimins *et al.*, Proc. Natl. Acad. Sci. USA 78:993 [1981]) and 3' (Lusky *et al.*, Mol. Cell Bio. 3:1108 [1983]) to the transcription unit, within an intron (Banerji *et al.*, Cell 33:729 [1983]), as well as within the coding sequence itself (Osborne *et al.*, Mol. Cell Bio. 4:1293 [1984]). Many enhancer sequences are now known from mammalian genes (globin, elastase, albumin, α-fetoprotein, and insulin). Typically, however, one will use an enhancer from a eukaryotic cell virus. Examples include the SV40 enhancer on the late side of the replication origin (bp 100-270), the cytomegalovirus early promoter enhancer, the polyoma enhancer on the late side of the replication origin, and adenovirus enhancers. See also Yaniv, Nature 297:17-18 (1982) on enhancing elements for activation of eukaryotic promoters. The enhancer may be spliced into the vector at a position 5' or 3' to the rPTK or receptor construct-encoding sequence, but is preferably located at a site 5' from the promoter.

Expression vectors used in eukaryotic host cells will also contain sequences necessary for the termination of transcription and for stabilizing the mRNA. Such sequences are commonly available from the 5' and, occasionally 3', untranslated regions of eukaryotic or viral DNAs or cDNAs. These regions contain nucleotide segments transcribed as polyadenylated fragments in the untranslated portion of the mRNA encoding the rPTK or receptor construct.

Construction of suitable vectors containing one or more of the above listed components employs standard ligation techniques. Isolated plasmids or DNA fragments are cleaved, tailored, and religated in the form desired to generate the plasmids required.

For analysis to confirm correct sequences in plasmids constructed, the ligation mixtures are used to transform *E. coli* K12 strain 294 (ATCC 31,446) and successful transformants selected by ampicillin or tetracycline resistance where appropriate. Plasmids from the transformants are prepared, analyzed by restriction endonuclease digestion, and/or sequenced

by the method of Messing *et al.*, Nucleic Acids Res. **9**:309 (1981) or by the method of Maxam *et al.*, Methods in Enzymology **65**:499 (1980).

Other methods, vectors, and host cells suitable for adaptation to the synthesis of the rPTK or receptor construct in recombinant vertebrate cell culture are described in Gething *et al.*, Nature **293**:620-625 (1981); Mantei *et al.*, Nature **281**:40-46 (1979); Levinson *et al.*; EP 117,060; and EP 117,058. A particularly useful plasmid for mammalian cell culture expression of rPTK or receptor construct DNA is pRK5 (EP 307,247) or pSVI6B (PCT pub. no. WO 91/08291 published 13 June 1991).

Examples of suitable eukaryotic cell lines for transformation include *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe* (Beach and Nurse, Nature **290**:140 [1981]; EP 139,383 published 2 May 1985); *Kluyveromyces* hosts (U.S. 4,943,529; Fleer *et al.*, Bio/Technology 9:968-975 [1991]) and *Aspergillus* hosts such as *A. nidulans* (Ballance *et al.*, Biochem. Biophys. Res. Commun. **112**:284-289 [1983]; Tilburn *et al.*, Gene **26**:205-221 [1983]; Yelton *et al.*, Proc. Natl. Acad. Sci. USA **81**:1470-1474 [1984]) and *A. niger* (Kelly and Hynes, EMBO J. **4**:475-479 [1985]), among lower eukaryotic host microorganisms.

Examples of useful animal host cell lines for transformation include monkey kidney CV1 line transformed by SV40 (COS-7, ATCC CRL 1651); human embryonic kidney line (293 or 293 cells subcloned for growth in suspension culture, Graham *et al.*, J. Gen Virol. **36**:59 [1977]); baby hamster kidney cells (BHK, ATCC CCL 10); Chinese hamster ovary cells/-DHFR (CHO, Urlaub and Chasin, Proc. Natl. Acad. Sci. USA **77**:4216 [1980]); mouse sertoli cells (TM4, Mather, Biol. Reprod. **23**:243-251 [1980]); monkey kidney cells (CV1 ATCC CCL 70); African green monkey kidney cells (VERO-76, ATCC CRL-1587); human cervical carcinoma cells (HELA, ATCC CCL 2); canine kidney cells (MDCK, ATCC CCL 34); buffalo rat liver cells (BRL 3A, ATCC CRL 1442); human lung cells (W138, ATCC CCL 75); human liver cells (Hep G2, HB 8065); mouse mammary tumor (MMT 060562, ATCC CCL51); TRI cells (Mather *et al.*, Annals N.Y. Acad. Sci. **383**:44-68 [1982]); MRC 5 cells; FS4 cells; and a human hepatoma line (Hep G2).

Host cells are transformed with the above-described expression or cloning vectors of this invention and cultured in conventional nutrient media modified as appropriate for inducing promoters, selecting transformants, or amplifying the genes encoding the desired sequences. Transformation means introducing DNA into an organism so that the DNA is replicable, either as an extrachromosomal element or as a chromosomal

integrant. Depending on the host cell used, transformation is done using standard techniques appropriate to such cells. Successful transformation is generally recognized when any indication of the operation of this vector occurs within the host cell.

For mammalian cells, the calcium phosphate precipitation method of Graham and Van der Eb, Virology 52:456-457 (1978) is preferred. General aspects of mammalian cell host system transformations have been described by Axel in U.S. 4,399,216 issued 16 August 1983. Transformations into yeast are typically carried out according to the method of Van Solingen *et al.*, J. Bact. 130:946 (1977) and Hsiao *et al.*, Proc. Natl. Acad. Sci. (USA) 76:3829 (1979). However, other methods for introducing DNA into cells, such as by nuclear microinjection, electroporation, bacterial protoplast fusion with intact cells, or polycations, *e.g.*, polybrene, polyornithine, *etc.*, may also be used. For various techniques for transforming mammalian cells, see Keown *et al.*, Methods in Enzymology (1989), Keown *et al.*, Methods in Enzymology 185:527-537 (1990), and Mansour *et al.*, Nature 336:348-352 (1988).

The mammalian host cells used to produce the rPTK or receptor construct may be cultured in a variety of media. Commercially available media such as Ham's F10 (Sigma), Minimal Essential Medium ([MEM], Sigma), RPMI-1640 (Sigma), and Dulbecco's Modified Eagle's Medium ([DMEM], Sigma) are suitable for culturing the host cells. In addition, any of the media described in Ham and Wallace, Meth. Enz. 58:44 (1979), Barnes and Sato, Anal. Biochem. 102:255 (1980), U.S. 4,767,704; 4,657,866; 4,927,762; or 4,560,655; WO 90/03430; WO 87/00195; U.S. Patent Re. 30,985; or U.S. Patent No. 5,122,469,

may be used as culture media for the host cells. Any of these media may be supplemented as necessary with hormones and/or other growth factors (such as insulin, transferrin, or epidermal growth factor), salts (such as sodium chloride, calcium, magnesium, and phosphate), buffers (such as HEPES), nucleosides (such as adenosine and thymidine), antibiotics (such as Gentamycin™ drug), trace elements (defined as inorganic compounds usually present at final concentrations in the micromolar range), and glucose or an equivalent energy source. Any other necessary supplements may also be included at appropriate concentrations that would be known to those skilled in the art. The culture conditions, such as temperature, pH, and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan.

In general, principles, protocols, and practical techniques for maximizing the productivity of mammalian cell cultures can be found in Mammalian Cell Biotechnology: a Practical Approach, M. Butler, ed., IRL Press, 1991.

Gene amplification and/or expression may be measured in a sample directly, for example, by conventional Southern blotting, Northern blotting to quantitate the transcription of mRNA (Thomas, Proc. Natl. Acad. Sci. USA 77:5201-5205 [1980]), dot blotting (DNA analysis), or *in situ* hybridization, using an appropriately labeled probe, based on the sequences provided herein. Various labels may be employed, most commonly radioisotopes, particularly ^{32}P. However, other techniques may also be employed, such as using biotin-modified nucleotides for introduction into a polynucleotide. The biotin then serves as the site for binding to avidin or antibodies, which may be labeled with a wide variety of labels, such as radionuclides, fluorophores, enzymes, or the like. Alternatively, antibodies may be employed that can recognize specific duplexes, including DNA duplexes, RNA duplexes, and DNA-RNA hybrid duplexes or DNA-protein duplexes. The antibodies in turn may be labeled and the assay may be carried out where the duplex is bound to a surface, so that upon the formation of duplex on the surface, the presence of antibody bound to the duplex can be detected.

Gene expression, alternatively, may be measured by immunological methods, such as immunohistochemical staining to quantitate directly the expression of gene product.

B. Selecting cells for use in the assay

As mentioned above, the cells to be subjected to the assay can be (a) cells having an endogenous receptor, (b) cells which have been transformed with a rPTK, or (c) cells transformed with a receptor construct. The suitability of the cells for use in the assay is investigated.

Cells having the endogenous rPTK can be subjected to a test-run KIRA ELISA using a known ligand to the PTK (*e.g.* an agonist antibody) and a control (*e.g.* the diluent for the agonist antibody). A range of ligand concentrations such as those used herein (see Examples 1, 2 and 3) will be used to determine whether sufficient numbers of the receptor are present in the cells being tested. In order to discover whether a cell line is unsuitable because the receptor is constitutively phosphorylated, the cell line can be subjected to the KIRA ELISA disclosed herein, wherein it is exposed to both positive and negative controls (*e.g.* a known agonist ligand

in cell culture media as described herein as a positive control and the cell culture media without the agonist ligand as the negative control). If phosphorylation of the receptor is detected for both positive and negative controls, this may be indicative that constitutive phosphorylation of the receptor is occurring. However, it is possible that a constituent of the serum in the cell culture media is activating the receptor. Thus, the cells can be "starved" in serum-free media for about 2-12 hours (depending on cell survival) and then the assay is repeated using the positive and negative controls. If activation is detected for both controls, the cell line may be considered unsuitable and another cell line can be tested.

If the cell line is transformed with the receptor (without the flag polypeptide) a strategy similar to that depicted in Figure 4 can be used to discover whether or not the cell line is suitable for use in the assay. As a first step, successful transformation and expression of the nucleic acid encoding the rPTK is determined (see Figure 4, step b). In order to identify whether the ECD of the rPTK is present on the surface of the cells, flow cytometric analysis can be performed using an antibody to the ECD of the receptor. The antibody can be made using the techniques for generating antibodies discussed herein. Flow cytometric analysis can be carried out using the techniques described in Current Protocols in Immunology, Ed. Coligen *et al.*, Wiley publishers, Vols. 1 and 2, for example. Briefly, flow cytometric analysis involves incubating intact cells (having the receptor) with antibodies to the ECD thereof, followed by washing. The antibody-bound cells are then incubated with species specific anti-antibody antibodies conjugated to a fluorochrome. Following washing, the labeled cells are analyzed by fluorescence-activated flow cytometry to detect whether the ECD is present on the surface of the cells.

In the following step, *i.e.* Figure 4, step (c), the ability of the cell-bound receptor to be activated is tested. In order to determine this, the transformed cells are exposed to a known agonist to the receptor (*e.g.* the endogenous ligand or an agonist antibody for the receptor). Following exposure, the cells are lysed in a suitable buffer (*e.g.* sodium dodecylbenzenesulfonate in phosphate buffered saline; SDS in PBS) and subjected to Western blotting with anti-phosphotyrosine antibodies as described in Wang, Molecular and Cellular Biology **5(12)**:3640-3643 (1985); Glenney *et al.*, Journal of Immunological Methods **109**:277-285 (1988); Kamps, Methods in Enzymology **201**:101-110 (1991); Kozma *et al.*, Methods in

Enzymology 201:28-43 (1991); Holmes *et al.*, Science 256:1205-10 (1992); or Corfas *et al.*, PNAS, USA 90:1624-1628 (1993), for example.

Assuming the Western blotting step indicates that the rPTK can be activated, a KIRA ELISA test run can be performed, see Figure 4 step (d), to further establish whether or not the transformed cell line can be used in the assay.

In the preferred embodiment of the invention, the KIRA ELISA is a "generic" assay insofar as any rPTK of interest can be studied regardless of the availability of receptor-specific reagents (*i.e.*, capture agent). This embodiment employs a receptor construct having a flag polypeptide at either the amino or carboxyl terminus of the receptor.

If the flag polypeptide is provided at the NH_2-terminus (see, *e.g.*, the gD.trk A, B and C receptor constructs disclosed in Example 3), the procedure for selecting a transformed cell line for use in the assay summarized in Figure 4 can be performed. In this embodiment, the cells are transformed with the flag polypeptide-receptor construct as described earlier herein. See step (a). In step (b), successful transformation of the receptor and flag polypeptide (*i.e.* the receptor construct) is confirmed. In order to study this, two-dimensional flow cytometric analysis can be performed using antibodies to both the flag polypeptide and the ECD of the receptor. Techniques for two-dimensional flow cytometric analysis are disclosed in Current Protocols in Immunology, *supra*. Assuming successful transformation of the receptor construct is demonstrated, steps (c) and (d) of Figure 4 are then performed. See the discussion above, for an explanation of steps (c) to (d) of Figure 4.

A technique for identification of cells which have been successfully transformed with the receptor construct having a C-terminal flag polypeptide and which cells are also suitable for use in the assay is illustrated in Figure 5. Following cell transformation [step (a)], successful transformation of the receptor is determined by flow cytometric analysis using an antibody directed against the ECD of the receptor of interest, for example. Flow cytometric analysis can be performed *substantially as* described above. This forms step (b) of the procedure outlined in Figure 5.

Following step (b), successful transformation of the entire receptor construct (including the COOH-terminal flag polypeptide) is analyzed in step (c). This can be achieved by lysing the cells (using techniques for lysing cells disclosed herein) and immunoprecipitating the membrane extract

with an antibody against the receptor of interest. This immunoprecipitated membrane extract is then subjected to Western blot analysis with antibodies specific for the flag polypeptide. Alternatively, rPTK-specific ELISA analysis of anti-flag polypeptide captured membrane lysate can be carried out. Briefly, this involves coating ELISA wells with appropriate flag specific capture agent. The wells are blocked, washed, and the lysate is then incubated in the wells. Unbound receptor construct is removed by washing. The wells are then reacted with receptor-specific antibody or antibodies, either directly or indirectly conjugated to HRPO. The wells are washed and the HRPO is then exposed to the chromogenic substrate (*e.g.*, TMB).

Steps (d) and (e), *i.e.*, detecting receptor activation and KIRA ELISA test run, are essentially the same as those steps described above.

Once useful cells are identified, they are subjected to the KIRA stage of the instantly claimed assay.

C. Coating the first solid phase with the cells

The first solid phase (*e.g.* a well of a first assay plate) is coated with cells having the endogenous receptor or cells which have been transformed pursuant to the preceding sections.

Preferably, an adherent cell line is chosen, so that the cells naturally adhere to the first solid phase. However, use of an adherent cell line is not essential. For example, non-adherent cells (*e.g.* red blood cells) can be added to round bottomed wells of an assay plate such as that sold by Becton Dickinson Labware, Lincoln Park, New Jersey, for example. The assay plate is then placed in a plate carrier and centrifuged so as to create a pellet of cells adhering to the base of the wells. The cell culture supernatants are removed using a pipette. Thus, use of an adherent cell is clearly advantageous over non-adherent cells since it reduces variability in the assay (*i.e*, the cells in the pellet of the round bottom wells may be taken up with the supernatant when the alternative method is used).

The cells to be added to the wells of the first assay plate may be *maintained in* tissue culture flasks and utilized when cells densities of about 70-90% confluency are achieved. Then, generally between about 1×10^4 to 3×10^5 (and preferably 5×10^4 to 1×10^5) cells are seeded per flat-bottom well, using a pipette, for example. It has been found that, contrary to expectations, addition of cell concentrations mentioned above is sufficient to enable activation of the rPTK to be measured in the ELISA

stage of the assay, without the need to concentrate or clarify the cells or cell lysate prior thereto. Often, the cells are diluted in culture medium prior to seeding them in the wells of the microtiter plate to achieve the desired cell densities.

Usually, the cells are cultured in the microtiter plates for a sufficient period of time to optimize adherence to the wells thereof, but not too long such that the cells begin to deteriorate. Thus, incubation for about 8 to 16 hours at a temperature which is the physiological optimum for the cells (usually about 37°C) is preferred. Suitable media for culturing the cells are described in Section 1A above. Culturing in 5% CO_2 is recommended.

Following incubation overnight, the well supernatants are decanted and excess supernatant may be further removed by lightly tamping the microtiter plates with an absorbent substrate, *e.g.*, a paper towel, but a sponge works equally well. Thus, a substantially homogeneous layer of adhering cells remains on the internal surfaces of the individual wells of the microtiter plate. These adhering cells are then exposed to the analyte.

D. Preparation and addition of the analyte

As mentioned above, the analyte may comprise an agonist ligand (or suspected agonist) or an antagonist (or suspected antagonist) for the rPTK of interest. The ligand may be an endogenous polypeptide, or a synthetic molecule, such as an inorganic or organic molecule. Usually, the ligand is a polypeptide. This assay is useful for screening molecules which activate (or antagonize activation) of the tyrosine kinase receptor of interest. Thus, the assay can be used for developing therapeutically effective molecules.

Where the ligand is an agonist, the molecule can comprise the native growth factor *e.g.*, heregulin (HRG), insulin, insulin-like growth factors I and II (IGF-I and IGF-II), epidermal growth factor (EGF), interleukins (*e.g.*, IL-8), macrophage colony-stimulating factor (M-CSF), erythropoietin (EPO), platelet-derived growth factor (PDGF), transforming growth factors *alpha and beta* (TGF-α and TGF-β), hepatocyte growth factor (HGF), fibroblast growth factor (FGF) and nerve growth factor (NGF). Many of these growth factors are available commercially. Alternatively, the growth factor can be made by peptide synthesis or recombinant techniques which are described herein. Synthetic small molecule agonists can similarly be

generated by those skilled in the art using conventional chemical synthesis techniques.

Where the ligand is present in a biological fluid, the analyte can be prepared using techniques which are well known in the art. Body fluid such as blood or amniotic fluid may be used directly, however concentration may be required. If the analyte to be tested comprises a particular tissue, the cells thereof can be grown in cell culture and the supernatant can be tested for secreted ligand.

Often, the ligand is diluted in an aqueous diluent (such as cell culture media) so that a standard curve can be generated. However, the ligand may be present in a cell or a cell component (*e.g.*, the cell membrane). In particular, it has been found that the assay can be used to detect the presence of a ligand in the cell membrane of a selected cell line. This is clearly useful for discovering a novel endogenous ligand for a known rPTK.

The ligand composition is added to each well which contains the adhering cells using a pipette, for example. At least one control well (*e.g.* to which the aqueous diluent for the ligand is added) is included in the assay.

The adhering cells are usually stimulated for a sufficient period of time to optimize the signal, but not too long such that the signal decreases as a consequence of dephosphorylation of the rPTK by endogenous phosphatases. A suitable stimulation period is between about 10 to 60 minutes, preferably about 30 minutes at a physiologically optimal temperature for the cells (usually about 37°C).

Following activation, well supernatants are decanted and the plates can then be lightly tamped with an absorbent substrate to remove excess supernatant.

The assay can be used to detect antagonist ligands for the rPTK of interest. Antagonists generally fall into two categories (a) ones which bind to the rPTK and thereby block binding and/or activation of the rPTK by an agonist thereto (the antagonist may bind to the ECD, but this is not necessarily the case) and (b) those which bind to the agonist and thus prevent activation of the rPTK by the agonist.

In order to detect antagonist molecules from category (a) above, the cells are exposed to the suspected antagonist ligand substantially as mentioned above. Following exposure to the antagonist, the well supernatants are decanted and the plates are lightly tamped. Then, a known

agonist (*e.g.*, the endogenous growth factor) is added to the washed cells essentially as discussed in the preceding paragraphs, following which, the well supernatants are decanted and plates are lightly tamped. Alternatively, a composition comprising both the antagonist and agonist can be added to the adhering cells substantially as discussed above. Ability of the suspected antagonist to block binding and/or activation of the rPTK can subsequently be measured by ELISA as discussed below.

To detect antagonist molecules from category (b) above, a known agonist is pre-incubated with the suspected antagonist prior to the KIRA stage of the assay. This incubation is carried out for a sufficient period of time to enable a complex of the antagonist-agonist to form; from 30 min. to 12 hours, for example. This complex is then subjected to the assay with the non-complexed agonist and antagonist used as controls.

Following exposure to the agonist (and optionally the antagonist) ligand, the cells are lysed, as discussed below.

E. Solubilizing the cells

In this step of the assay, the cells are lysed so as to solubilize the rPTK such that it remains activated (*i.e.*, the tyrosine residues remain phosphorylated) for the ELISA stage of the assay. Thus, the cells are lysed using a lysis buffer as described above which serves to solubilize the rPTK or receptor construct, yet does not dephosphorylate or denature the rPTK.

Where microtiter plates are used as mentioned above, about 75 to 200µl of lysis buffer is added to each well. The plates can then be agitated gently using a plate shaker (*e.g.*, such as that sold by Bellco Instruments, Vineland, NJ) for about 1 to 2 hours. Shaking can be carried out at room temperature.

2. <u>Enzyme-Linked Immunosorbent Assay - ELISA</u>

The second stage of the assay involves a sandwich ELISA performed in the second assay plate. In order to carry out the ELISA, a capture agent is prepared.

A. Preparation of the capture agent

As mentioned above, the capture agent often comprises a polyclonal antibody (usually an affinity purified polyclonal antibody) or monoclonal antibody. Other capture agents are envisaged and are discussed in the definitions section above. The capture agent either binds specifically to the kinase receptor, or to the flag polypeptide (*i.e.* the antigen).

Polyclonal antibodies to the antigen (either the receptor or the flag polypeptide) generally are raised in animals by multiple subcutaneous (sc)

or intraperitoneal (ip) injections of the antigen or an antigenic fragment thereof (often the ECD of the rPTK) and an adjuvant. It may be useful to conjugate the antigen or a fragment containing the target amino acid sequence to a protein that is immunogenic in the species to be immunized (*e.g.*, keyhole limpet hemocyanin), using a bifunctional or derivatizing agent.

The route and schedule for administration of immunogen to the host animal or cultured antibody-producing cells therefrom are generally in keeping with established and conventional techniques for antibody stimulation and production. While mice are frequently employed as the test model, it is contemplated that any mammalian subject including human subjects or antibody-producing cells obtained therefrom can be manipulated according to the processes of this invention to serve as the basis for production of mammalian, including human, hybrid cell lines.

Animals are typically immunized against the immunogenic conjugates or derivatives by combining 1 mg or 1 μg of conjugate (for rabbits or mice, respectively) with 3 volumes of Freund's complete adjuvant and injecting the solution intradermally at multiple sites. One month later the animals are boosted with 1/5 to 1/10 the original amount of conjugate in Freund's complete adjuvant (or other suitable adjuvant) by subcutaneous injection at multiple sites. 7 to 14 days later animals are bled and the serum is assayed for anti-antigen titer. Animals are boosted until the titer plateaus. Preferably, the animal is boosted with the conjugate of the same antigen, but conjugated to a different protein and/or through a different cross-linking agent. Conjugates also can be made in recombinant cell culture as protein fusions. Also, aggregating agents such as alum are used to enhance the immune response.

After immunization, monoclonal antibodies can be prepared by recovering immune cells (typically spleen cells or lymphocytes from lymph node tissue) from immunized animals and immortalizing the cells in conventional fashion, *e.g.*, by fusion with myeloma cells or by Epstein-Barr (EB)-virus transformation and screening for clones producing the desired *antibody*. The hybridoma technique described originally by Kohler and Milstein, Eur. J. Immunol. 6:511 (1976), and also described by Hammerling *et al.*, In: Monoclonal Antibodies and T-Cell Hybridomas, Elsevier, N.Y., pp. 563-681 (1981) has been widely applied to produce hybrid cell lines that secrete high levels of monoclonal antibodies against many specific antigens.

It is possible to fuse cells of one species with another. However, it is preferable that the source of the immunized antibody producing cells and the myeloma be from the same species.

The hybrid cell lines can be maintained in culture in cell culture media. The cell lines of this invention can be selected and/or maintained in a composition comprising the continuous cell line in hypoxanthine-aminopterin-thymidine (HAT) medium. In fact, once the hybridoma cell line is established, it can be maintained on a variety of nutritionally adequate media. Moreover, the hybrid cell lines can be stored and preserved in any number of conventional ways, including freezing and storage under liquid nitrogen. Frozen cell lines can be revived and cultured indefinitely with resumed synthesis and secretion of monoclonal antibody.

The secreted antibody is recovered from tissue culture supernatant by conventional methods such as precipitation, ion exchange chromatography, affinity chromatography, or the like. The antibodies described herein are also recovered from hybridoma cell cultures by conventional methods for purification of IgG or IgM, as the case may be, that heretofore have been used to purify these immunoglobulins from pooled plasma, *e.g.*, ethanol or polyethylene glycol precipitation procedures. The purified antibodies are then sterile filtered. Where the antibody is a polyclonal antibody, it is generally affinity purified using an affinity column generated from the antigen of interest so as to provide a substantially specific capture antibody. Affinity chromatography is usually preceded by other purification techniques, such as liquid chromatography.

In a further embodiment, antibodies or antibody fragments can be isolated from antibody phage libraries generated via the techniques described in McCafferty *et al.*, Nature, **348**:552-554 (1990), using the flag polypeptide, rPTK, or a fragment thereof, to select for a suitable antibody or antibody fragment. Clackson *et al.*, Nature, **352**:624-628 (1991) and Marks *et al.*, J. Mol. Biol., **222**:581-597 (1991) describe the isolation of murine and human antibodies, respectively, using phage libraries. Subsequent *publications describe* the production of high affinity (nM range) human antibodies by chain shuffling (Mark *et al.*, Bio/Technol. **10**:779-783 [1992]), as well as combinatorial infection and *in vivo* recombination as a strategy for constructing very large phage libraries (Waterhouse *et al.*, Nuc. Acids Res., **21**:2265-2266 [1993]). Thus, these techniques are viable alternatives to traditional monoclonal antibody hybridoma techniques for

isolation of "monoclonal" antibodies which are encompassed by the present invention.

DNA encoding the monoclonal antibodies of the invention is readily isolated and sequenced using conventional procedures (*e.g.*, by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of murine antibodies). The hybridoma cells of the invention serve as a preferred source of such DNA. Once isolated, the DNA may be placed into expression vectors, which are then transfected into host cells such as simian COS cells, Chinese hamster ovary (CHO) cells, or myeloma cells that do not otherwise produce immunoglobulin protein, to obtain the synthesis of monoclonal antibodies in the recombinant host cells. The DNA also may be modified, for example, by substituting the coding sequence for human heavy and light chain constant domains in place of the homologous murine sequences, Morrison *et al.*, Proc. Nat. Acad. Sci. **81**, 6851 (1984), or by covalently joining to the immunoglobulin coding sequence all or part of the coding sequence for a non-immunoglobulin polypeptide. In that manner, "chimeric" or "hybrid" antibodies are prepared that have the binding specificity of an anti-rPTK or anti-flag polypeptide monoclonal antibody herein. Thus, the antibody may be made by recombinant DNA methods (Cabilly *et al.*, U.S. Pat. No. 4,816,567).

Binding of the capture agent is not affected by the presence or absence of a ligand bound to the receptor and the capture agent does not sterically block access to the phosphorylated tyrosine(s) by the anti-phosphotyrosine antibody. Furthermore, the capture agent does not, of course, activate the receptor of interest. In order to screen for an antibody having these characteristics, the procedure outlined in Figure 3 can be carried out.

First, once the capture agent (*e.g.* an antibody or streptavidin) has been chosen, binding to either the receptor or the flag polypeptide (where a receptor construct is to be used in the assay) is confirmed. This can be determined by flow cytometric analysis, immuno-precipitation or antigen-coat *ELISA*, for example. Flow cytometric analysis has been described above. Immunoprecipitation usually involves lysing the cells (having the receptor or receptor construct) in non-ionic detergent (*e.g.* 0.5% Triton X-100) in a suitable buffer (*e.g.* PBS) and the cell lysates thus obtained are then incubated with the potential anti-receptor or anti-flag polypeptide capture agent. The immune complexes are precipitated with

either (a) anti-capture agent antibodies in the presence of polyethylene glycol (PEG) which enhances precipitation of the immune complex or with (b) insoluble (*e.g.* agarose bound) protein A or protein G. The immunoprecipitated material is then analyzed by polyacrylamide gel electrophoresis (PAGE). For antigen-coat ELISA, ELISA wells are coated overnight with either the purified receptor, purified flag polypeptide or purified receptor construct. The coated wells are then exposed to the potential capture agent and screened with HRPO-conjugated species specific anti-capture agent antibody.

The ability of the capture agent to bind to the receptor or flag polypeptide in the presence of a ligand to the receptor is also confirmed. This can be analyzed by incubating the receptor or receptor construct with a known ligand for the receptor (*e.g.* the endogenous growth factor or an agonist antibody thereto). Flow cytometric analysis, immunoprecipitation or antigen-coat ELISA can then be performed substantially as described above to investigate binding of the capture agent.

Assuming the capture agent is suitable as determined by the preceding two steps, it is then shown that the capture agent does not induce receptor activation (*i.e.* autophosphorylation) either before or after cell lysis. Thus, the cell-bound receptor or receptor construct is exposed to either the potential capture agent or a negative control (*e.g.* a control antibody which does not activate the receptor). Following cell lysis, the receptor or receptor construct can be subjected to Western blot analysis using labeled anti-phosphotyrosine antibodies. See, *e.g.*, Glenney *et al.*, Journal of Immunological Methods **109**:277-285 (1988); Kamps, Methods in Enzymology **201**:101-110 (1991); Kozma *et al.*, Methods in Enzymology **201**:28-43 (1991); or Holmes *et al.*, Science **256**:1205-10 (1992). To establish whether the capture agent induces receptor activation following cell lysis, a trial run of the KIRA ELISA (with both the capture agent and a negative control as discussed above) can be performed.

Finally, the ability of an anti-phosphotyrosine antibody (*e.g.* biotinylated anti-phosphotyrosine antibody) to bind the activated receptor in the presence of the potential capture agent is confirmed by a trial run in the KIRA ELISA disclosed herein.

Assuming the capture agent meets all the criteria specified above, it has good potential for use in the KIRA ELISA.

Once a suitable capture agent has been prepared, the second solid phase is coated therewith. Between about 0.1 to 10 μg/ml of capture agent

can be added to each well of the second assay plate using a pipette, for example. The capture agent is often provided in a buffer at a high pH (*e.g.*, between about 7.5 to 9.6) so that it has an increased overall charge and therefore displays enhanced binding to the second assay plate. Usually, the capture agent will be incubated in the wells for between about 8 to 72 hours to enable a sufficient coating of the capture agent to form on the inside walls of the wells. This incubation is generally carried out at low temperatures (*e.g.*, between about 3-8°C) to avoid or reduce degradation of the capture agent.

Following incubation, the wells of the plate are decanted and tamped lightly with an absorbent substrate. Non-specific binding is then blocked. In order to achieve this, a block buffer, is added to the wells. For example, a block buffer containing bovine serum albumin (BSA) such as that sold by Intergen Company, Purchase, NY, is suitable. It has been found that addition of between about 100 to 200µl of block buffer to each well followed by gentle agitation at room temperature for between about 1-2 hours is sufficient to block non-specific binding. It is also possible to add the block buffer directly to the cell lysate obtained in the previous step rather than to the second assay plate.

Following this, the capture agent-coated plates are washed several times (usually between about 3-8 times) with a wash buffer. The wash buffer can comprise phosphate buffered saline (PBS) at pH 7.0 to 7.5, for example. However, other wash buffers are available which can also be used. Conveniently, an automated plate washer, such as the ScanWasher 300 (Skatron Instruments, Inc., Sterling, VA) can be used for this, and other, washing steps of the assay.

B. Measuring tyrosine phosphorylation

The activated, solubilized rPTK (or receptor construct) is then added to the wells having the capture agent adhering thereto. As a general proposition, about 80% of cell lysate obtained as mentioned under Section 1E above can be added to each well (*i.e.*, about 60 to 160 µl depending on the original volume of the wells). The lysate is incubated with the capture agent for an adequate period of time to enable the rPTK to be captured in the wells, *e.g.*, from 1 to 3 hours. Incubation can be carried out at room temperature.

Unbound cell lysate is then removed by washing with wash buffer. Following this washing step, an amount of the anti-phosphotyrosine antibody which is equal to, or less than, the amount of block buffer added

previously, is added to each well. For example, about 50 to 200µl of an anti-phosphotyrosine antibody preparation having between about 0.3 to 0.5 µg/ml of antibody in a suitable buffer (*e.g.*, PBS with a detergent such as those included in the lysis buffer) is added to the well. This is followed by a washing step to remove unbound anti-phosphotyrosine antibody.

Tyrosine phosphorylation is then quantified by the amount of anti-phosphotyrosine antibody binding to the second solid phase. Many systems for detecting the presence of an antibody are available to those skilled in the art. Some examples follow.

Generally, the anti-phosphotyrosine antibody will be labelled either directly or indirectly with a detectable label. Numerous labels are available which can be generally grouped into the following categories:

(a) Radioisotopes, such as ^{35}S, ^{14}C, ^{125}I, ^{3}H, and ^{131}I. The antibody can be labeled with the radioisotope using the techniques described in Current Protocols in Immunology, *supra*, for example and radioactivity can be measured using scintillation counting.

(b) Fluorescent labels such as rare earth chelates (europium chelates) or fluorescein and its derivatives, rhodamine and its derivatives, dansyl, Lissamine, phycoerythrin and Texas Red are available. The fluorescent labels can be conjugated to the antibody using the techniques disclosed in Current Protocols in Immunology, *supra*, for example. Fluorescence can be quantified using a fluorimeter (Dynatech).

(c) Various enzyme-substrate labels are available and U.S. Patent No. 4,275,149 provides a review of some of these. The enzyme generally catalyses a chemical alteration of the chromogenic substrate which can be measured using various techniques. For example, the enzyme may catalyze a color change in a substrate, which can be measured spectrophotometrically. Alternatively, the enzyme may alter the fluorescence or chemiluminescence of the substrate. Techniques for quantifying a change in fluorescence are described above. The chemiluminescent substrate becomes electronically excited by a chemical reaction and may then emit light which can be measured (using a Dynatech ML3000 chemiluminometer, for example) or donates energy to a fluorescent acceptor. Examples of enzymatic labels include luciferases (*e.g.*, firefly luciferase and bacterial luciferase; U.S. Patent No. 4,737,456), luciferin, 2,3-dihydrophthalazinediones, malate dehydrogenase, urease, peroxidase such as horseradish peroxidase (HRPO), alkaline phosphatase, β-galactosidase, glucoamylase, lysozyme, saccharide oxidases (*e.g.*, glucose oxidase, galactose oxidase, and glucose-6-phosphate

dehydrogenase), heterocyclic oxidases (such as uricase and xanthine oxidase), lactoperoxidase, microperoxidase, and the like. Techniques for conjugating enzymes to antibodies are described in O'Sullivan *et al.*, Methods for the Preparation of Enzyme-Antibody Conjugates for use in Enzyme Immunoassay, in Methods in Enzym. (ed J. Langone & H. Van Vunakis), Academic press, New York, 73: 147-166 (1981) and Current Protocols in Immunology, *supra*.

Examples of enzyme-substrate combinations include, for example:

(i) Horseradish peroxidase (HRPO) with hydrogen peroxidase as a substrate, wherein the hydrogen peroxidase oxidizes a dye precursor (*e.g.* orthophenylene diamine [OPD] or 3,3',5,5'-tetramethyl benzidine hydrochloride [TMB]).

(ii) alkaline phosphatase (AP) with para-Nitrophenyl phosphate as chromogenic substrate.

(iii) β-D-galactosidase (β-D-Gal) with a chromogenic substrate (*e.g.* p-nitrophenyl-β-D-galactosidase) or fluorogenic substrate 4-methylumbelliferyl-β-D-galactosidase.

Numerous other enzyme-substrate combinations are available to those skilled in the art. For a general review of these, see U.S. Patent Nos. 4,275,149 and 4,318,980.

Sometimes, the label is indirectly conjugated with the antibody. The skilled artisan will be aware of various techniques for achieving this. For example, the antibody can be conjugated with biotin and any of the three broad categories of labels mentioned above can be conjugated with avidin, or *vice versa*. Biotin binds selectively to avidin and thus, the label can be conjugated with the antibody in this indirect manner. See, Current Protocols in Immunology, *supra*, for a review of techniques involving biotin-avidin conjugation. Alternatively, to achieve indirect conjugation of the label with the antibody, the antibody is conjugated with a small hapten (*e.g.* digoxin) and one of the different types of labels mentioned above is conjugated with an anti-hapten antibody (*e.g.* anti-digoxin antibody). Thus, indirect conjugation of the label with the antibody can be achieved.

In another embodiment of the invention, the anti-phosphotyrosine antibody need not be labeled, and the presence thereof can be detected using a labeled anti-antiphosphotyrosine antibody (*e.g.* anti-mouse anti-phosphotyrosine antibody conjugated with HRPO).

In the preferred embodiment, the anti-phosphotyrosine antibody is labeled with an enzymatic label which catalyzes a color change of a substrate (such as tetramethyl benzimidine [TMB], or orthaphenylene diamine [OPD]). Thus, the use of radioactive materials is avoided. A color change of the reagent can be determined spectrophotometrically at a suitable wavelength (*e.g.* 450nm for TMB and 490nm for OPD, with a reference wavelength of 650 nm).

3. Intracellular Kinase Activity

The assay described herein is also useful for measuring phosphorylation and/or activation of intracellular kinases (*e.g.* cytoplasmic tyrosine kinases and/or cytoplasmic serine-threonine kinases). Phosphorylation of these molecules can occur as a consequence of trans-phosphorylation of the intracellular kinase by a kinase receptor or "receptor complex" (which comprises one or more kinase receptors residing in a cell membrane). Examples of intracellular tyrosine kinases include insulin receptor substrate I (IRS-1), Shc, Ras and GRB2, for example. Antibodies to human Shc, human Ras and GRB2 can be obtained commercially from UBI, NY, which can be used as capture agents for these tyrosine kinases. Examples of intracellular serine-threonine kinases include MEK and MAPK.

In order to measure phosphorylation of these kinases, the procedure is essentially as described above except that a chimera of the intracellular kinase and the flag polypeptide is normally formed (*i.e.* a "kinase construct"). Alternatively, the cell has an endogenous intracellular kinase or is transformed with nucleic acid encoding an intracellular kinase of interest. Generally, a eukaryotic cell will be transformed with nucleic acid encoding a kinase construct. Upon expression of the nucleic acid, the kinase or kinase construct will reside intracellularly (*i.e.* in the cytoplasm). The cells comprising the kinase or kinase construct are subjected to the KIRA as discussed above. Exposure to the agonist may result in trans-phosphorylation of the intracellular kinase which can be quantified in the ELISA as elaborated above. The capture agent in the ELISA binds to either the intracellular kinase or to the flag polypeptide.

4. Serine-Threonine Kinase Activity

This assay is further useful for measuring phosphorylation and/or activation of serine-threonine kinases. The term "serine-threonine kinase" refers to a kinase which phosphorylates a substrate which has at least one

phosphate accepting alcohol group. The serine-threonine kinase is usually a "receptor" insofar as it has a ligand-binding ECD, TM domain and ICD. The ICD usually comprises a catalytic kinase domain and generally has one or more phosphate accepting serine and/or threonine residues. Examples of intracellular serine-threonine kinases include MEK and MAPK. See section 3 above for a discussion as to measuring phosphorylation of intracellular serine-threonine kinases. Examples of serine-threonine kinase receptors include *daf*-1, activin type II receptor (ActR-II), activin type IIB receptor (ActR-IIB), TGF-β type II receptor (TβR-II), activin receptor-like kinase (ALK) -1, -2, -3, -4 and TGF-β type I receptor (TβR-1)/ALK-5. See ten Dijke *et al.*, *supra*. The serine-threonine kinase assay is essentially the same as described above for tyrosine kinases, except that phosphorylation is quantified using anti-phosphoserine and/or anti-phosphothreonine antibodies. Anti-phosphoserine and anti-phosphothreonine monoclonal antibodies can be purchased from Sigma Immuno Chemicals, St Louis, MO, for example.

5. Phosphatase Activity

Phosphatase activity can similarly be measured using the assay described herein. Phosphatase enzymes are able to dephosphorylate phosphorylated tyrosine, serine and/or threonine residues (*i.e.* liberate inorganic phosphate from phosphoric esters of such amino acid residues). Generally the phosphatase enzyme is specific for either tyrosine residues or serine-threonine residues but sometimes can dephosphorylate tyrosine, serine and threonine residues. Sometimes "endogenous" phosphatase activity is measured and this refers to the activity of phosphatase enzyme(s) which exist in nature in a selected cell.

In order to quantify endogenous phosphatase activity, cells possessing at least one phosphatase are stimulated in the presence and absence of one or more phosphatase inhibitors. Examples of protein tyrosine phosphatase (PTPase) inhibitors include sodium orthovanadate and sodium molybdate (Sigma Chemical Co., St. Louis, MO). ICN Biochemicals supply okadaic acid which is a serine-threonine phosphatase inhibitor. As a general proposition, between about 1-10μM phosphatase inhibitor can be added to each well of the assay plate. In all other respects, the assay is performed essentially as discussed above. Thus, the ability of endogenous phosphatases to dephosphorylate a kinase in the selected cell can be quantified.

In the preferred embodiment, a phosphatase enzyme of interest can be studied. Examples of protein tyrosine phosphatases (PTPases) include PTP1B, PTPMEG, PTP1c, Yop51, VH1, cdc25, CD45, HLAR, PTP18, HPTPα and DPTP10D. See Zhang and Dixon, Adv. Enzym. 68: 1-36 (1994). Examples of protein serine-threonine phosphatases include PP1, PP2A, PP2B and PP2C. See Meth. Enzym., ed Hunter & Sefton, Academic press, New York, 201:389-398 (1991). These proteins can be purchased commercially or made using the recombinant techniques described herein. To measure phosphatase activity, the KIRA ELISA can be performed essentially as described above with the following modifications. Following capture of the kinase or kinase construct (*e.g.* receptor construct) to the second solid phase and the washing step (to remove unbound cell lysate), the phosphatase of interest is added to the wells of the second assay plate and incubated with the adhering kinase or kinase construct. For example, between about 50-200 μl of the phosphatase in a suitable dilution buffer (see Meth. Enzym., ed Hunter & Sefton, Academic press, New York, 201:416-440 [1991]) can be added to each well. This is generally followed by gentle agitation at room temperature (or 37°C) for between about 30 min to 2 hours to allow the phosphatase to dephosphorylate the kinase. Following washing to remove the phosphatase, the decreased degree of phosphorylation of the kinase relative to the control (*i.e.* no phosphatase added) is quantified by ELISA as described earlier herein.

6. Kits

As a matter of convenience, the reagents can be provided in a kit, *i.e.*, a packaged combination of reagents, for combination with the analyte in assaying the ability of the analyte to activate or prevent activation of a rPTK of interest. The components of the kit will be provided in predetermined ratios. Thus, a kit will comprise the specific second solid phase for the assay as well as the anti-flag polypeptide capture agent either packaged separately or captured to the second solid phase (*e.g.* a microtiter plate). Usually, other reagents, such as the anti-phosphotyrosine antibody labelled directly or indirectly with an enzymatic label will also be provided in the kit. Where the detectable label is an enzyme, the kit will include substrates and cofactors required by the enzyme (*e.g.* a substrate precursor which provides the detectable chromophore or fluorophore). In addition, other additives may be included such as stabilizers, buffers (*e.g.* a block buffer and a lysis buffer) and the like. Conveniently, the kit can also supply the homogeneous population

of cells which have been transformed with the receptor construct. The relative amounts of the various reagents may be varied widely to provide for concentrations in solution of the reagents which substantially optimize the sensitivity of the assay. Particularly, the reagents may be provided as dry powders, usually lyophilized, including excipients which on dissolution will provide a reagent solution having the appropriate concentration. The kit also suitably includes instructions for carrying out the KIRA ELISA.

7. Uses for the Assay

This application provides two assays which are useful for reliable, sensitive and quantitative detection of kinase activation. The first assay can be used where a kinase receptor-specific capture antibody having the desired characteristics herein described is available or has been prepared. The second assay is a generic assay which enables activation of any kinase receptor to be measured via the use of a flag polypeptide and a capture agent which binds specificity thereto.

These assays are useful for identifying novel agonists/antagonists for a selected kinase receptor. Also, the assay provides a means for studying ligand-receptor interactions (*i.e.*, mechanism studies). Also the presence of an endogenous receptor in a selected cell line can be quantified using the assay. The assays are further useful for identifying the presence of a ligand for a selected kinase receptor in a biological sample and, *e.g.*, establishing whether a growth factor has been isolated following a purification procedure. It is desirable to have an assay for measuring the ability of these growth factors to activate their respective receptors.

The assay also has clinical applications for detecting the presence of a ligand for a selected rPTK (*e.g.* the insulin receptor) in a biological sample taken from a human and thus patients having elevated or depressed levels of the ligand can be identified. This is particularly desirable where elevated or depressed levels of the ligand cause a pathological condition. Accordingly, candidates for administration of the selected ligand (*e.g.* insulin) can be identified through this diagnostic method. It is possible, using the assay disclosed herein, to assay the pK of agonists or antagonists administered to a patient. This assay also facilitates the detection of shed receptor in a biological sample.

The assay is also useful to quantify phosphatase activity of endogenous phosphatases or, in the preferred embodiment, a phosphatase of

interest. This can be used for screening phosphatase inhibitors, for example.

Below are examples of specific embodiments for carrying out the present invention. The examples are offered for illustrative purposes only, and are not intended to limit the scope of the present invention in any way.

EXAMPLE 1

KIRA ELISA of the HER2 Receptor

The assay system described in this example was developed to measure the extent of autophosphorylation as a result of the interactions between the HER2 receptor and its specific activator, heregulin (HRG). The overexpression of $p185^{HER2}$ has been correlated with poor clinical outcome in a number of epithelial-derived cancers. Heregulin and its rodent homologue, *neu* differentiation factor (NDF), were originally purified based on their ability to stimulate the autophosphorylation of a 185 kDa protein in the breast carcinoma cell lines MCF-7 and MDA-453, respectively. In this embodiment of the invention, the cell line expressing the tyrosine kinase receptor DNA (either endogenous or transformed) is adherent and there is an antibody (*e.g.* monoclonal or affinity purified polyclonal) capable of specifically binding the receptor such that it neither stimulates autophosphorylation in the absence of ligand nor suffers impaired binding due to the presence of bound ligand. Standard curve preparations and many samples may easily be run simultaneously in replicate and at several dilutions using this assay, readily allowing quantitation of ligand activity in a large number of unknown samples.

(i) Capture agent preparation

Polyclonal anti-HER2 antibody was isolated from pooled immune sera from New Zealand White rabbits immunized with the extracellular domain of the HER2 molecule (Fendly *et al.*, Journal of Biological Response Modifiers 9:449-455 [1990]). The rHER2 ECD specific antibodies were affinity purified using an FPLC (Pharmacia Biotech, Inc, Piscataway, NJ) with an affinity column generated from rHER2 ECD conjugated to Avidgel F (Bioprobe International, Inc, Tustin, CA). The resulting purified antibody stock was

0.829 mg/ml in phosphate buffered saline (PBS), pH 7.4, and was stored as 0.5 ml aliquots at -20°C.

(ii) Anti-phosphotyrosine antibody preparation

Monoclonal anti-phosphotyrosine, clone 4G10, was purchased from Upstate Biologicals, Inc (UBI, Lake Placid, NY) and biotinylated using long-arm biotin-N-hydroxysuccinamide (Biotin-X-NHS, Research Organics, Cleveland, OH).

(iii) Ligand

The recombinant truncated form of β1heregulin (MW= 7.88 Kd) corresponding to residues 177-244 ($HRG\beta1_{177-244}$) was produced in *E. coli* and purified to homogeneity as described in Holmes *et al.*, Science, **256**: 1205-1210 (1992) and was stored at 4°C as an 89.7 μM stock solution in 50 mM Tris/HCl, pH 7.5.

(iv) Adherent Cells

MCF-7 (ATCC-HTB 22), an adherent cell line isolated from a human breast adenocarcinoma, was obtained from American Type Culture Collection (ATCC, Rockville, MD). MCF-7 cells have been shown to produce measurable levels of surface $p185^{HER2}$ by both FACS and ELISA analysis. The cells were maintained in 150 cm^2 tissue culture flasks (Corning Inc, Corning, NY) and utilized when at cell densities of 60 % to 75 % confluency. For the assay, 2×10^5 cells were seeded per well in flat-bottom microtiter plates (Falcon 3072, Becton Dickinson Labware, Lincoln Park, NJ) cultured overnight at 37°C in 5 % CO_2. Cells were grown in F12/DMEM 50:50 Gibco as a custom formulation (Gibco/BRL, Life Technologies, Grand Island, NY). The medium was supplemented with 10 % FBS (HyClone, Logan, Utah), 25 mM HEPES (Gibco) and 2 mM L-glutamine (Gibco).

(v) KIRA ELISA

MCF-7 cells (2×10^5) in 100 μl media were added to each well in a flat-bottom-96 well culture plate and cultured overnight at 37°C in 5% CO_2. The following morning the well supernatants were decanted, and the plates were lightly tamped on a paper towel. 50 μl of media containing either experimental samples or the recombinant $HRG\beta1_{177-244}$ standards (3000, 1000, 333, 111, 37, 12, 4, and 0 pM) was then added to each well. The cells were stimulated at 37°C for 30 min., the well supernatants were decanted, and the plates were once again lightly tamped on a paper towel. To lyse the cells and solubilize the receptors, 100 μl of lysis buffer was added to each well. Lysis buffer consisted of 150 mM NaCl containing 50 mM HEPES (Gibco), 0.5 % Triton-X 100 (Gibco), 0.01 % thimerosal, 30 KIU/ml aprotinin

(ICN Biochemicals, Aurora, OH), 1mM 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride (AEBSF; ICN Biochemicals), 50 μM leupeptin (ICN Biochemicals), and 2 mM sodium orthovanadate (Na_3VO_4, Sigma Chemical Co, St. Louis, MO), pH 7.5. The plate was then agitated gently on a plate shaker (Bellco Instruments, Vineland, NJ) for 60 min. at room temperature.

While the cells were being solubilized, an ELISA microtiter plate (Nunc Maxisorp, Inter Med, Denmark) coated overnight at 4°C with the affinity-purified polyclonal anti-HER2 ECD (1.0 μg/ml in 50 mM carbonate buffer, pH 9.6, 100 μl/well) was decanted, tamped on a paper towel and blocked with 150 μl/well of Block Buffer [PBS containing 0.5 % BSA (Intergen Company, Purchase, NY) and 0.01 % thimerosal] for 60 min. at room temperature with gentle agitation. After 60 minutes, the anti-HER2 ECD coated plate was washed 6 times with wash buffer (PBS containing 0.05 % Tween-20 and 0.01 % thimerosal) using an automated plate washer (ScanWasher 300, Skatron Instruments, Inc, Sterling, VA).

The lysate containing solubilized $p185^{HER2}$ from the cell-culture microtiter well was transferred (85 μl/well) to anti-rHER2 ECD coated and blocked ELISA well and was incubated for 2 h at room temperature with gentle agitation. The unbound receptor was removed by washing with wash buffer and 100 μl of biotinylated 4G10 (anti-phosphotyrosine) diluted 1:2000 in dilution buffer (PBS containing 0.5 % BSA, 0.05 % Tween-20, 5 mM EDTA, and 0.01 % thimerosal), *i.e.* 400pg/ml, was added to each well. After incubation for 2 h at room temperature the plate was washed and 100 μl of HRPO-conjugated streptavidin (Zymed Laboratories, S. San Francisco, CA) diluted 1:10000 in dilution buffer was added to each well. The plate was incubated for 30 minutes at room temperature with gentle agitation. The free avidin-conjugate was washed away and 100 μl freshly prepared substrate solution (tetramethyl benzidine [TMB]; 2-component substrate kit; Kirkegaard and Perry, Gaithersburg, MD) was added to each well. The reaction was allowed to proceed for 10 minutes, after which the color development was stopped by the addition of 100 μl/well 1.0 M H_3PO_4. The absorbance at 450 nm was read with a reference wavelength of 650 nm ($ABS_{450/650}$), using a *vmax* plate reader (Molecular Devices, Palo Alto, CA) controlled with a Macintosh Centris 650 (Apple Computers, Cupertino, CA) and DeltaSoft software (BioMetallics, Inc, Princeton, NJ).

The standard curve shown in Figure 7 was generated by stimulating MCF-7 cells with 3000, 1000, 333, 111, 37, 12, 4, or 0 pM $HRG\beta1_{177-244}$ and presented as pM $HRG\beta1_{177-244}$ vs. mean $ABS_{450/650}$ ± sd using the DeltaSoft

program. Sample concentrations were obtained by interpolation of their absorbance on the standard curve and are expressed in terms of pM $HRG\beta1_{177-244}$ activity.

When the data were fitted to a 4-parameter nonlinear least squares equation, they resulted in a correlation coefficient of 0.9998. For the data shown in Figure 7, the EC_{50} of receptor activation by $HRG\beta1_{177-244}$ was 373 pM. To demonstrate the highly reproducible nature of the $p185^{HER2}$ KIRA ELISA, seven standard curves were generated over the period of one month and the EC_{50}'s are averaged. This gives an $EC_{50}ave$ for $HRG\beta1_{177-244}$ of 360 ± 40 pM (average ± SD).

(vi) Intra- and inter-assay precision and assay specificity

The intra-assay variability was determined by performing the $p185^{HER2}$ KIRA ELISA on three separate days. For each test, the standard curve is run in triplicate. Controls with $HRG\beta1_{177-244}$ corresponding to high (1000 pM), mid (200 pM) and low (40 pM) were assayed in 24 replicates. The $ABS_{450/650}$ of the individual test samples were converted to pM $HRG\beta1_{177-244}$ activity and the 24 converted values for each test concentration were averaged. The data are expressed as *averaged value* and *% coefficient of variation (%cv;* [(intra-assay standard deviation/intra-assay averaged calculated value) x 100]. See Table 1A below.

Table 1

Intra- and Inter-assay Variation

A. Intra-assay Precision (n-24 per test)

	High Value[a]		Mid Value		Low Value	
	Average Value (pM)	% cv[b]	Average Value (pM)	% cv	Average Value (pM)	% cv
Test #1	1256	19.5%	209	10.8%	33	12.3%
Test #2	1078	10.0%	196	5.1%	38	7.5%
Test #3	999	14.3%	196	6.3%	35	11.3%

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B. Inter-assay Precision (n=3)

Average Value (pM)	%cv[c]	Average Value (pM)	% cv	Average Value (pM)	% cv
1100	4.3%	200	6.3%	34	9.0%

[a]Expected high value: 1000 pM; mid value: 200 pM; low value: 40 pM
[b]Intra-assay % cv determined as intra-assay sd/intra-assay average x 100
[c]Inter-assay % cv determined as inter-assay sd/inter-assay average x 100

The intra-assay variability of the KIRA ELISA was within acceptable limits despite the fact that the assay actually consists of both bioassay and ELISA components. The coefficients of variance (%) for the highest values were under 20% and for the mid and low values were at or under 10%.

The inter-assay variability was determined by averaging the values from upper-most three adjacent wells (of the 24 wells run) for a given sample concentration from each run. The three separate averages for each test concentration were then averaged. The data were expressed as *averaged value* and *%cv* [(inter-assay standard deviation/inter-assay averaged value) x 100]. See Table 1B. above. The inter-assay variability of the KIRA ELISA was within acceptable limits.

In order to confirm the specificity of the assay, MCF-7 cells were stimulated with either $HRG\beta1_{177-244}$ at 3000, 1000, 333, 111, 37, 12, 4 or 0 pM or insulin-like growth factor-1 (IGF-1), epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), or insulin at 30000, 10000, 3333, 1111, 370, 120, 40 or 0 pM. The $p185^{HER2}$ KIRA ELISA was then performed as described above. The results are depicted in Figure 8.

The $p185^{HER2}$ KIRA ELISA was clearly specific for heregulin. While $HRG\beta1_{177-244}$ induced normal receptor stimulation and autophosphorylation, the closely related EGF gives only a slight stimulation ($OD_{450/650}$ = 0.239) at the highest concentration tested (100 nM). Since EGF-R is produced in MCF-7 cells, this signal is likely due to EGF receptor transphosphorylation of $p185^{HER2}$. Neither insulin-like growth factor-1 (IGF-1), vascular endothelial growth factor (VEGF) nor insulin have any detectable effect on the MCF-7 $p185^{HER2}$ KIRA ELISA, the latter despite the fact that MCF-7 cells produce active insulin receptors.

The results presented in this example demonstrate that the KIRA ELISA is a useful method for assaying ligand activation of a kinase receptor, *e.g.*, heregulin activation of the $p185^{HER2}$ receptor. Levels of receptor activation in terms of tyrosine phosphorylation are easily quantified and an EC_{50} for a given ligand is readily determined. One potential use for this

assay would be to screen compounds for receptor agonist or antagonist activities. The potential throughput for this assay greatly surpasses that of Western blot analysis. Since the cell-culture portion of the assay is conducted in 96-well plates, many samples may be run in replicate at different dilutions at one time in a one-day assay.

EXAMPLE 2

KIRA ELISA of the Rse Receptor

Mark *et al.*, Journal of Biological Chemistry **269(14)**:10720-10728 (1994) describe isolation of the Rse receptor protein tyrosine kinase from human and murine tissues. This Rse receptor with a carboxyl-terminal flag polypeptide (*i.e.* Rse.gD) was subjected to the KIRA ELISA described herein. The experimental procedure is outlined below.

(i) Capture agent preparation

Monoclonal anti-gD (clone 5B6) was produced against a peptide from Herpes simplex virus glycoprotein D (Paborsky *et al.*, Protein Engineering **3(6)**:547-553 [1990]). The purified stock preparation was adjusted to 3.0mg/ml in phosphate buffered saline (PBS), pH 7.4 and 1.0ml aliquots were stored at -20°C.

(ii) Anti-phosphotyrosine antibody preparation

Monoclonal anti-phosphotyrosine, clone 4G10, was purchased from Upstate Biologicals, Inc (UBI, Lake Placid, NY) and biotinylated using long-arm biotin-N-hydroxysuccinamide (Biotin-X-NHS, Research Organics, Cleveland, OH).

(iii) Ligand

Since the endogenous ligand for the Rse receptor was not available, an agonist antibody for the Rse receptor was prepared which forms the ligand for the KIRA ELISA described in this Example. To generate the agonist antibody, a Rse.IgG chimera was generated. Briefly, the coding sequence of the ECD of Rse was fused to that of the human IgG-γ1 heavy chain in a multi-step process. PCR was used to generate a fragment with a unique BstEII site 3' to the coding sequences of the Rse amino acid 428. The PCR product was joined to the human IgG-γ_1 heavy chain cDNA through a unique BstEII site in that construct (Mark *et al.*, J. Cell. Biol., **267**: 26166-26171 [1992]). The resulting construct (termed pRK.bpTK3.IgG.fusion) contained the coding sequences for amino acids 375-428 of Rse joined to those encoding human IgG-γ_1 heavy chain. The remaining portion of the Rse

ECD (amino acids 1-374) was then added by linkage through the Bam HI site in pRK.bpTK3.IgG.fusion to yield pRK.Rse.IgG.

To generate stable cell populations expressing Rse.IgG, the cDNA encoding Rse.IgG was subcloned into the episomal CMV-driven expression plasmid pCIS.EBON, a pRK5 derivative disclosed in Cachianes *et al.*, Bio. Techniques, **15**: 225-259 (1993). Human fetal kidney 293 cells (obtained from ATCC, 12301 Parklawn Drive, Rockville, MD, USA) were transfected by the calcium phosphate technique. Cell monolayers were incubated for four hours in the presence of the DNA precipitate, glycerol shocked, and cultured in F12:DMEM (1:1) containing 2mM glutamine, 10% fetal bovine serum, penicillin and streptomycin. After 48 hours, populations were replated in media containing G418 to select for a stable population of cells. Conditioned media was collected from cells expressing Rse.IgG nucleic acid that have been cultured in serum-free media for 72 hours in the absence of G418.

Rse.IgG was purified by affinity chromatography on a protein A column using procedures as described by Chamow, S.M., *et al.*, Biochemistry, **29**:9885-9891 (1990) with the following minor modifications. Conditioned media collected from cells expressing the Rse.IgG was adjusted to 0.1 M citrate pH 6.0 and loaded directly onto a protein A column (Repligen). The column was washed with 0.1 M citrate, pH 6.0, and was eluted with 3 M $MgCl_2$ with 10% glycerol. Fractions were pooled and desalted on a PD-10 column, dialyzed and concentrated against PBS. Protein concentrations were determined by an ELISA against human IgG (Fc). The protein was analyzed for purity by Coomassie staining of PAGE gels.

Polyclonal antibodies were generated in New Zealand white rabbits against the Rse.IgG formed as described above. 4μg of Rse.IgG in 100μL PBS was emulsified with 100μL Freund's adjuvant (complete adjuvant for the primary injection and incomplete adjuvant for all boosts). For the primary immunization and the first boost, the protein was injected directly into the popliteal lymph nodes (Sigel *et al.*, Methods Enzymol., **93**, 3-12 [1983]). For subsequent boosts, the protein was injected into subcutaneous and intramuscular sites. 1.3 μg protein/kg body weight was injected every 3 weeks with bleeds taken 1 and 2 weeks following each boost. The polyclonal antisera generated was then precipitated in 50% ammonium sulphate.

The resultant, purified polyclonal antisera is called "19B" herein. To confirm the ability of the 19B antisera to induce autophosphorylation of the Rse receptor, serum starved 3T3.gD.R11 cells (transformed with

nucleic acid encoding the Rse receptor with an amino terminal gD flag polypeptide [*i.e.* gD.Rse] using the techniques described in Mark *et al.*, Journal of Biological Chemistry **269(14)**:10720-10728 [1994]) or NIH3T3 cells were exposed to pre-immune serum or 19B polyclonal antisera at a 1:200 dilution for 10 minutes. The gD.Rse protein was immunoprecipitated from extracts using the anti-gD monoclonal antibody 5B6. Proteins were fractionated on 7% SDS-PAGE under reducing conditions and transferred to nitrocellulose. Phosphorylation of Rse was detected with labelled anti-phosphotyrosine antibody. Treatment of the 3T3.gD.R11 cells with 19B antisera stimulated the phosphorylation of the 140kD gD.Rse protein. This increase was not observed in cells treated with pre-immune sera.

The purified 19B polyclonal antisera was stored at 4°C as an 2.8mg/ml stock solution in PBS, pH 7.5.

(iv) Preparation of Rse.gD nucleic acid

Synthetic double stranded oligonucleotides were used to reconstitute the coding sequence for the C-terminal 10 amino acids (880 - 890) of human Rse and add an additional 21 amino acids containing an epitope for the antibody 5B6 and a stop codon. The final sequence of the synthetic portion of the fusion gene was:

coding strand:

5'-TGCAGCAAGGGCTACTGCCACACTCGAGCTGCGCAGATGCTAGCCTCAAGATGGCT G
ATCCAAATCGATTCCGCGGCAAAGATCTTCCGGTCCTGTAGAAGCT-3' (SEQ ID NO: 10)

noncoding (anti-sense) strand:

5'-AGCTTCTACAGGACCGGAAGATCTTTGCCGCGGAATCGATTTGGATCAGCCATCTT G
AGGCTAGCATCTGCGCAGCTCGAGTGTGGCAGTAGCCCTTGCTGCA-3'(SEQ ID NO: 11).

The synthetic DNA was ligated with the cDNA encoding amino acids 1-880 of human Rse at the PstI site beginning at nucleotide 2644 of the published human Rse cDNA sequence (Mark *et al.*, Journal of Biological Chemistry **269(14)**:10720-10728 [1994]) and HindIII sites in the polylinker of the expression vector pSVI7.ID.LL (See Figure 16; SEQ ID NO: 9) to create the expression plasmid pSV.ID.Rse.gD. Briefly, the expression plasmid comprises a dicistronic primary transcript which contains sequence encoding DHFR bounded by 5' splice donor and 3' splice acceptor intron splice sites, followed by sequence that encodes the Rse.gD. The full length (non-spliced) message contains DHFR as the first open reading frame and therefore generates DHFR protein to allow selection of stable transformants.

(v) Cell transformation

dp12.CHO cells (EP 307,247 published 15 March 1989) were electroporated with 20 μgs of pSV.ID.Rse.gD which had been linearized at a unique NotI site in the plasmid backbone. The DNA was ethanol precipitated after phenol/chloroform extraction and was resuspended in 20μl 1/10 Tris EDTA. Then, 10μg of DNA was incubated with 10^7 CHO.dp12 cells in 1 ml of PBS on ice for 10 min. before electroporation at 400 volts and 330μf. Cells were returned to ice for 10 min. before being plated into non-selective medium. After 24 hours cells were fed nucleoside-free medium to select for stable DHFR+ clones.

(vi) Selection of transformed cells for use in the KIRA ELISA

To identify a cell line that expresses Rse.gD nucleic acid, candidate clones were screened by fluorescence activated cell sorting (FACS) analysis using the polyclonal antiserum 19B generated as described above, which recognizes epitopes in the extracellular domain of Rse. See Figure 5, step (b).

To confirm that clones that scored positive in the FACS assay express full-length Rse.gD nucleic acid, cell lysates were prepared (Lokker *et al.*, EMBO J, 11:2503-2510 [1992]) and solubilized Rse.gD was immunoprecipitated with the 19B antisera. The immunoprecipitated proteins were fractionated under reducing conditions using 7% PAGE, blotted onto nitrocellulose and then probed with the anti-gD 5B6 antibody which was detected with a horseradish peroxidase conjugated anti-mouse IgG antibody. See Figure 5, step (c). The ability of Rse.gD in cell clones to be activated to undergo autophosphorylation in response to the 19B agonistic antibody was determined. Briefly, serum starved dp.CHO cells transformed with Rse.gD nucleic acid as described above were exposed to pre-immune or 19B antisera at a 1:200 dilution for 10 min. The Rse.gD protein was immunoprecipitated from extracts using the anti-gD 5B6 monoclonal antibody. Proteins were fractionated on 7% SDS-PAGE under reducing conditions and transferred to nitrocellulose. Phosphorylation of Rse was detected with labelled antiphosphotyrosine antibody. See Figure 5, step (d).

(vii) Media

Cells were grown in F12/DMEM 50:50 (Gibco/BRL, Life Technologies, Grand Island, NY). The media was supplemented with 10% diafiltered FBS (HyClone, Logan, Utah), 25mM HEPES and 2mM L-glutamine.

(viii) KIRA ELISA

Rse.gD transformed dp12.CHO cells (EP 307,247 published 15 March 1989) were seeded ($5x10^4$ per well) in the wells of a flat-bottom-96 well culture plate in 100 µl media and cultured overnight at 37°C in 5% CO_2. The following morning the well supernatants were decanted, and the plates were lightly tamped on a paper towel. 100µl of media containing either experimental samples or 1:100, 1:200, 1:400, 1:800, 1:1600, 1:3200 or 0 diluted, anti-Rse agonist polyclonal antibody (19B pAb) was then added to each well. The cells were stimulated at 37°C for 30 min., the well supernatants were decanted, and the plates were once again lightly tamped on a paper towel. To lyse the cells and solubilize the receptors, 100 µl of lysis buffer was added to each well. Lysis buffer consisted of 150 mM NaCl containing 50 mM HEPES (Gibco), 0.5 % Triton-X 100 (Gibco), 0.01 % thimerosal, 30 KIU/ml aprotinin (ICN Biochemicals, Aurora, OH), 1mM 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride (AEBSF; ICN Biochemicals), 50 µM leupeptin (ICN Biochemicals), and 2 mM sodium orthovanadate (Na_3VO_4; Sigma Chemical Co, St. Louis, MO), pH 7.5. The plate was then agitated gently on a plate shaker (Bellco Instruments, Vineland, NJ) for 60 min. at room temperature.

While the cells were being solubilized, an ELISA microtiter plate (Nunc Maxisorp, Inter Med, Denmark) coated overnight at 4°C with the 5B6 monoclonal anti-gD antibody (0.5 µg/ml in 50 mM carbonate buffer, pH 9.6, 100 µl/well) was decanted, tamped on a paper towel and blocked with 150 µl/well of Block Buffer [PBS containing 0.5 % BSA (Intergen Company, Purchase, NY) and 0.01 % thimerosal] for 60 min. at room temperature with gentle agitation. After 60 minutes, the anti-gD 5B6 coated plate was washed 6 times with wash buffer (PBS containing 0.05 % Tween-20 and 0.01 % thimerosal) using an automated plate washer (ScanWasher 300, Skatron Instruments, Inc, Sterling, VA).

The lysate containing solubilized Rse.gD from the cell-culture microtiter well was transferred (85 µl/well) to anti-gD 5B6 coated and blocked ELISA well and was incubated for 2 h at room temperature with gentle agitation. The unbound Rse.gD was removed by washing with wash buffer and 100 µl of biotinylated 4G10 (anti-phosphotyrosine) diluted 1:2000 in dilution buffer (PBS containing 0.5 % BSA, 0.05 % Tween-20, 5 mM EDTA, and 0.01 % thimerosal), *i.e.* 400 pg/ml was added to each well. After incubation for 2 h at room temperature the plate was washed and 100 µl of HRPO-conjugated streptavidin (Zymed Laboratories, S. San Francisco, CA)

diluted 1:10000 in dilution buffer was added to each well. The plate was incubated for 30 minutes at room temperature with gentle agitation. The free avidin-conjugate was washed away and 100 µl freshly prepared substrate solution (tetramethyl benzidine [TMB]; 2-component substrate kit; Kirkegaard and Perry, Gaithersburg, MD) was added to each well. The reaction was allowed to proceed for 10 minutes, after which the color development was stopped by the addition of 100 µl/well 1.0 M H_3PO_4. The absorbance at 450 nm was read with a reference wavelength of 650 nm ($ABS_{450/650}$), using a *vmax* plate reader (Molecular Devices, Palo Alto, CA) controlled with a Macintosh Centris 650 (Apple Computers, Cupertino, CA) and DeltaSoft software (BioMetallics, Inc, Princeton, NJ).

The standard curve shown in Figure 10 was generated by stimulating Rse.gD transformed CHO cells with 1:100, 1:200, 1:400, 1:800, 1:1600, 1:3200 or 0 diluted, anti-Rse agonist antibody (19B) and presented as 1/dilution anti-Rse agonist antibody (19B) vs. mean $ABS_{450/650}$ ± sd using the DeltaSoft program.

The results presented in this example demonstrate that the KIRA ELISA is a useful method for assaying ligand activation of a receptor construct having a carboxyl terminal flag polypeptide, *e.g.*, activation of Rse.gD. Levels of receptor activation in terms of tyrosine phosphorylation are easily quantified and an EC_{50} for a given ligand (*e.g.* an agonist antibody for the receptor) is readily determined.

EXAMPLE 3

KIRA ELISA of the trk A, B and C Receptors

Neurotrophins belong to a family of small, basic proteins which play a crucial role in the development and maintenance of the nervous system. The first identified and probably best understood member of this family is nerve growth factor (NGF). See U.S. Patent No. 5,169,762, issued December 8, 1992. Recently, sequentially related but distinct polypeptides with similar functions to NGF have been identified. For example, brain-derived neurotrophic factor (BDNF), now also referred to as neurotrophin-2 (NT2), was cloned and sequenced by Leibrock *et al.* (Nature, **341**: 149-152 [1989]). Several groups identified a neurotrophic factor originally called neuronal factor (NF), and now referred to as neurotrophin-3 (NT3). (Ernfors *et al.*, Proc. Natl. Acad. Sci. USA, **87**: 5454-5458 [1990]; Höhn *et al.*, Nature, **344**: 339 [1990]; Maisonpierre *et al.*, Science, **247**: 1446 [1990]; Rosenthal *et al.*, Neuron, **4**: 767 [1990]; Jones and Reichardt, Proc. Natl. Acad. Sci.

USA, 87: 8060-8064 [1990]; Kaisho *et al.*, FEBS Lett., 266: 187 [1990]). Neurotrophins-4 and -5 (NT4 and NT5) have been recently added to the family (Hallbook *et al.*, Neuron, 6: 845-858 [1991]; Berkmeier *et al.*, Neuron, 7: 857-866 [1991]; Ip *et al.*, Proc. Natl. Acad. Sci. USA, 89: 3060-3064 [1992]).

Neurotrophins, similarly to other polypeptide growth factors, affect their target cells through interactions with cell surface rPTKs (called Trk receptors). The first member of the trk receptor family, trkA, was initially identified as the result of an oncogenic transformation caused by the translocation of tropomyosin sequences onto its catalytic domain. Later work identified trkA as a signal transducing receptor for NGF. Subsequently, two other related receptors, mouse and rat trkB (Klein *et al.*, EMBO J., 8: 3701-3709 [1989]; Middlemas *et al.*, Mol. Cell. Biol., 11: 143-153 [1991]; EP 455,460 published 6 November 1991) and porcine, mouse and rat trkC (Lamballe *et al.*, Cell, 66: 967-979 [1991]; EP 522,530 published 13 January 1993), were identified as members of the trk receptor family. The structures of the trk receptors are quite similar, but alternate splicing increases the complexity of the family by giving rise to two known forms of trkA, three known forms of trkB (two without functional tyrosine kinase domains) and at least four forms of trkC (several without functional tyrosine kinase domain, and two with small inserts in the tyrosine kinase domain). Human trk A, B and C receptor sequences are disclosed in Canadian Patent application SN 2,184,059 filed March 17, 1995.

The following KIRA ELISA was performed using trk A, B and C receptor constructs having amino-terminal flag polypeptides.

(i) Capture agent preparation

Monoclonal anti-gD (clone 5B6) was produced against a peptide from Herpes simplex virus glycoprotein D as discussed above in Example 2. The purified stock preparation was adjusted to 3.0mg/ml in phosphate buffered saline (PBS), pH 7.4 and 1.0ml aliquots were stored at -20°C.

(ii) Anti-phosphotyrosine antibody preparation

Monoclonal anti-phosphotyrosine, clone 4G10, was purchased from Upstate Biologicals, Inc (UBI, Lake Placid, NY) and biotinylated using long-arm biotin-N-hydroxysuccinamide (Biotin-X-NHS, Research Organics, Cleveland, OH).

(iii) Ligands

Nerve growth factor (NGF), neurotrophin 3 (NT3), and neurotrophin 5 (NT5) were prepared by recombinant techniques using the sequence data provided for each of these proteins in the above-mentioned references. The purified NGF, NT3 and NT5 were stored at 4°C as stock solutions (180μM, 8.8μM and 26.9μM, respectively) in PBS, pH 7.5.

(iv) Preparation of gD.trk nucleic acid

In order to express the various trk receptors with gD flags (*i.e.* gD.trk constructs), DNA constructs were made which encoded the signal and epitope of gD (see Paborsky *et al.*, *supra*) fused to the amino terminus of the various trk receptors. These were made by inserting the trk receptor and gD sequences into pRK5 or pRK7 (Suva *et al.*, Science, **237**: 893-896 [1987]) using standard molecular biology techniques, to generate the constructs shown in Figures 12-14. In addition to the gD.trk constructs, constructs were also made to express gD tagged trk.IgG fusion proteins (*i.e.*, gD.trk.IgG). DNA constructs encoding the chimeras of trk extracellular domain and IgG-1 Fc domains were made with the Fc region clones of human IgG-1 (Ashkenazi *et al.*, Immunoadhesins Intern. Rev. Immunol., **10**: 219-227 [1993]). More specifically, the source of the IgG-1 encoding sequence was the CD4-IgG-1 expression plasmid $pRKCD4_2Fc_1$ (Capon *et al.*, Nature, **334**: 525 [1989]; Byrn *et al.*, Nature, **344**: 667 [1990]) containing a cDNA sequence encoding a hybrid polypeptide consisting of residues 1-180 of the mature human CD4 protein fused to human IgG-1 sequences beginning at aspartic acid 216 (taking amino acid 114 as the first residue of the heavy chain constant region; Kabat *et al.*, Sequences of Proteins of Immunological Interest 4th ed. [1987]), which is the first residue of the IgG-1 hinge after the cysteine residue involved in heavy-light chain bonding, and ending with residues 441 to include the CH2 and CH3 Fc domains of IgG-1. The CD4-encoding sequence was deleted from the expression plasmid $pRKCD4_2Fc_1$ and the vector was fused to DNA encoding the trk receptors, with the splice between aspartate 216 of the IgG-1 and valine 402 of trkA, threonine 422 of trkB, or threonine 413 of trkC. The gD tag was added to the amino terminus of each trk.IgG in the same way as for the gD.trk constructs.

(v) Cell transformation

Human embryonic kidney 293 cells (obtained from ATCC, Rockville, MD) were transiently transfected with the nucleic acid encoding gD.trk.IgG using a calcium phosphate protocol (Gorman, DNA Cloning: A Practical Approach [Glover, D., ed.] Vol II: 143-190, IRL Press, Washington DC).

After twelve hours, the transformed cells were rinsed three times with serum free F12/DMEM 50:50 media (Gibco) and then serum free media was added for a 48 hour collection.

Cell lines stably expressing each of the gD.trk constructs were made by co-transfecting dp12.CHO cells (EP 307,247 published 15 March 1989) with the pRK plasmids encoding the gD tagged trk receptors and a plasmid encoding DHFR, again using calcium phosphate mediated transfection.

The media mentioned above (having the gD.trk.IgG) was used without further purification in binding assays to assess the effects of the presence of the gD flag polypeptide on neurotrophin binding to the gD.trk.IgG polypeptides. DNA encoding untagged trk.IgG polypeptide was run in parallel as a control. trk.IgG and gD tagged trk.IgG containing cell supernatants were prepared as described and used in competitive displacement assays with the appropriate iodinated neurotrophin. NGF is used as ligand for trkA, NT5 is used as ligand for trkB, and NT3 is used as a ligand for trkC. A summary of the results obtained is shown in the following table.

TABLE 1

Binding of Neurotrophins to trk.IgG

	IC50 without gD	IC50 with gD
trkA	68.4+/-11.9 pM	68.8+/-3.0 pM
trkB	31.1+/-15.6 pM	12.1+/-18 pM
trkC	31.1+/-1.1 pM	30.2+/-0.7 pM

(vi) Selection of transformed cells for use in the KIRA ELISA

It was apparent from the preceding experiment that there was no observable change in the affinity of interaction of neurotrophins with their receptor due to the presence of the gD flag polypeptide on the amino terminus. Based on this result, cells were transformed with the gD.trk constructs for use in the KIRA ELISA using the techniques described in the previous section.

After two days, dp12.CHO cells (EP 307,247 published 15 March 1989) transformed with gD.trk constructs were selected for by growth in media without GHT, and after two weeks, growing cells were sorted by FACS analysis using the 5B6 monoclonal to select cells expressing the gD flag polypeptide on their surface. gD positive cells were cloned by plating at limiting dilution and resultant colonies were then rescreened by FACS

analysis (using the anti-gD 5B6 monoclonal antibody), neurotrophin binding (as discussed above), tyrosine phosphorylation indicated by Western blot using an anti-phosphotyrosine antibody, gD expression by Western blot using th anti-gD 5B6 antibody, and immunocytochemistry using the 5B6 antibody. Clones which were positive were then recloned by limiting dilution and were subjected to the KIRA ELISA as described below.

(vii) Media

Cells were grown in F12/DMEM 50:50 (Gibco/BRL, Life Technologies, Grand Island, NY). The media was supplemented with 10% diafiltered FBS (HyClone, Logan, Utah), 25mM HEPES and 2mM L-glutamine.

(viii) KIRA ELISA

gD.trk transformed dp12.CHO cells (EP 307,247 published 15 March 1989) were seeded (5 x 10^4 per well) in a flat-bottom-96 well culture plate in 100 μl media and cultured overnight at 37°C in 5% CO_2. The following morning the well supernatants were decanted, and the plates were lightly tamped on a paper towel. 100μl of media containing either experimental samples or the recombinant purified NGF, NT3, or NT5 standards (3000, 1000, 333, 111, 37, 12, 4, and 0 pM) was then added to each well. The cells were stimulated at 37°C for 30 min., the well supernatants were decanted, and the plates were once again lightly tamped on a paper towel. To lyse the cells and solubilize the receptors, 100 μl of lysis buffer was added to each well. Lysis buffer consisted of 150 mM NaCl containing 50 mM HEPES (Gibco), 0.5 % Triton-X 100 (Gibco), 0.01 % thimerosal, 30 KIU/ml aprotinin (ICN Biochemicals, Aurora, OH), 1mM 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride (AEBSF; ICN Biochemicals), 50 μM leupeptin (ICN Biochemicals), and 2 mM sodium orthovanadate (Na_3VO_4; Sigma Chemical Co, St. Louis, MO). pH 7.5. The plate was then agitated gently on a plate shaker (Bellco Instruments, Vineland, NJ) for 60 min. at room temperature.

While the cells were being solubilized, an ELISA microtiter plate (Nunc Maxisorp, Inter Med, Denmark) coated overnight at 4°C with the 5B6 monoclonal anti-gD antibody (0.5 μg/ml in 50 mM carbonate buffer, pH 9.6, 100 μl/well) was decanted, tamped on a paper towel and blocked with 150 μl/well of Block Buffer [PBS containing 0.5 % BSA (Intergen Company, Purchase, NY) and 0.01 % thimerosal] for 60 min. at room temperature with gentle agitation. After 60 minutes, the anti-gD 5B6 coated plate was washed 6 times with wash buffer (PBS containing 0.05 % Tween-20 and 0.01 % thimerosal) using an automated plate washer (ScanWasher 300, Skatron Instruments, Inc, Sterling, VA).

The lysate containing solubilized gD.trk from the cell-culture microtiter well was transferred (85 µl/well) to anti-gD 5B6 coated and blocked ELISA well and was incubated for 2 h at room temperature with gentle agitation. The unbound gD.trk was removed by washing with wash buffer and 100 µl of biotinylated 4G10 (anti-phosphotyrosine) diluted 1:2000 in dilution buffer (PBS containing 0.5 % BSA, 0.05 % Tween-20, 5 mM EDTA, and 0.01 % thimerosal), *i.e.*, 400pg/ml, was added to each well. After incubation for 2 h at room temperature the plate was washed and 100 µl of HRPO-conjugated streptavidin (Zymed Laboratories, S. San Francisco, CA) diluted 1:10000 in dilution buffer was added to each well. The plate was incubated for 30 minutes at room temperature with gentle agitation. The free avidin-conjugate was washed away and 100 µl freshly prepared substrate solution (tetramethyl benzidine; 2-component substrate kit; Kirkegaard and Perry, Gaithersburg, MD) was added to each well. The reaction was allowed to proceed for 10 minutes, after which the color development was stopped by the addition of 100 µl/well 1.0 M H_3PO_4. The absorbance at 450 nm was read with a reference wavelength of 650 nm ($ABS_{450/650}$), using a *vmax* plate reader (Molecular Devices, Palo Alto, CA) controlled with a Macintosh Centris 650 (Apple Computers, Cupertino, CA) and DeltaSoft software (BioMetallics, Inc, Princeton, NJ).

The standard curves shown in Figures 15A-15C were generated by stimulating gD.trk transformed CHO cells with 3000, 1000, 333, 111, 37, 12, 4, and 0 pM NGF, NT3 or NT5 and were presented as pM neurotrophin vs. mean $ABS_{450/650}$ ± sd using the DeltaSoft program. Sample concentrations were obtained by interpolation of their absorbance on the standard curve and are expressed in terms of pM neurotrophin activity.

The results presented in this example demonstrate that the KIRA ELISA is a useful method for assaying ligand activation of a receptor construct having an amino terminal flag polypeptide, *e.g.*, activation of gD.trk receptor constructs. Levels of receptor activation in terms of tyrosine phosphorylation are easily quantified and an EC_{50} for a given ligand is readily determined.

EXAMPLE 4

KIRA ELISA of the *MPL*/Rse Chimeric Receptor

The human *MPL* receptor has been disclosed by Vigon *et al.*, PNAS, USA 89:5640-5644 (1992). A chimeric receptor comprising the ECD of the *MPL* receptor and the TM and ICD of Rse (Mark *et al.*, *supra*) with a carboxyl-

terminal flag polypeptide (*i.e.* Rse.gD; see Example 2) was subjected to the KIRA ELISA described herein. The experimental procedure is outlined below. See also Figs. 16 and 17.

(i) Capture agent preparation

Monoclonal anti-gD (clone 5B6) was produced against a peptide from Herpes simplex virus glycoprotein D (Paborsky *et al.*, Protein Engineering 3(6):547-553 [1990]). The purified stock preparation was adjusted to 3.0mg/ml in phosphate buffered saline (PBS), pH 7.4 and 1.0ml aliquots were stored at -20¤C.

(ii) Anti-phosphotyrosine antibody preparation

Monoclonal anti-phosphotyrosine, clone 4G10, was purchased from UBI (Lake Placid, NY) and biotinylated using long-arm biotin-N-hydroxysuccinamide (biotin-X-NHS, Research Organics, Cleveland, OH).

(iii) Ligand

The *MPL* ligand [de Sauvage *et al.*, Nature 369: 533-538 (1994)] was prepared by recombinant techniques. The purified *MPL* ligand was stored at 4¤C as a stock solution.

(iv) Preparation of MPL/Rse.gD nucleic acid

The expression plasmid pSV.ID.Rse.gD produced as described in Example 2 above was modified to produce plasmid pSV.ID.M.tmRd6 which contained the coding sequence of the EDC of human *MPL* (amino acids 1-491) fused to the transmembrane domain and intracellular domain of Rse.gD (amino acids 429-911). Synthetic oligonucleotides were used to join the coding sequence of a portion of the extracellular domain of human MPL to a portion of the Rse coding sequence in a two step PCR cloning reaction as described by Mark *et al.* in J. Biol. Chem. 267:26166-26171 (1992). Primers used for the first PCT reaction were M1 (5'-TCTCGCTACCGTTTACAG - SEQ ID NO:12) and M2 (5'-CAGGTACCCACCAGGCGGTCTCGGT - SEQ ID NO. 13) with a MPL cDNA template and R1 (5'-GGGCCATGACACTGTCAA - SEQ ID NO:14) and R2 (5'-GACCGCCACCGAGACCGCCTGGTGGGTACCTGTGGTCCTT - SEQ ID NO:15) with a Rse cDNA template. The PvuII-SmaI portion of this fusion junction was used for the construction of the full-length chimeric receptor.

(v) Cell transformation

dp12.CHO cells (EP 307,247 published 15 March 1989) were electroporated with pSV.ID.M.tmRd6 which had been linearized at a unique NotI site in the plasmid backbone. The DNA was ethanol precipitated after phenol/chloroform extraction and was resuspended in 20µl 1/10 Tris EDTA. Then, 10µg of DNA was incubated with 10^7 CHO.dp12 cells in 1 ml of PBS on

ice for 10 min. before electroporation at 400 volts and 330μf. Cells were returned to ice for 10 min. before being plated into non-selective medium. After 24 hours cells were fed nucleoside-free medium to select for stable DHFR+ clones.

(vi) Selection of transformed cells for use in the KIRA ELISA

Clones expressing *MPL*/Rse.gD were identified by western-blotting of whole cell lysates post-fractionation by SDS-PAGE using the antibody 5B6 which detects the gD epitope tag.

(vii) Media

Cells were grown in F12/DMEM 50:50 (Gibco/BRL, Life Technologies, Grand Island, NY). The media was supplemented with 10% diafiltered FBS (HyClone, Logan, Utah), 25mM HEPES and 2mM L-glutamine.

(viii) KIRA ELISA

MPL/Rse.gD transformed dp12.CHO cells were seeded ($3x10^4$ per well) in the wells of a flat-bottom-96 well culture plate in 100 μl media and cultured overnight at 37°C in 5% CO_2. The following morning the well supernatants were decanted, and the plates were lightly tamped on a paper towel. 50μl of media containing either experimental samples or 200, 50, 12.5, 3.12, 0.78, 0.19, 0.048 or 0 ng/ml *MPL* ligand was then added to each well. The cells were stimulated at 37°C for 30 min., the well supernatants were decanted, and the plates were once again lightly tamped on a paper towel. To lyse the cells and solubilize the chimeric receptors, 100 μl of lysis buffer was added to each well. Lysis buffer consisted of 150 mM NaCl containing 50 mM HEPES (Gibco), 0.5 % Triton-X 100 (Gibco), 0.01 % thimerosal, 30 KIU/ml aprotinin (ICN Biochemicals, Aurora, OH), 1mM 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride (AEBSF; ICN Biochemicals), 50 μM leupeptin (ICN Biochemicals), and 2 mM sodium orthovanadate (Na_3VO_4; Sigma Chemical Co, St. Louis, MO), pH 7.5. The plate was then agitated gently on a plate shaker (Bellco Instruments, Vineland, NJ) for 60 min. at room temperature.

While the cells were being solubilized, an ELISA microtiter plate (Nunc Maxisorp, Inter Med, Denmark) coated overnight at 4°C with the 5B6 monoclonal anti-gD antibody (5.0 μg/ml in 50 mM carbonate buffer, pH 9.6, 100 μl/well) was decanted, tamped on a paper towel and blocked with 150 μl/well of Block Buffer [PBS containing 0.5 % BSA (Intergen Company, Purchase, NY) and 0.01 % thimerosal] for 60 min. at room temperature with gentle agitation. After 60 minutes, the anti-gD 5B6 coated plate was washed 6 times with wash buffer (PBS containing 0.05 % Tween-20 and 0.01 %

thimerosal) using an automated plate washer (ScanWasher 300, Skatron Instruments, Inc, Sterling, VA).

The lysate containing solubilized *MPL*/Rse.gD from the cell-culture microtiter well was transferred (85 µl/well) to anti-gD 5B6 coated and blocked ELISA well and was incubated for 2 h at room temperature with gentle agitation. The unbound *MPL*/Rse.gD was removed by washing with wash buffer and 100 µl of biotinylated 4G10 (anti-phosphotyrosine) diluted 1:18000 in dilution buffer (PBS containing 0.5 % BSA, 0.05 % Tween-20, 5 mM EDTA, and 0.01 % thimerosal), *i.e.* 56 ng/ml was added to each well. After incubation for 2 h at room temperature the plate was washed and 100 µl of HRPO-conjugated streptavidin (Zymed Laboratories, S. San Francisco, CA) diluted 1:60000 in dilution buffer was added to each well. The plate was incubated for 30 minutes at room temperature with gentle agitation. The free avidin-conjugate was washed away and 100 µl freshly prepared substrate solution (tetramethyl benzidine [TMB]; 2-component substrate kit; Kirkegaard and Perry, Gaithersburg, MD) was added to each well. The reaction was allowed to proceed for 10 minutes, after which the color development was stopped by the addition of 100 µl/well 1.0 M H_3PO_4. The absorbance at 450 nm was read with a reference wavelength of 650 nm ($ABS_{450/650}$), using a *vmax* plate reader (Molecular Devices, Palo Alto, CA) controlled with a Macintosh Centris 650 (Apple Computers, Cupertino, CA) and DeltaSoft software (BioMetallics, Inc, Princeton, NJ).

The results demonstrated that *MPL* ligand was able to activate the *MPL*/Rse.gD chimeric receptor in a concentration-dependent and ligand-specific manner.

CLAIMS

1. A method for measuring autophosphorylation of a tyrosine kinase receptor comprising the steps of:

(a) coating a first solid phase with a homogeneous population of eukaryotic cells so that the cells adhere to the first solid phase, wherein, positioned in their membranes, the cells have a receptor construct comprising a flag polypeptide and the receptor, wherein the flag polypeptide provides an epitope to which a capture agent can specifically bind but does not interfere with the activity of the receptor;

(b) exposing the adhering cells to an analyte;

(c) solubilizing the adhering cells, thereby releasing cell lysate therefrom;

(d) coating a second solid phase with the capture agent so that the capture agent adheres to the second solid phase;

(e) exposing the adhering capture agent to the cell lysate obtained in step (c) so that the receptor construct adheres to the second solid phase;

(f) washing the second solid phase so as to remove unbound cell lysate;

(g) exposing the adhering receptor construct to an anti-phosphotyrosine antibody which identifies phosphorylated tyrosine residues in the receptor; and

(h) measuring binding of the anti-phosphotyrosine antibody to the adhering receptor construct.

2. The method of claim 1 wherein the cells are transformed with nucleic acid encoding the receptor construct prior to step (a).

3. *The method of* claim 1 wherein the cells comprise a mammalian cell line.

4. The method of claim 1 wherein the cells naturally adhere to the first solid phase.

5. The method of claim 1 wherein the capture agent is a capture antibody.

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6. The method of claim 1 wherein the first solid phase comprises a well of a first assay plate.

7. The method of claim 6 wherein the first assay plate is a microtiter plate.

8. The method of claim 6 wherein between about 1×10^4 to 3×10^5 cells are added to the well in step (a).

9. The method of claim 1 wherein the second solid phase comprises a well of a second assay plate.

10. The method of claim 1 wherein the cell lysate is not concentrated or clarified prior to step (e).

11. The method of claim 6 wherein step (c) comprises adding a lysis buffer to the well of the first assay plate and gently agitating the first assay plate.

12. The method of claim 11 wherein the lysis buffer comprises a solubilizing detergent.

13. The method of claim 1 wherein the anti-phosphotyrosine antibody is labelled.

14. The method of claim 13 wherein the label comprises an enzyme which is exposed to a color reagent and the color change of the color reagent is determined in step (h).

15. The method of claim 1 wherein the flag polypeptide is fused to the amino terminus of the receptor.

16. The method of claim 15 wherein the receptor is a trk A receptor, trk B receptor or trk C receptor.

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17. The method of claim 1 wherein the flag polypeptide is fused to the carboxyl terminus of the receptor.

18. The method of claim 17 wherein the receptor is the Rse receptor.

19. The method of claim 17 wherein the receptor construct comprises the extracellular domain of a receptor of interest and the intracellular domain of the Rse receptor.

20. The method of claim 19 wherein the receptor of interest is the MPL receptor.

21. The method of claim 20 wherein the receptor construct further comprises the transmembrane domain of the Rse receptor and the flag polypeptide comprises the Herpes Simplex virus glycoprotein D (gD) polypeptide.

22. The method of claim 1 wherein the analyte comprises an agonist for the receptor.

23. The method of claim 1 wherein the analyte comprises an antagonist for the receptor.

24. The method of claim 23 wherein the antagonist competitively inhibits binding or activation of the receptor by an agonist thereto and step (b) is followed by a step wherein the adhering cells are exposed to the agonist.

25. The method of claim 1 wherein the analyte is a composition which comprises an antagonist and an agonist for the receptor and the assay measures the ability of the antagonist to bind to the agonist and thereby reduce activation of the receptor by the agonist.

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26. The method of claim 1 wherein a buffer solution is added to the second solid phase following step (d), wherein the buffer solution contains at least one blocking compound which binds to exposed surfaces of the second solid phase which are not coated with the capture agent.

27. A method for measuring autophosphorylation of a tyrosine kinase receptor comprising the steps of:

(a) coating a well of a first assay plate with a homogeneous population of adherent cells so that the cells adhere to the well, wherein the cells have a tyrosine kinase receptor positioned in the cell membranes thereof;

(b) exposing the adhering cells to an analyte;

(c) solubilizing the adhering cells thereby releasing cell lysate therefrom;

(d) coating a well of a second assay plate with a capture agent which binds specifically to the tyrosine kinase receptor so that the capture agent adheres to the well;

(e) exposing the cell lysate obtained in step (c) to the adhering capture agent so that the tyrosine kinase receptor adheres to the well;

(f) washing the well so as to remove unbound cell lysate;

(g) exposing the adhering tyrosine kinase receptor to an anti-phosphotyrosine antibody which binds selectively to phosphorylated tyrosine residues in the tyrosine kinase receptor;

(h) measuring binding of the anti-phosphotyrosine antibody to the adhering tyrosine kinase receptor.

28. The method of claim 27 wherein the tyrosine kinase receptor comprises the HER2 receptor.

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29. A polypeptide comprising a flag polypeptide fused to the carboxyl terminus of the intracellular domain of the Rse receptor, wherein the flag polypeptide provides an epitope to which a capture agent can specifically bind, but does not interfere with the activity of the receptor.

30. The polypeptide of claim 29 further comprising the transmembrane domain of the Rse receptor.

31. The polypeptide of claim 30 further comprising the extracellular domain of a receptor protein tyrosine kinase, other than the Rse receptor.

32. The polypeptide of claim 29 wherein the flag polypeptide comprises the Herpes Simplex virus glycoprotein D (gD) polypeptide.

33. A method for measuring phosphorylation of a kinase comprising the steps of:

(a) coating a first solid phase with a homogeneous population of eukaryotic cells so that the cells adhere to the first solid phase, wherein the cells comprise a kinase construct comprising a flag polypeptide and the kinase, wherein the flag polypeptide provides an epitope to which a capture agent can specifically bind, but does not interfere with the activity of the receptor;

(b) exposing the adhering cells to an analyte;

(c) solubilizing the adhering cells, thereby releasing cell lysate therefrom;

(d) coating a second solid phase with the capture agent which binds specifically to the flag polypeptide so that the capture agent adheres to the second solid phase;

(e) exposing the adhering capture agent to the cell lysate obtained in step (c) so that the kinase construct adheres to the second solid phase;

(f) washing the second solid phase so as to remove unbound cell lysate;

(g) exposing the adhering kinase construct to an antibody which identifies phosphorylated residues in the kinase construct; and

(h) measuring binding of the antibody to the adhering kinase construct.

34. The method of claim 33 wherein the kinase is a receptor.

35. The method of claim 33 wherein the kinase is a serine-threonine kinase.

36. The method of claim 33 which measures phosphatase activity.

37. The method of claim 36 wherein the cells further comprise a phosphate and the assay further comprises the step of exposing the eukaryotic cells to a phosphatase inhibitor prior to step (c).

38. The method of claim 36 which further comprises the steps in between steps (f) and (g) of exposing the adhering kinase construct to a phosphatase and then washing the second solid phase so as to remove unbound phosphatase.

FIG. 1 A

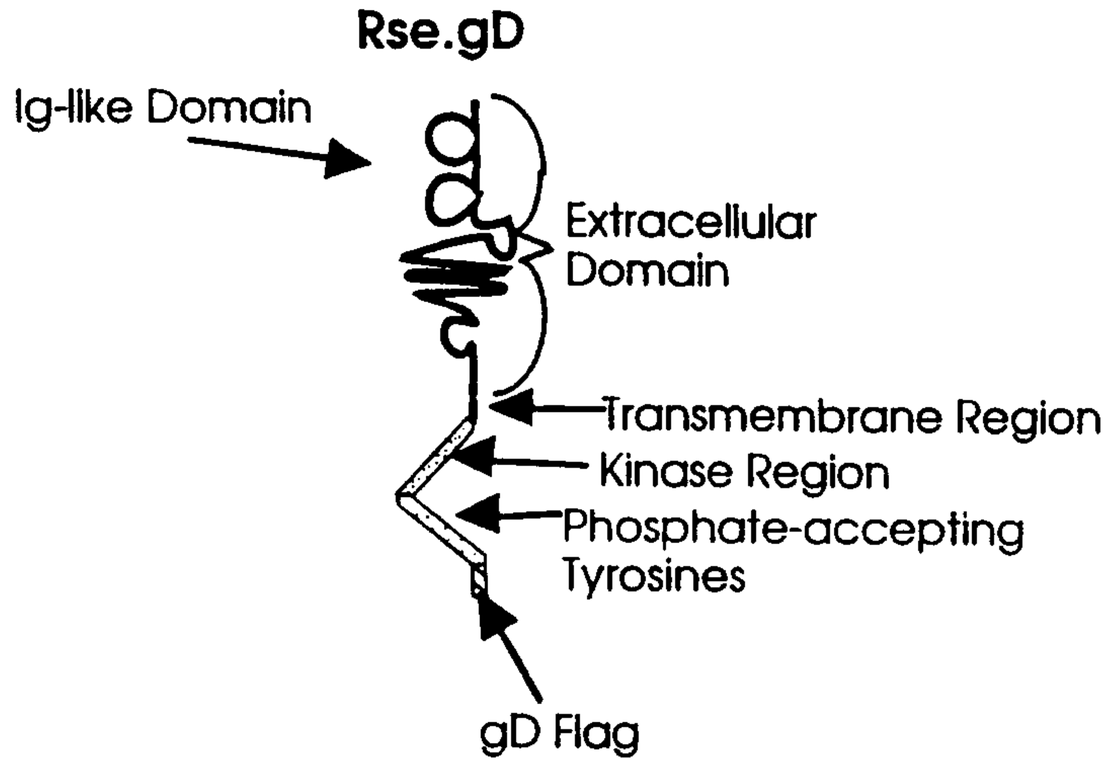


FIG. 1 B

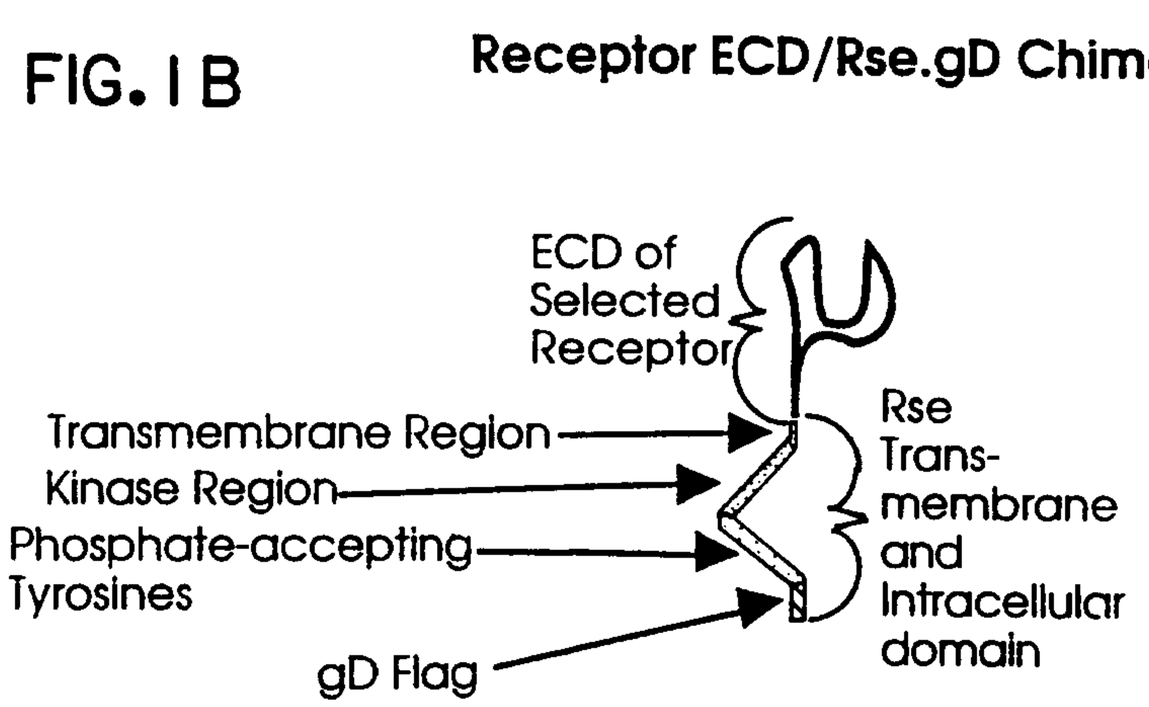
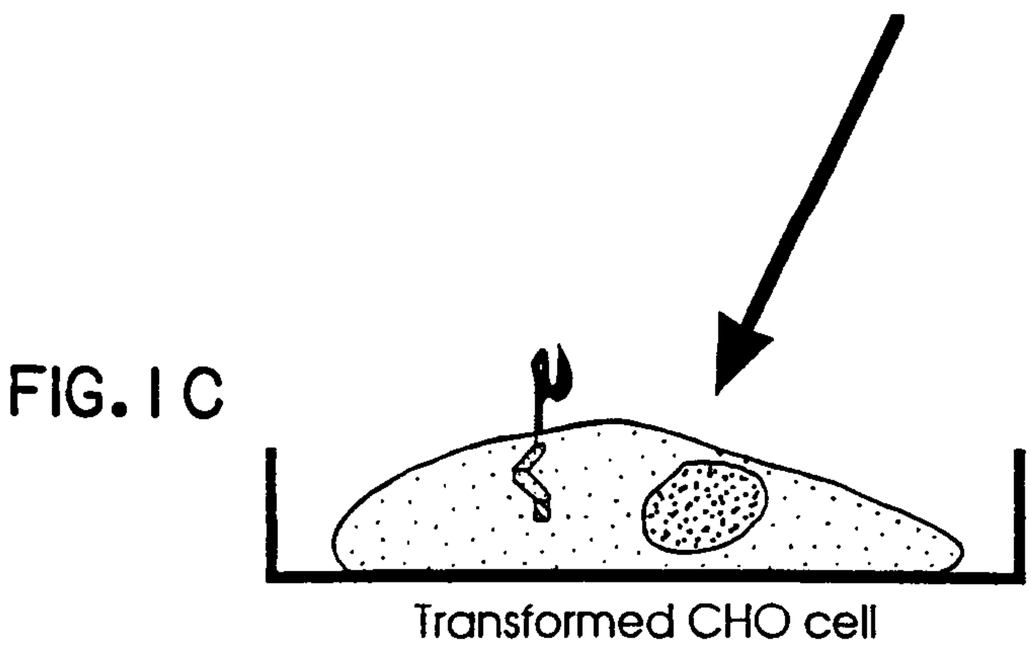


FIG. 1 C



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   signal sequence
   * * * *  * * *  * * *  * * * *  * * *  * * *  * * * *  * * *  * * *  * * * *
  1 M  A  L  R   R  S  M   G  R  P   G  L  P  P   L  P  L   P  P  P   P  R  L  G   L  L  L   A  A  L   A  S  L  L
  1 ATGGCGCTGA GGCGGAGCAT GGGGCGGCCG GGGCTCCCGC CGCTGCCGCT GCCGCCGCCA CCGCGGCTCG GGCTGCTGCT GGCGGCTCTG GCTTCTCTGC
     * * *  * * *  extracellular domain
 35   L  P  E   S  A  A   A  G  L  K   L  M  G   A  P  V   K  L  T  V   S  Q  G   Q  P  V   K  L  N  C   S  V  E
101 TGCTCCCGGA GTCCGCCGCC GCAGGTCTGA AGCTCATGGG AGCCCCGGTG AAGCTGACAG TGTCTCAGGG GCAGCCGGTG AAGCTCAACT GCAGTGTGGA

 68  G  M  E   E  P  D  I   Q  W  V   K  D  G   A  V  V  Q   N  L  D   Q  L  Y   I  P  V  S   E  Q  H   W  I  G
201 GGGGATGGAG GAGCCTGACA TCCAGTGGGT GAAGGATGGG GCTGTGGTCC AGAACTTGGA CCAGTTGTAC ATCCCAGTCA GCGAGCAGCA CTGGATCGGC

101 F  L  S  L   K  S  V   E  R  S   D  A  G  R   Y  W  C   Q  V  E   D  G  G  E   T  E  I   S  Q  P   V  W  L  T
301 TTCCTCAGCC TGAAGTCAGT GGAGCGCTCT GACGCCGGCC GGTACTGGTG CCAGGTGGAG GATGGGGGTG AAACCGAGAT CTCCCAGCCA GTGTGGCTCA

135   V  E  G   V  P  F   F  T  V  E   P  K  D   L  A  V   P  P  N  A   P  F  Q   L  S  C   E  A  V  G   P  P  E
401 CGGTAGAAGG TGTGCCATTT TTCACAGTGG AGCCAAAAGA TCTGGCAGTG CCACCCAATG CCCCTTTCCA ACTGTCTTGT GAGGCTGTGG GTCCCCCTGA

168  P  V  T   I  V  W  W   R  G  T   T  K  I   G  G  P  A   P  S  P   S  V  L   N  V  T  G   V  T  Q   S  T  M
501 ACCTGTTACC ATTGTCTGGT GGAGAGGAAC TACGAAGATC GGGGGACCCG CTCCCTCTCC ATCTGTTTTA AATGTAACAG GGGTGACCCA GAGCACCATG

201 F  S  C  E   A  H  N   L  K  G   L  A  S  S   R  T  A   T  V  H   L  Q  A  L   P  A  A   P  F  N   I  T  V  T
601 TTTTCCTGTG AAGCTCACAA CCTAAAAGGC CTGGCCTCTT CTCGCACAGC CACTGTTCAC CTTCAAGCAC TGCCTGCAGC CCCCTTCAAC ATCACCGTGA

235   K  L  S   S  S  N   A  S  V  A   W  M  P   G  A  D   G  R  A  L   L  Q  S   C  T  V   Q  V  T  Q   A  P  G
701 CAAAGCTTTC CAGCAGCAAC GCTAGTGTGG CCTGGATGCC AGGTGCTGAT GGCCGAGCTC TGCTACAGTC CTGTACAGTT CAGGTGACAC AGGCCCCAGG

268  G  W  E   V  L  A  V   V  V  P   V  P  P   F  T  C  L   L  R  D   L  V  P   A  T  N  Y   S  L  R   V  R  C
801 AGGCTGGGAA GTCCTGGCTG TTGTGGTCCC TGTGCCCCCC TTTACCTGCC TGCTCCGGGA CCTGGTGCCT GCCACCAACT ACAGCCTCAG GGTGCGCTGT

301 A  N  A  L   G  P  S   P  Y  A   D  W  V  P   F  Q  T   K  G  L   A  P  A  S   A  P  Q   N  L  H   A  I  R  T
901 GCCAATGCCT TGGGGCCCTC TCCCTATGCT GACTGGGTGC CCTTTCAGAC CAAGGGTCTA GCCCCAGCCA GCGCTCCCCA AAACCTCCAT GCCATCCGCA

335   D  S  G   L  I  L   E  W  E  E   V  I  P   E  A  P   L  E  G  P   L  G  P   Y  K  L   S  W  V  Q   D  N  G
1001 CAGATTCAGG CCTCATCTTG GAGTGGGAAG AAGTGATCCC CGAGGCCCCT TTGGAAGGCC CCCTGGGACC CTACAAACTG TCCTGGGTTC AAGACAATGG
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FIG.2A

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368  T  Q  D   E  L  T  V   E  G  T   R  A  N   L  T  G  W   D  P  Q   K  D  L   I  V  R  V   C  V  S   N  A  V
1101 AACCCAGGAT GAGCTGACAG TGGAGGGGAC CAGGGCCAAT TTGACAGGCT GGGATCCCCA AAAGGACCTG ATCGTACGTG TGTGCGTCTC CAATGCAGTT
                                                                                                  transmembrane domain
401  G  C  G  P   W  S  Q   P  L  V   V  S  S  H   D  R  A   G  Q  Q   G  P  P  H   S  R  T   S  W  V   P  V  V  L
1201 GGCTGTGGAC CCTGGAGTCA GCCACTGGTG GTCTCTTCTC ATGACCGTGC AGGCCAGCAG GGCCCTCCTC ACAGCCGCAC ATCCTGGGTA CCTGTGGTCC
                                                                intracellular domain
435   G  V  L   T  A  L   V  T  A  A   A  L  A   L  I  L   L  R  K  R   R  K  E   T  R  F   G  Q  A  F   D  S  V
1301 TTGGTGTGCT AACGGCCCTG GTGACGGCTG CTGCCCTGGC CCTCATCCTG CTTCGAAAGA GACGGAAAGA GACGCGGTTT GGGCAAGCCT TTGACAGTGT

468  M  A  R   G  E  P  A   V  H  F   R  A  A   R  S  F  N   R  E  R   P  E  R   I  E  A  T   L  D  S   L  G  I
1401 CATGGCCCGG GGAGAGCCAG CCGTTCACTT CCGGGCAGCC CGGTCCTTCA ATCGAGAAAG GCCCGAGCGC ATCGAGGCCA CATTGGACAG CTTGGGCATC

501  S  D  E  L   K  E  K   L  E  D   V  L  I  P   E  Q  Q   F  T  L   G  R  M  L   G  K  G   E  F  G   S  V  R  E
1501 AGCGATGAAC TAAAGGAAAA ACTGGAGGAT GTGCTCATCC CAGAGCAGCA GTTCACCCTG GGCCGGATGT TGGGCAAAGG AGAGTTTGGT TCAGTGCGGG

535   A  Q  L   K  Q  E   D  G  S  F   V  K  V   A  V  K   M  L  K  A   D  I  I   A  S  S   D  I  E  E   F  L  R
1601 AGGCCCAGCT GAAGCAAGAG GATGGCTCCT TTGTGAAAGT GGCTGTGAAG ATGCTGAAAG CTGACATCAT TGCCTCAAGC GACATTGAAG AGTTCCTCAG

568  E  A  A   C  M  K  E   F  D  H   P  H  V   A  K  L  V   G  V  S   L  R  S   R  A  K  G   R  L  P   I  P  M
1701 GGAAGCAGCT TGCATGAAGG AGTTTGACCA TCCACACGTG GCCAAACTTG TTGGGGTAAG CCTCCGGAGC AGGGCTAAAG GCCGTCTCCC CATCCCCATG

601  V  I  L  P   F  M  K   H  G  D   L  H  A  F   L  L  A   S  R  I   G  E  N  P   F  N  L   P  L  Q   T  L  I  R
1801 GTCATCTTGC CCTTCATGAA GCATGGGGAC CTGCATGCCT TCCTGCTCGC CTCCCGGATT GGGGAGAACC CCTTTAACCT ACCCCTCCAG ACCCTGATCC

635   F  M  V   D  I  A   C  G  M  E   Y  L  S   S  R  N   F  I  H  R   D  L  A   A  R  N   C  M  L  A   E  D  M
1901 GGTTCATGGT GGACATTGCC TGCGGCATGG AGTACCTGAG CTCTCGGAAC TTCATCCACC GAGACCTGGC TGCTCGGAAT TGCATGCTGG CAGAGGACAT

668  T  V  C   V  A  D  F   G  L  S   R  K  I   Y  S  G  D   Y  Y  R   Q  G  C   A  S  K  L   P  V  K   W  L  A
2001 GACAGTGTGT GTGGCTGACT TCGGACTCTC CCGGAAGATC TACAGTGGGG ACTACTATCG TCAAGGCTGT GCCTCCAAAC TGCCTGTCAA GTGGCTGGCC
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FIG.2B

SUBSTITUTE SHEET (RULE 26)

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 701 L  E  S  L     A  D  N     L  Y  T     V  Q  S  D     V  W  A     F  G  V     T  M  W  E     I  M  T     R  G  Q     T  P  Y  A
2101 CTGGAGAGCC TGGCCGACAA CCTGTATACT GTGCAGAGTG ACGTGTGGGC GTTCGGGGTG ACCATGTGGG AGATCATGAC ACGTGGGCAG ACGCCATATG

 735    G  I  E     N  A  E     I  Y  N  Y     L  I  G     G  N  R     L  K  Q  P     P  E  C     M  E  D     V  Y  D  L     M  Y  Q
2201 CTGGCATCGA AAACGCTGAG ATTTACAACT ACCTCATTGG CGGGAACCGC CTGAAACAGC CTCCGGAGTG TATGGAGGAC GTGTATGATC TCATGTACCA

 768  C  W  S     A  D  P  K     Q  R  P     S  F  T     C  L  R  M     E  L  E     N  I  L     G  Q  L  S     V  L  S     A  S  Q
2301 GTGCTGGAGT GCTGACCCCA AGCAGCGCCC GAGCTTTACT TGTCTGCGAA TGGAACTGGA GAACATCTTG GGCCAGCTGT CTGTGCTATC TGCCAGCCAG

 801 D  P  L  Y     I  N  I     E  R  A     E  E  P  T     A  G  G     S  L  E     L  P  G  R     D  Q  P     Y  S  G     A  G  D  G
2401 GACCCCTTAT ACATCAACAT CGAGAGAGCT GAGGAGCCCA CTGCGGGAGG CAGCCTGGAG CTACCTGGCA GGGATCAGCC CTACAGTGGG GCTGGGGATG

 835    S  G  M     G  A  V     G  G  T  P     S  D  C     R  Y  I     L  T  P  G     G  L  A     E  Q  P     G  Q  A  E     H  Q  P
2501 GCAGTGGCAT GGGGGCAGTG GGTGGCACTC CCAGTGACTG TCGGTACATA CTCACCCCCG GAGGGCTGGC TGAGCAGCCA GGGCAGGCAG AGCACCAGCC

                                                                                                  gD flag polypeptide
 868  E  S  P     L  N  E  T     Q  R  L     L  L  L     Q  Q  G  L     L  P  H     S  S  C     A  D  A  S     L  K  M     A  D  P
2601 AGAGAGTCCC CTCAATGAGA CACAGAGGCT TTTGCTGCTG CAGCAAGGGC TACTGCCACA CTCGAGCTGC GCAGATGCTA GCCTCAAGAT GGCTGATCCA

 901 N  R  F  R     G  K  D     L  P  V     L  O
2701 AATCGATTCC GCGGCAAAGA TCTTCCGGTC CTGTAGAAGC TT
```

FIG.2C

PCT/US94/13329

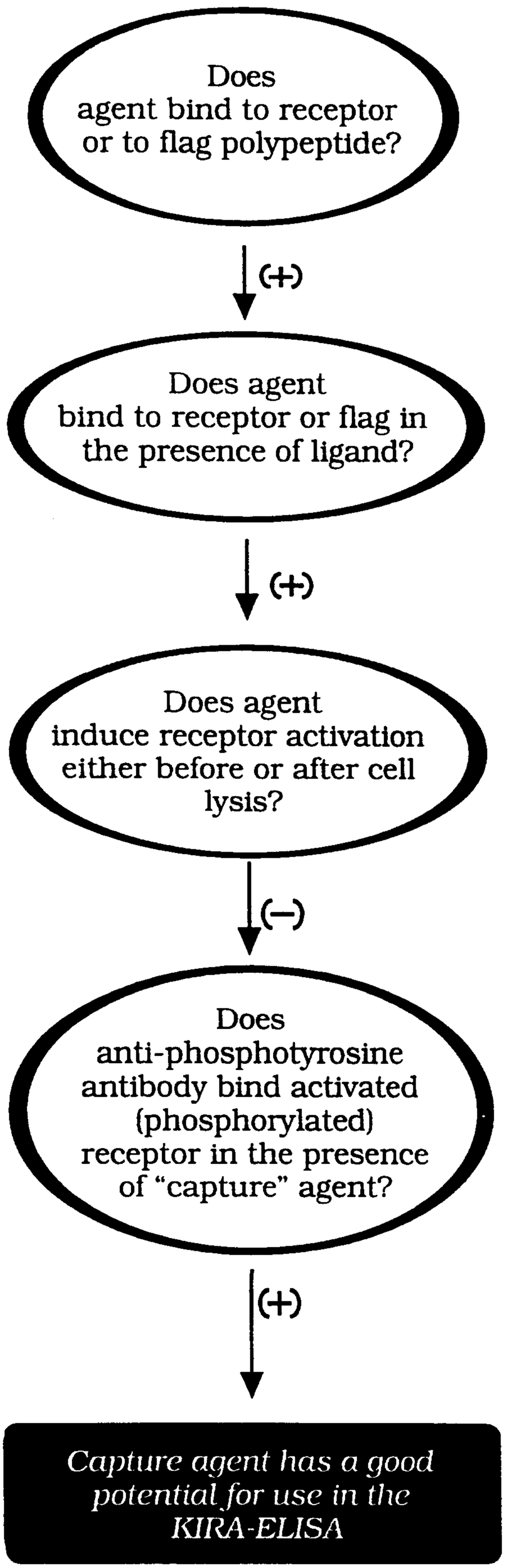
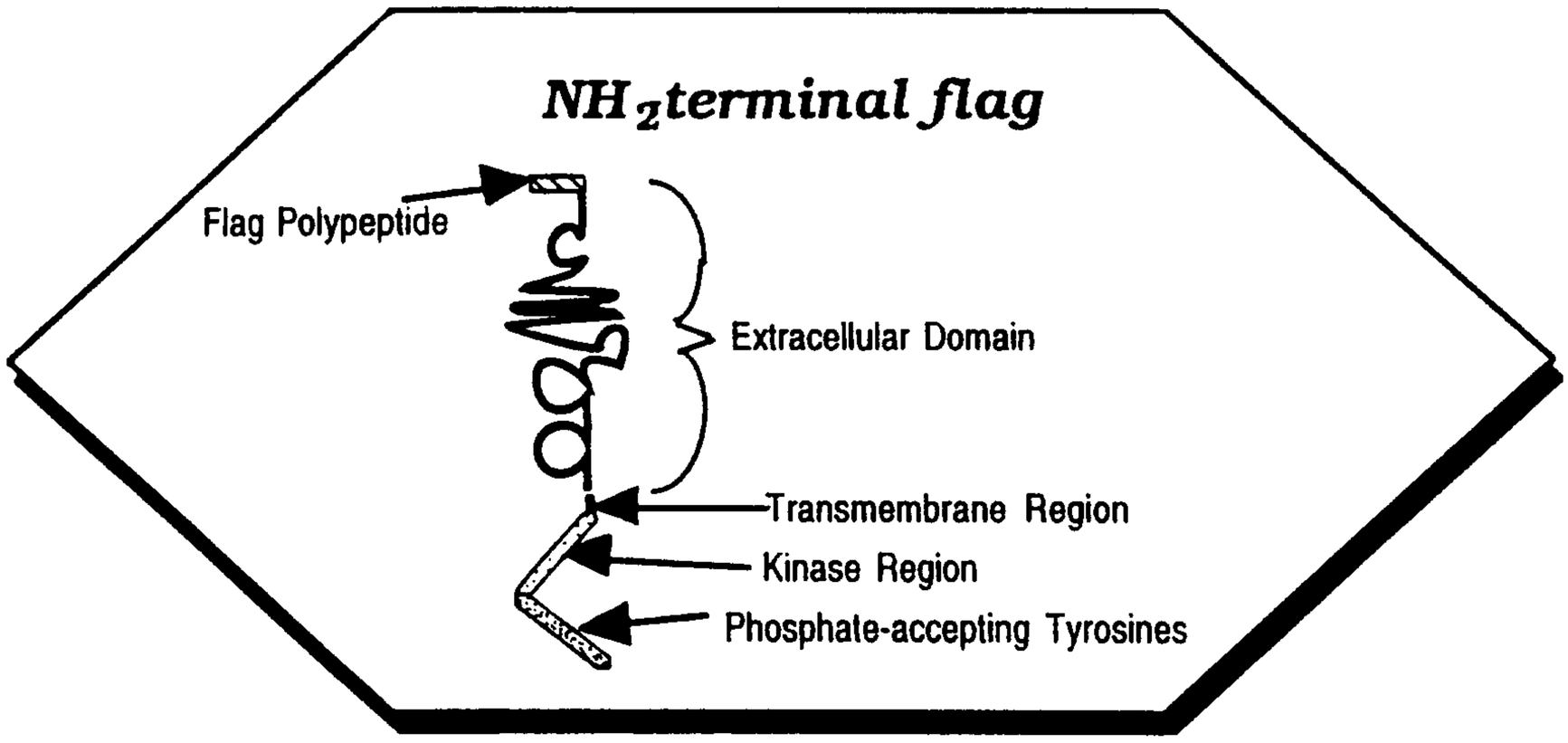

Does agent bind to receptor or to flag polypeptide?
(+)
Does agent bind to receptor or flag in the presence of ligand?
(+)
Does agent induce receptor activation either before or after cell lysis?
(–)
Does anti-phosphotyrosine antibody bind activated (phosphorylated) receptor in the presence of "capture" agent?
(+)
Capture agent has a good potential for use in the KIRA-ELISA

FIG.3



(a) Cell transformation

(b) Detecting successful transformation and expression of the receptor construct nucleic acid

(+)

(c) Detecting receptor activation

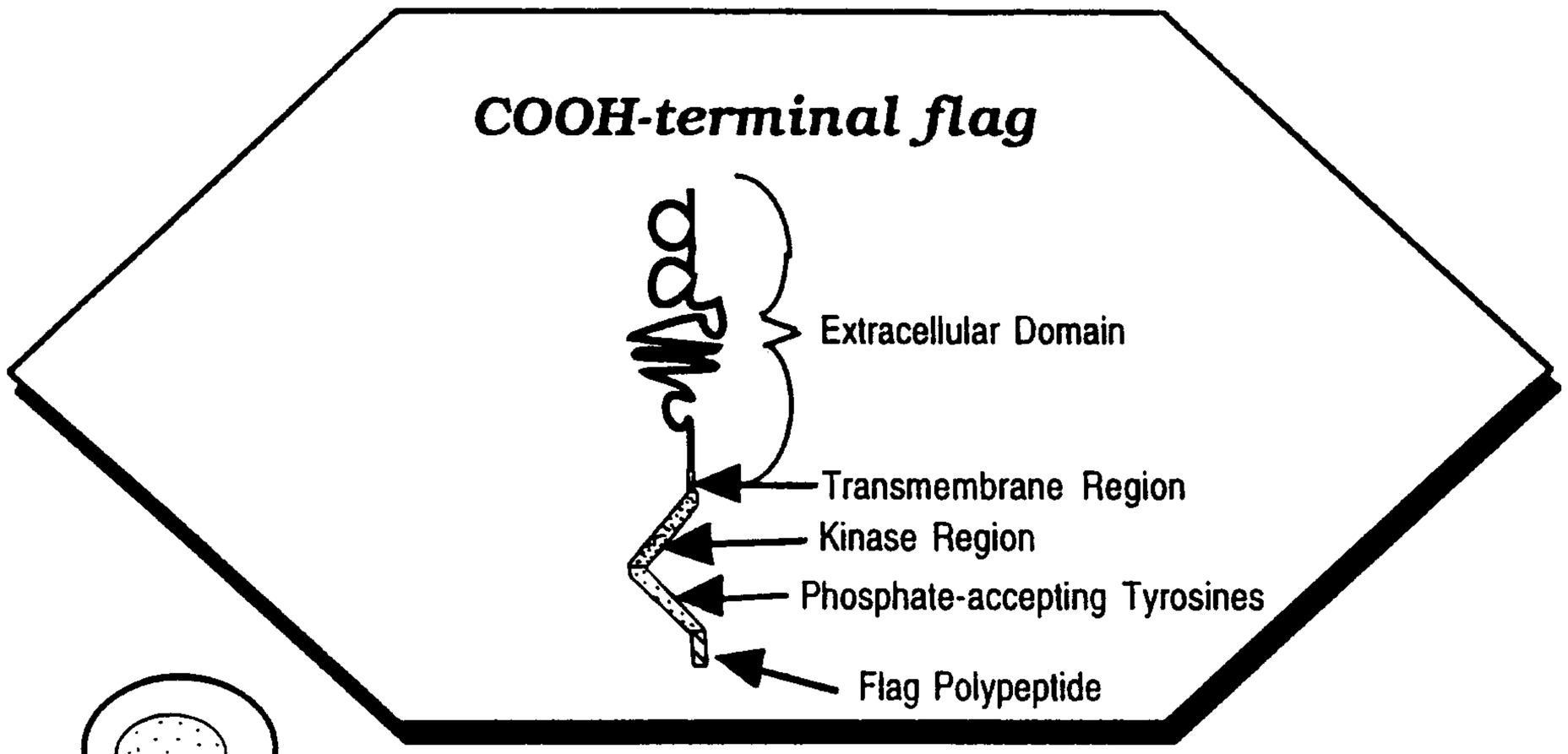
(+)

(d) KIRA-ELISA Test Run

(+)

For use in full KIRA-ELISA Analysis

FIG.4


COOH-terminal flag
Extracellular Domain
Transmembrane Region
Kinase Region
Phosphate-accepting Tyrosines
Flag Polypeptide

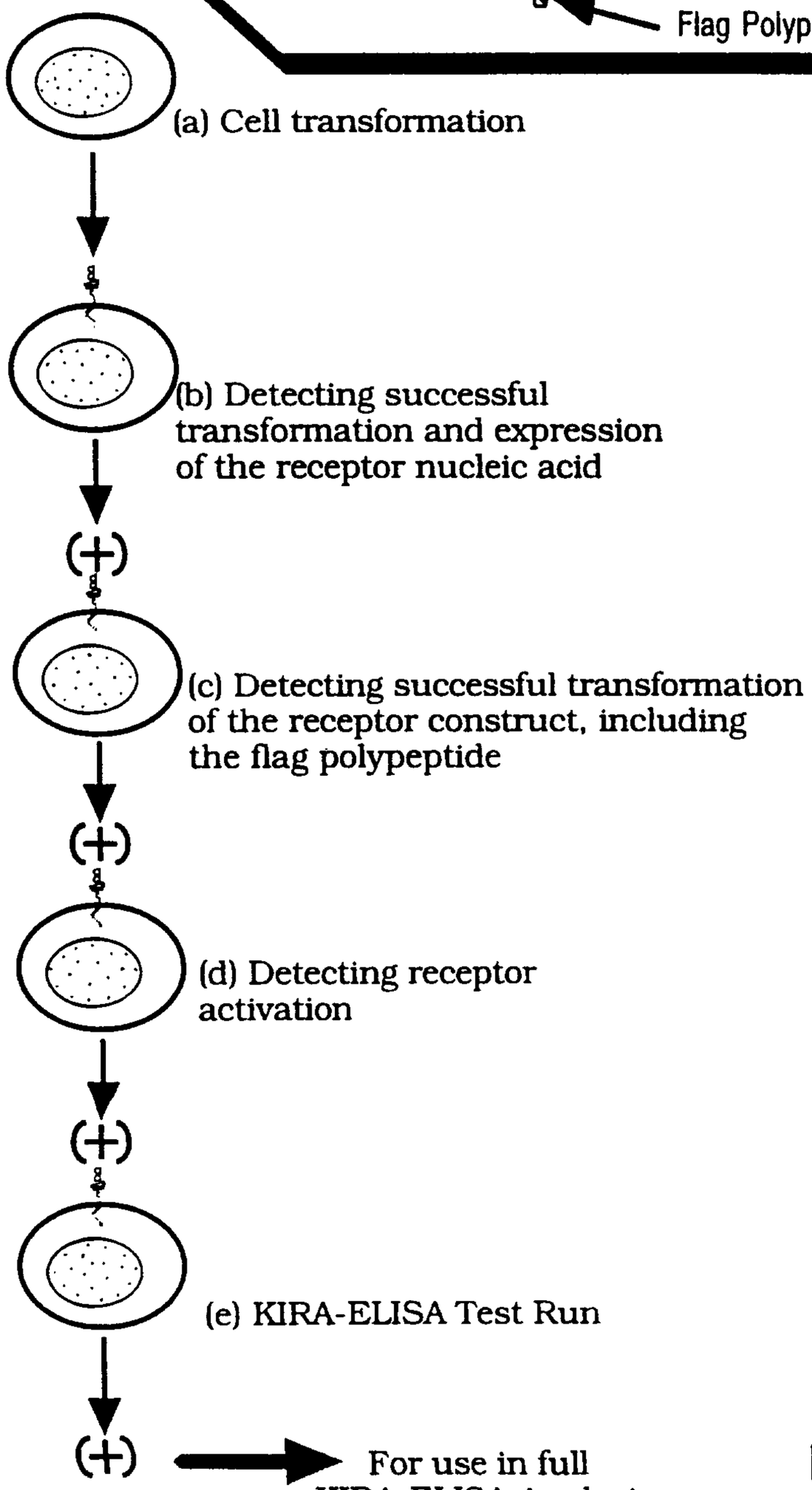

(a) Cell transformation
(b) Detecting successful transformation and expression of the receptor nucleic acid
(+)
(c) Detecting successful transformation of the receptor construct, including the flag polypeptide
(+)
(d) Detecting receptor activation
(+)
(e) KIRA-ELISA Test Run
(+)
For use in full KIRA-ELISA Analysis

FIG.5

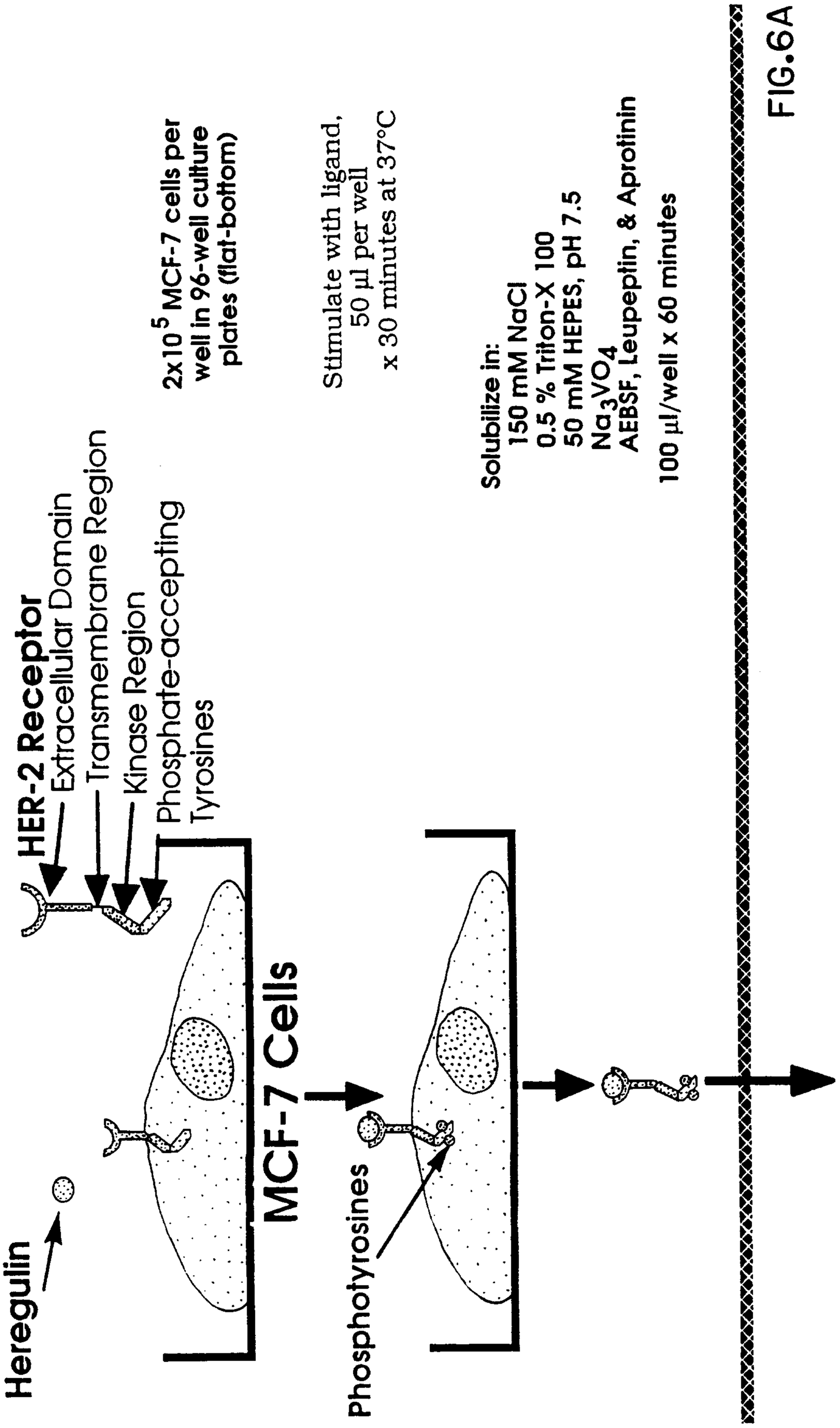

HER-2 Receptor
Extracellular Domain
Transmembrane Region
Kinase Region
Phosphate-accepting
Tyrosines
2x10^5 MCF-7 cells per well in 96-well culture plates (flat-bottom)
Stimulate with ligand, 50 µl per well x 30 minutes at 37°C
Solubilize in:
150 mM NaCl
0.5 % Triton-X 100
50 mM HEPES, pH 7.5
Na3VO4
AEBSF, Leupeptin, & Aprotinin
100 µl/well x 60 minutes
MCF-7 Cells
Heregulin
Phosphotyrosines

FIG.6A

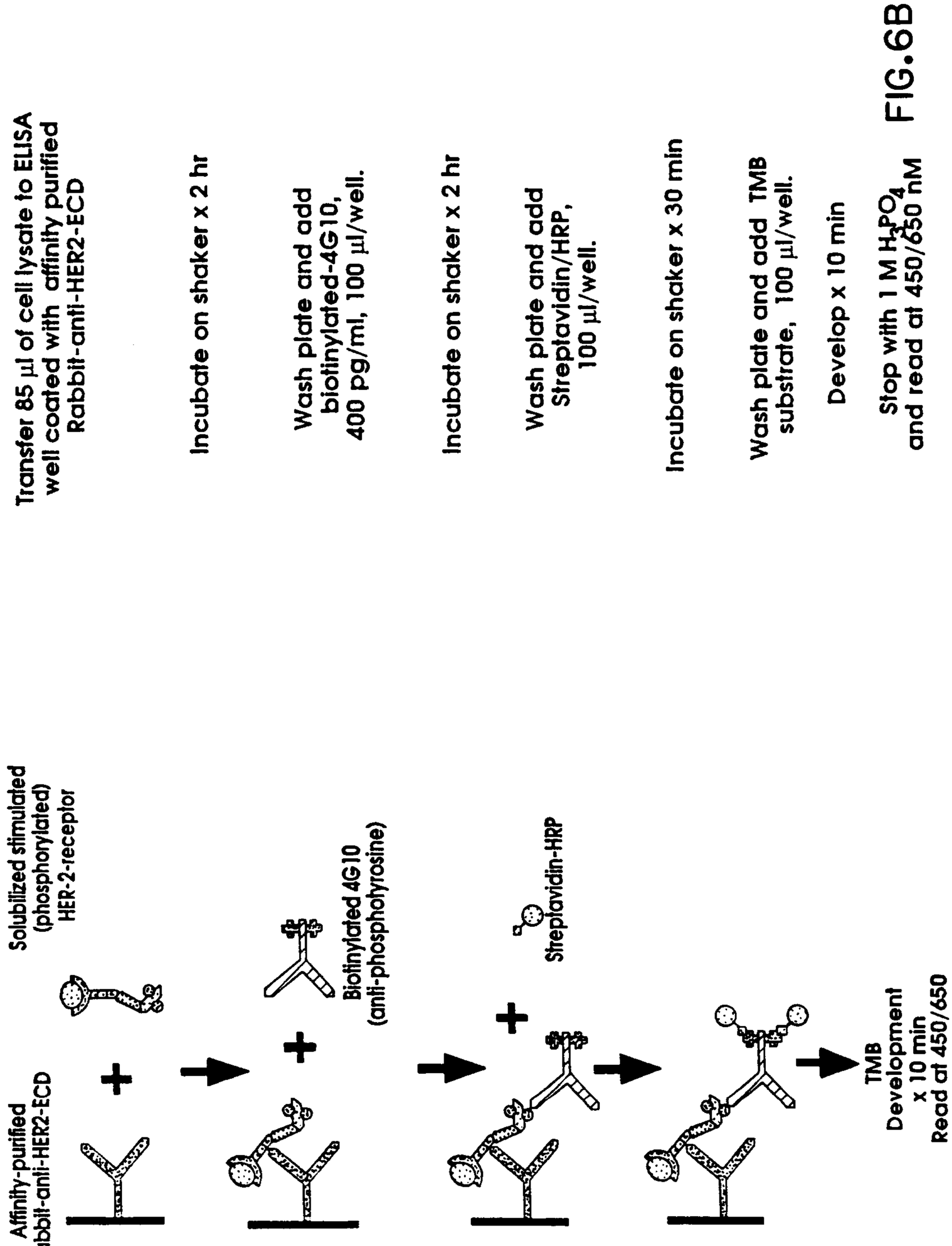

ELISA 96-well plate
Solubilized stimulated (phosphorylated) HER-2-receptor
Affinity-purified Rabbit-anti-HER2-ECD
Biotinylated 4G10 (anti-phosphotyrosine)
Streptavidin-HRP
TMB Development x 10 min Read at 450/650
Transfer 85 µl of cell lysate to ELISA well coated with affinity purified Rabbit-anti-HER2-ECD
Incubate on shaker x 2 hr
Wash plate and add biotinylated-4G10, 400 pg/ml, 100 µl/well.
Incubate on shaker x 2 hr
Wash plate and add Streptavidin/HRP, 100 µl/well.
Incubate on shaker x 30 min
Wash plate and add TMB substrate, 100 µl/well.
Develop x 10 min
Stop with 1 M H_3PO_4 and read at 450/650 nM

FIG.6B

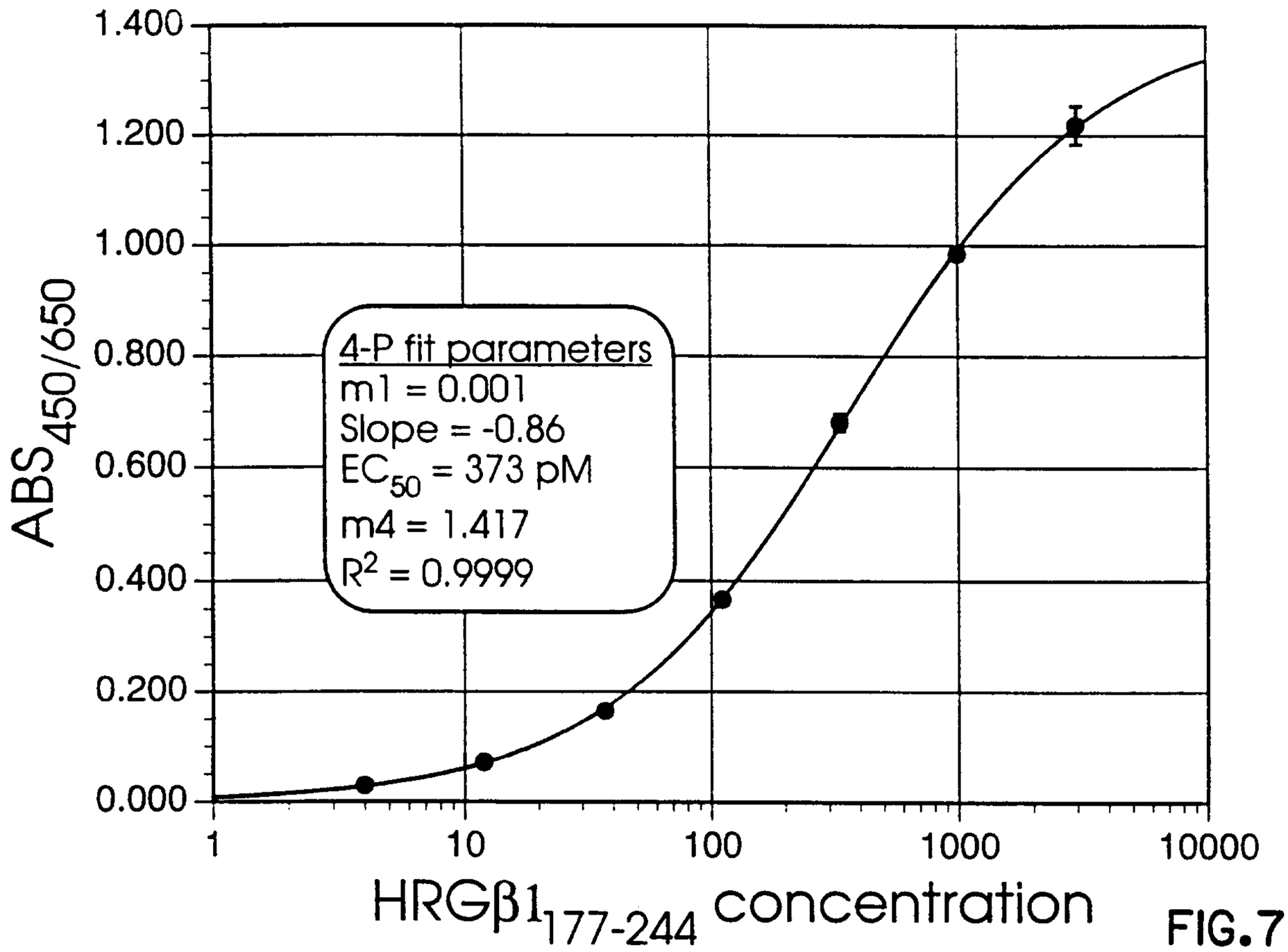

1.400
1.200
1.000
0.800
0.600
0.400
0.200
0.000
ABS450/650
4-P fit parameters
m1 = 0.001
Slope = -0.86
EC50 = 373 pM
m4 = 1.417
R2 = 0.9999
1
10
100
1000
10000
HRGβ1177-244 concentration
(pM)

FIG.7

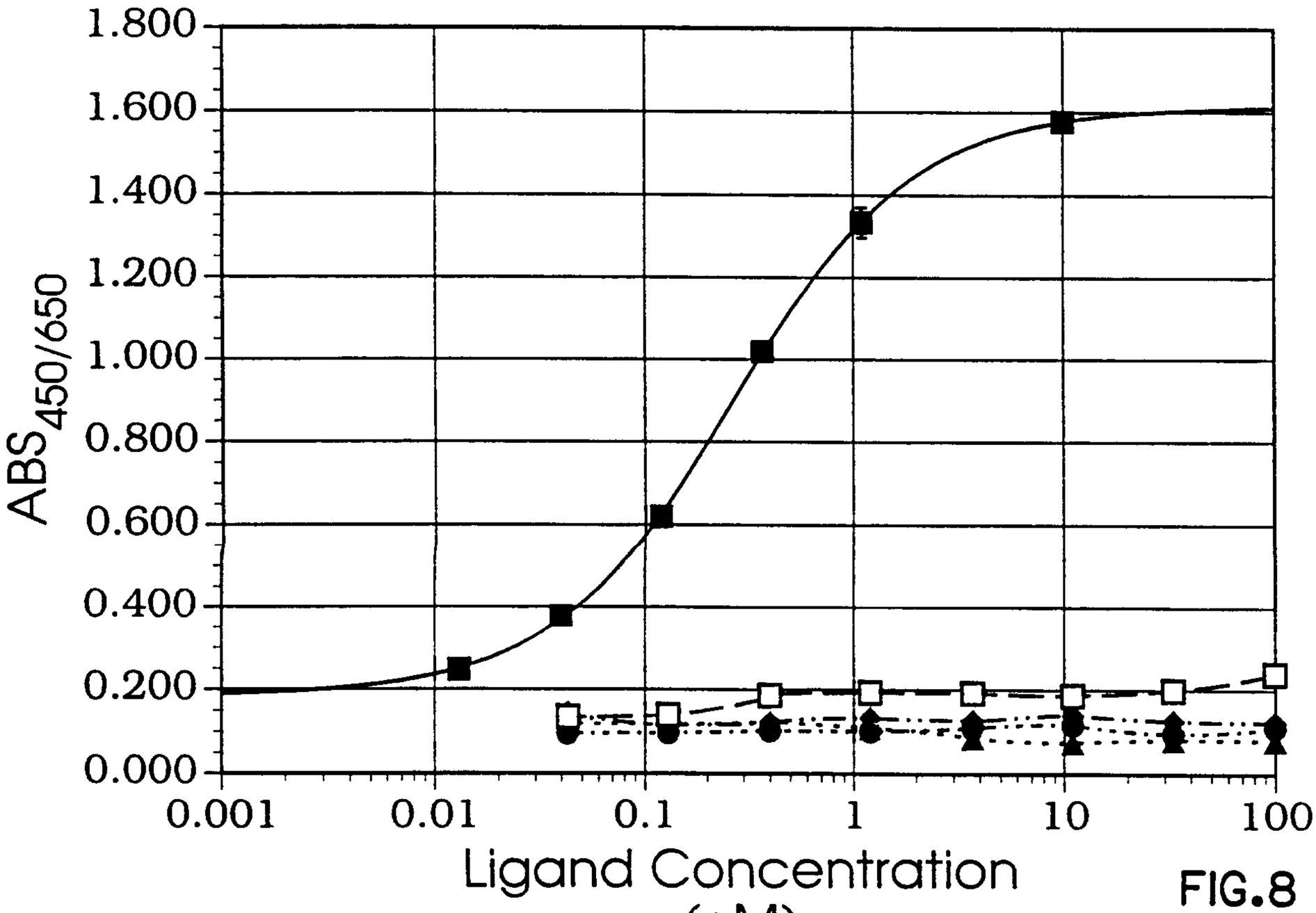

1.800
1.600
1.400
1.200
1.000
0.800
0.600
0.400
0.200
0.000
ABS450/650
0.001
0.01
0.1
1
10
100
Ligand Concentration
(nM)

FIG.8

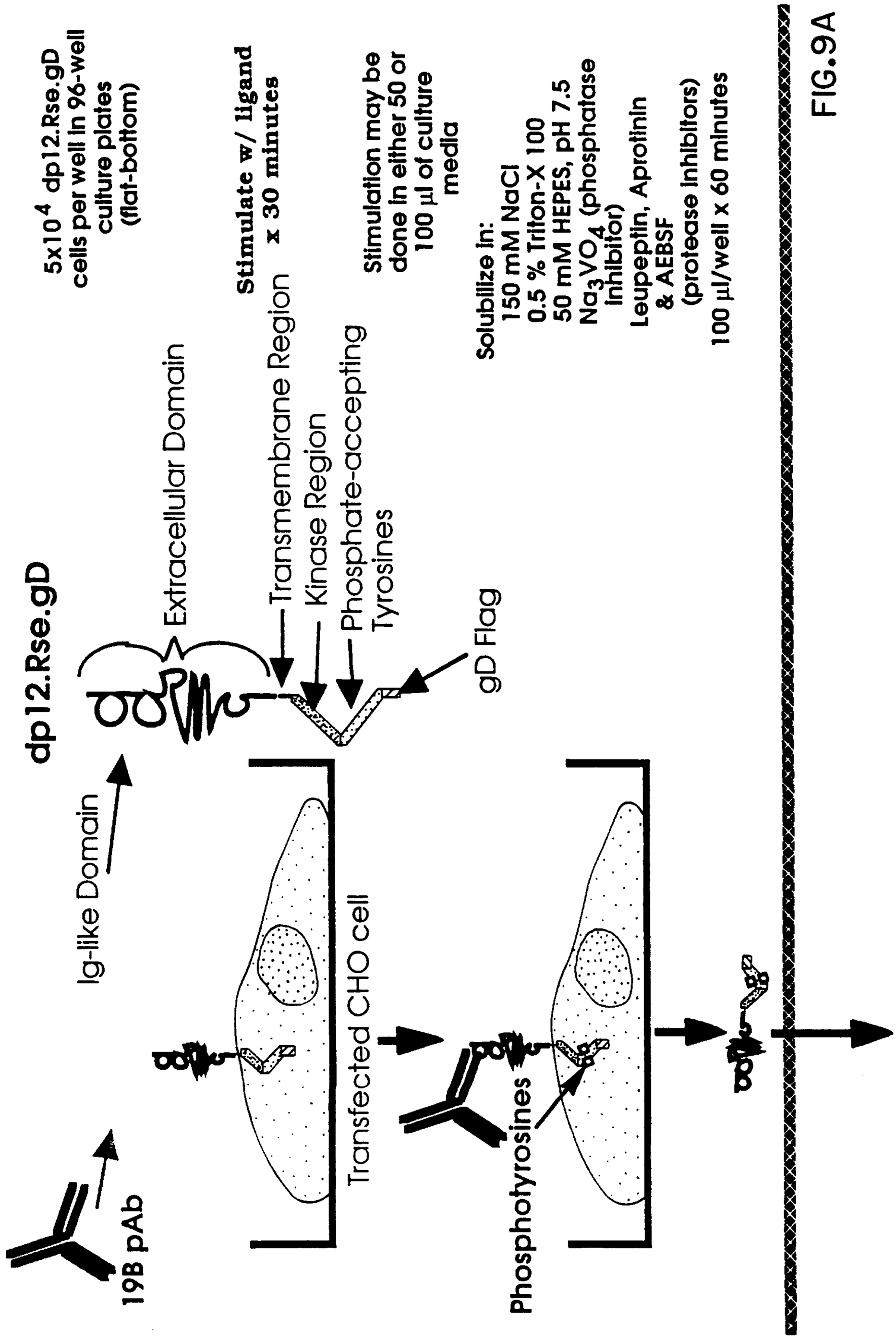
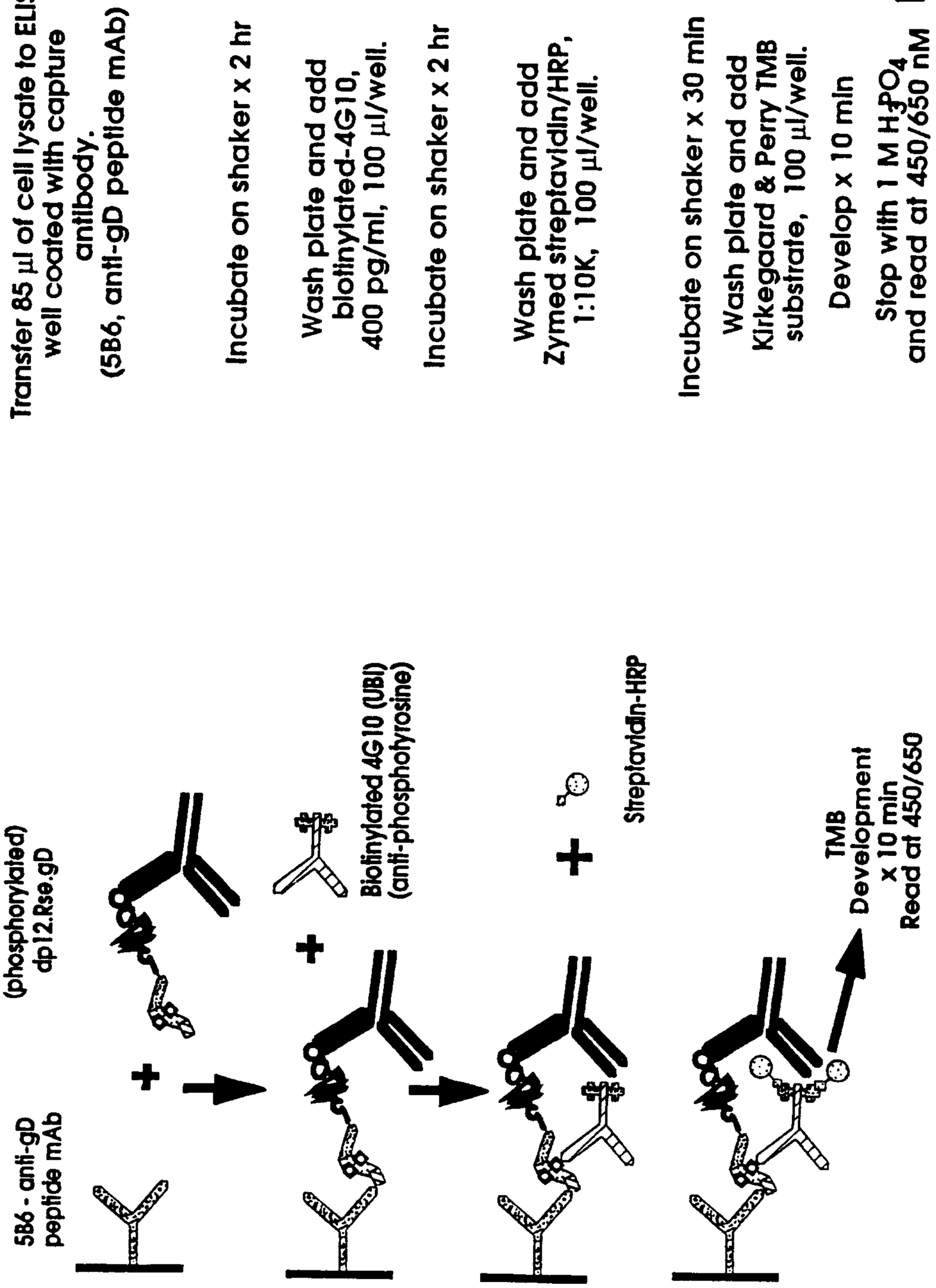

dp12.Rse.gD
Extracellular Domain
Transmembrane Region
Kinase Region
Phosphate-accepting Tyrosines
gD Flag
Ig-like Domain
19B pAb
Transfected CHO cell
Phosphotyrosines
5x10^4 dp12.Rse.gD cells per well in 96-well culture plates (flat-bottom)
Stimulate w/ ligand x 30 minutes
Stimulation may be done in either 50 or 100 µl of culture media
Solubilize in:
150 mM NaCl
0.5 % Triton-X 100
50 mM HEPES, pH 7.5
Na_3VO_4 (phosphatase inhibitor)
Leupeptin, Aprotinin & AEBSF
(protease inhibitors)
100 µl/well x 60 minutes

FIG.9A

FIG.9B

ELISA 96-well plate



Transfer 85 µl of cell lysate to ELISA well coated with capture antibody. (5B6, anti-gD peptide mAb)

Incubate on shaker x 2 hr

Wash plate and add biotinylated-4G10, 400 pg/ml, 100 µl/well.

Incubate on shaker x 2 hr

Wash plate and add Zymed streptavidin/HRP, 1:10K, 100 µl/well.

Incubate on shaker x 30 min

Wash plate and add Kirkegaard & Perry TMB substrate, 100 µl/well.

Develop x 10 min

Stop with 1 M H_3PO_4 and read at 450/650 nM

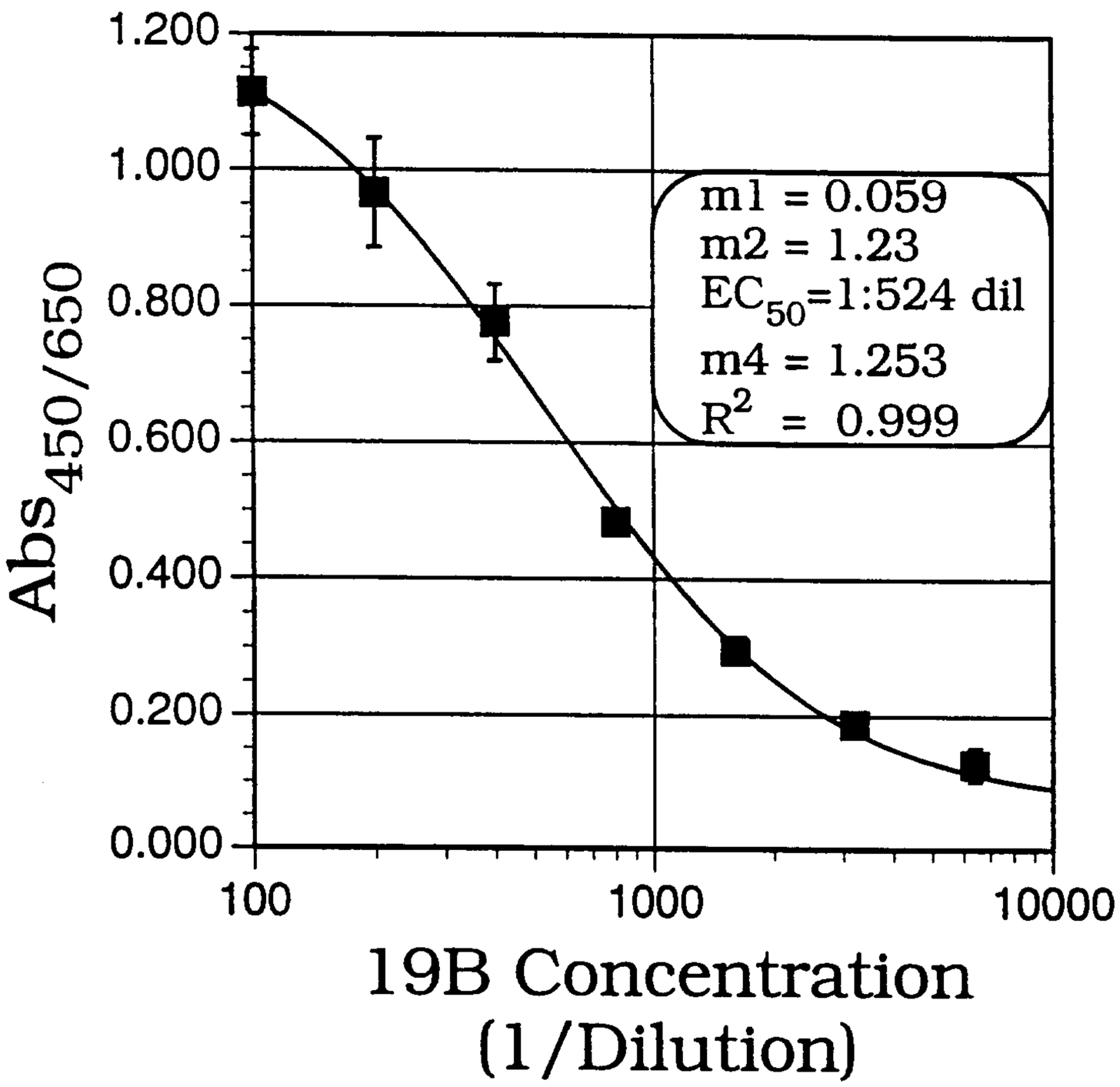

1.200
1.000
0.800
0.600
0.400
0.200
0.000
Abs450/650
100
1000
10000
m1 = 0.059
m2 = 1.23
EC50=1:524 dil
m4 = 1.253
R2 = 0.999
19B Concentration
(1/Dilution)

FIG. 10

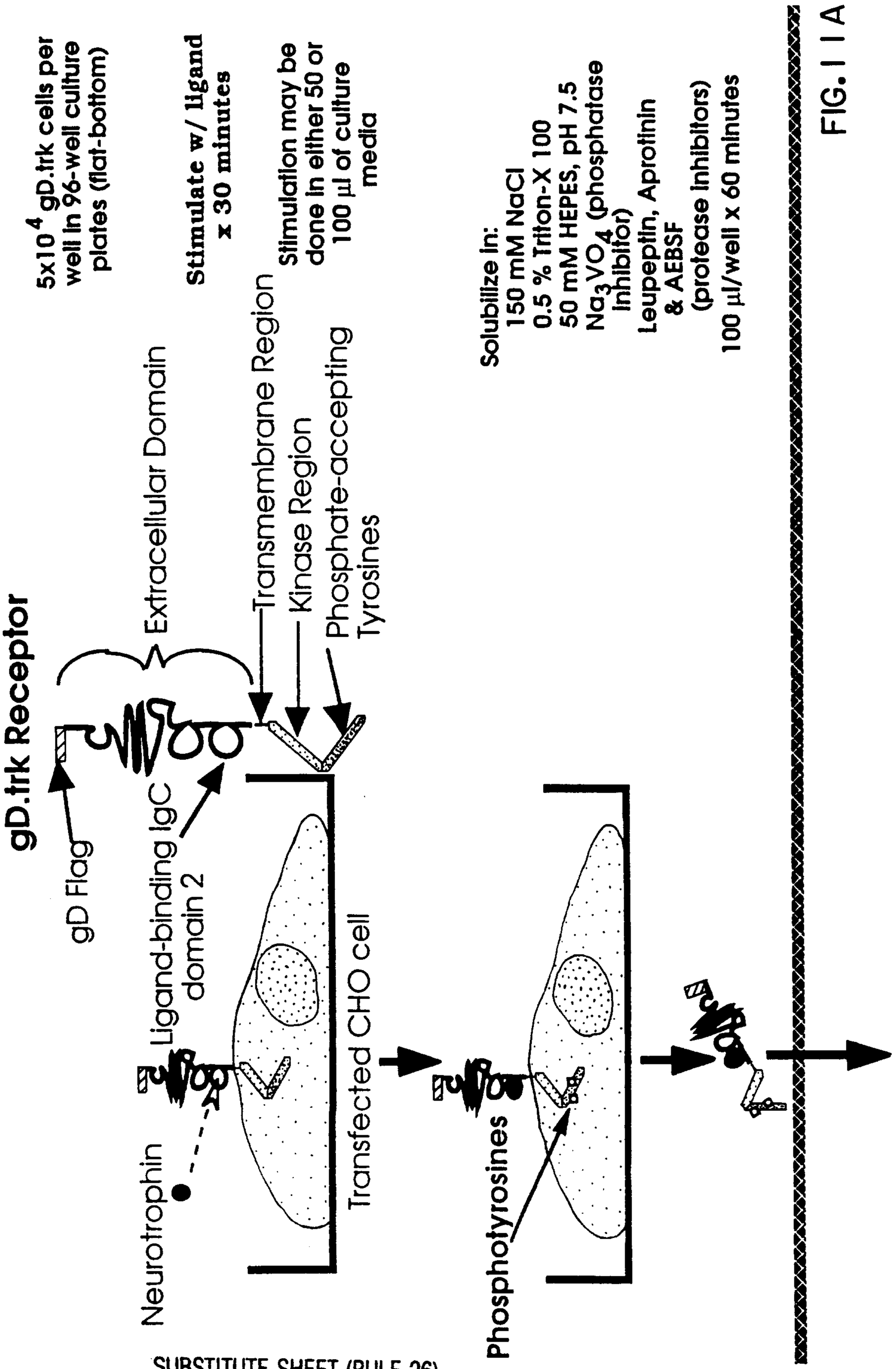
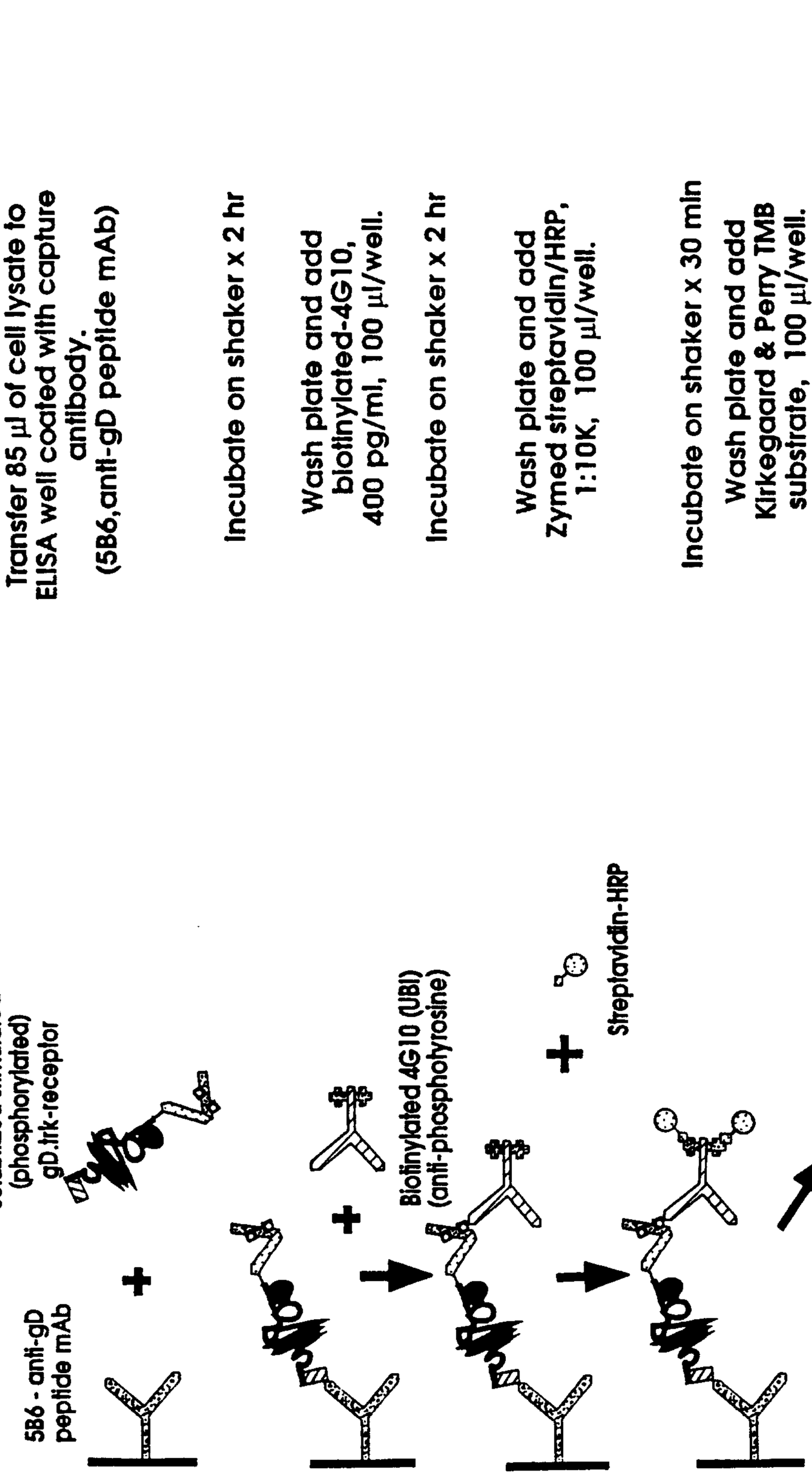

gD.trk Receptor
gD Flag
Ligand-binding IgC domain 2
Neurotrophin
Transfected CHO cell
Phosphotyrosines
Extracellular Domain
Transmembrane Region
Kinase Region
Phosphate-accepting Tyrosines
5x10^4 gD.trk cells per well in 96-well culture plates (flat-bottom)
Stimulate w/ ligand x 30 minutes
Stimulation may be done in either 50 or 100 µl of culture media
Solubilize in:
150 mM NaCl
0.5 % Triton-X 100
50 mM HEPES, pH 7.5
Na_3VO_4 (phosphatase inhibitor)
Leupeptin, Aprotinin & AEBSF (protease inhibitors)
100 µl/well x 60 minutes

FIG. 11A

FIG. I IB


ELISA 96-well plate
Transfer 85 µl of cell lysate to ELISA well coated with capture antibody. (5B6,anti-gD peptide mAb)
Incubate on shaker x 2 hr
Wash plate and add biotinylated-4G10, 400 pg/ml, 100 µl/well.
Incubate on shaker x 2 hr
Wash plate and add Zymed streptavidin/HRP, 1:10K, 100 µl/well.
Incubate on shaker x 30 min
Wash plate and add Kirkegaard & Perry TMB substrate, 100 µl/well.
Develop x 10 min
Stop with 1 M H_3PO_4 and read at 450/650 nM
Solubilized stimulated (phosphorylated) gD.trk-receptor
5B6 - anti-gD peptide mAb
Biotinylated 4G10 (UBI) (anti-phosphotyrosine)
Streptavidin-HRP
TMB Development x 10 min Read at 450/650

```
          ^sp6 RNA start
 841 TATAGAATAA CATCCACTTT GCCTTTCTCT CCACAGGTGT CCACTCCCAG GTCCAACTGC
     ATATCTTATT GTAGGTGAAA CGGAAAGAGA GGTGTCCACA GGTGAGGGTC CAGGTTGACG

       ^cloning linker
          ^R1 site mutated in
          ^begin gD from pchadII
 901 ACCTGAATTC CACTGCCTTC CACCAAGCTC TGCAGGATCC CAGAGTCAGG GGTCTGTATC
     TGGACTTAAG GTGACGGAAG GTGGTTCGAG ACGTCCTAGG GTCTCAGTCC CCAGACATAG

 961 TTCCTGCTGG TGGCTCCAGT TCAGGAACAG TAAACCCTGC TCCGAATATT GCCTCTCACA
     AAGGACGACC ACCGAGGTCA AGTCCTTGTC ATTTGGGACG AGGCTTATAA CGGAGAGTGT

1021 TCTCGTCAAT CTCCGCGAGG ACTGGGGACC CTGTGACAAG CTTCAGCGCG AACGACCAAC
     AGAGCAGTTA GAGGCGCTCC TGACCCCTGG GACACTGTTC GAAGTCGCGC TTGCTGGTTG

                                                          ^Start gD
   1                                                      M* G* G*
1081 TACCCCGATC ATCAGTTATC CTTAAGGTCT CTTTTGTGTG GTGCGTTCCG GTATGGGGGG
     ATGGGGCTAG TAGTCAATAG GAATTCCAGA GAAAACACAC CACGCAAGGC CATACCCCCC

   4  T* A* A*  R* L* G* A*  V* I* L*  F* V* V*  I* V* G* L*  H* G* V*
1141 GACTGCCGCC AGGTTGGGGG CCGTGATTTT GTTTGTCGTC ATAGTGGGCC TCCATGGGGT
     CTGACGGCGG TCCAACCCCC GGCACTAAAA CAAACAGCAG TATCACCCGG AGGTACCCCA

  24  R* G* K*  Y* A* L* A  D  A  S  L  K  M  A  D  P  N  R  F  R
1201 CCGCGGCAAA TATGCCTTGG CGGATGCCTC TCTCAAGATG GCCGACCCCA ATCGATTTCG
     GGCGCCGTTT ATACGGAACC GCCTACGGAG AGAGTTCTAC CGGCTGGGGT TAGCTAAAGC
```

FIG. 12A

2175892

FIG. 12B

^Xho and GTA mutated in
^begin mature trkA

```
44   G  K  D  L  P  V  L  D  Q  L  H  G  S  S  G  L  R  P  G  A  E  N  L  T  E  L  R  D  L  R  G  L  R  F  V  A  P  D  S  F  N  A  L  E  S  L  V  L  S  G  N  P
1261 CGGCAAAGAC CTTCCGGTCC TGGACCAGCT CACGGCTCCT CGGGACTGCG GTGCCGAGGA CCCGGCGCAG GGGCCGCGTC GAGCTCCGTG ATCTGAGGGG CTCGAGGCAC TTCCGTTTCG GGGCCAAAGC TCCTTCAACG AGGAAGTTGC CTGGTCCTGT GACCAGGACA
     GCCGTTTCTG GAAGGCCAGG ACCTGGTCGA GTGCCGAGGA GCCCTGACGC GGGTGGACTG GACTCTTTGG TAGACTCCCC GAGGCAAAAGC ACCGCGGTCT GAAGAGTGGT CTTCTCACCA GGACTTAGAG GAATGTCCTT GGGTCCTTGG GACCAGGACA
1381 CCACCACCTG CCACCACCTG AGAACCTGAC TCTTGGACTG TCGTCGTAGA AGCAGCATCT TCACCATCGT AGTGGTAGCA CCGAGTCAGC CTCTGGAGTC CTCTGGAGTC CGGGGAACCC GCCCCTTGGG ACGCGACCGA
1441 GCAGCATCTG CGTCGTAGAC ATCTGAGGGG TAGACTCCCC GACTCTTTGG AAGTGAGGAG ACCGCGGTCT AAGAGTGGGT CTTCTCACCA GGACTTAGAG GAATGTCCTT GGGTCCTTGG ACGCGACCGA
1501 GAAGAGTGGT CTTCTCACCA GGACTTAGAG GAATGTCCTT GGGTCCTTGG ACGCGACCGA GCAGCATCTG CGTCGTAGAC ATCTGAGGGG TAGACTCCCC GACTCTTTGG AAGTGAGGAG ACCGCGGTCT AAGAGTGGGT CTTCTCACCA GGACTTAGAG GAATGTCCTT GGGTCCTTGG ACGCGACCGA
1561 CCTGAATCTC GGACTTAGAG GAATGTCCTT GGGTCCTTGG ACGCGACCGA CCTGAATCTC GGACTTAGAG GAATGTCCTT GGGTCCTTGG ACGCGACCGA CCTGAATCTC GGACTTAGAG GAATGTCCTT GGGTCCTTGG ACGCGACCGA
1621 CTTACAGGAA GAATGTCCTT GGGTCCTTGG ACGCGACCGA CTTACAGGAA GAATGTCCTT GGGTCCTTGG ACGCGACCGA CTTACAGGAA GAATGTCCTT GGGTCCTTGG ACGCGACCGA
```

```
 184  Q  R  W   E  E  E  G   L  G  G   V  P  E   Q  K  L  Q   C  H  G
1681  ACAGCGCTGG GAGGAGGAGG GACTGGGCGG AGTGCCTGAA CAGAAGCTGC AGTGTCATGG
      TGTCGCGACC CTCCTCCTCC CTGACCCGCC TCACGGACTT GTCTTCGACG TCACAGTACC

 204  Q  G  P   L  A  H  M   P  N  A   S  C  G   V  P  T  L   K  V  Q
1741  GCAAGGGCCC CTGGCCCACA TGCCCAATGC CAGCTGTGGT GTGCCCACGC TGAAGGTCCA
      CGTTCCCGGG GACCGGGTGT ACGGGTTACG GTCGACACCA CACGGGTGCG ACTTCCAGGT

 224  V  P  N   A  S  V  D   V  G  D   D  V  L   L  R  C  Q   V  E  G
1801  GGTGCCCAAT GCCTCGGTGG ATGTGGGGGA CGACGTGCTG CTGCGGTGCC AGGTGGAGGG
      CCACGGGTTA CGGAGCCACC TACACCCCCT GCTGCACGAC GACGCCACGG TCCACCTCCC

 244  R  G  L   E  Q  A  G   W  I  L   T  E  L   E  Q  S  A   T  V  M
1861  GCGGGGCCTG GAGCAGGCCG GCTGGATCCT CACAGAGCTG GAGCAGTCAG CCACGGTGAT
      CGCCCCGGAC CTCGTCCGGC CGACCTAGGA GTGTCTCGAC CTCGTCAGTC GGTGCCACTA

 264  K  S  G   G  L  P  S   L  G  L   T  L  A   N  V  T  S   D  L  N
1921  GAAATCTGGG GGTCTGCCAT CCCTGGGGCT GACCCTGGCC AATGTCACCA GTGACCTCAA
      CTTTAGACCC CCAGACGGTA GGGACCCCGA CTGGGACCGG TTACAGTGGT CACTGGAGTT

 284  R  K  N   L  T  C  W   A  E  N   D  V  G   R  A  E  V   S  V  Q
1981  CAGGAAGAAC TTGACGTGCT GGGCAGAGAA CGATGTGGGC CGGGCAGAGG TCTCTGTTCA
      GTCCTTCTTG AACTGCACGA CCCGTCTCTT GCTACACCCG GCCCGTCTCC AGAGACAAGT

 304  V  N  V   S  F  P  A   S  V  Q   L  H  T   A  V  E  M   H  H  W
2041  GGTCAACGTC TCCTTCCCGG CCAGTGTGCA GCTGCACACG GCGGTGGAGA TGCACCACTG
      CCAGTTGCAG AGGAAGGGCC GGTCACACGT CGACGTGTGC CGCCACCTCT ACGTGGTGAC
```

FIG. 12C

```
 324  C  I  P   F  S  V  D   G  Q  P   A  P  S   L  R  W  L   F  N  G
2101 GTGCATCCCC TTCTCTGTGG ATGGGCAGCC GGCACCGTCT CTGCGCTGGC TCTTCAATGG
     CACGTAGGGG AAGAGACACC TACCCGTCGG CCGTGGCAGA GACGCGACCG AGAAGTTACC

 344  S  V  L   N  E  T  S   F  I  F   T  E  F   L  E  P  A   A  N  E
2161 CTCCGTGCTC AATGAGACCA GCTTCATCTT CACTGAGTTC CTGGAGCCGG CAGCCAATGA
     GAGGCACGAG TTACTCTGGT CGAAGTAGAA GTGACTCAAG GACCTCGGCC GTCGGTTACT

 364  T  V  R   H  G  C  L   R  L  N   Q  P  T   H  V  N  N   G  N  Y
2221 GACCGTGCGG CACGGGTGTC TGCGCCTCAA CCAGCCCACC CACGTCAACA ACGGCAACTA
     CTGGCACGCC GTGCCCACAG ACGCGGAGTT GGTCGGGTGG GTGCAGTTGT TGCCGTTGAT

 384  T  L  L   A  A  N  P   F  G  Q   A  S  A   S  I  M  A   A  F  M
2281 CACGCTGCTG GCTGCCAACC CCTTCGGCCA GGCCTCCGCC TCCATCATGG CTGCCTTCAT
     GTGCGACGAC CGACGGTTGG GGAAGCCGGT CCGGAGGCGG AGGTAGTACC GACGGAAGTA

 404  D  N  P   F  E  F  N   P  E  D   P  I  P   D  T  N  S   T  S  G
2341 GGACAACCCT TTCGAGTTCA ACCCCGAGGA CCCCATCCCT GACACTAACA GCACATCTGG
     CCTGTTGGGA AAGCTCAAGT TGGGGCTCCT GGGGTAGGGA CTGTGATTGT CGTGTAGACC

                                    ^begin TM
 424  D  P  V   E  K  K  D   E  T  P   F  G  V   S  V  A  V   G  L  A
2401 AGACCCGGTG GAGAAGAAGG ACGAAACACC TTTTGGGGTC TCGGTGGCTG TGGGCCTGGC
     TCTGGGCCAC CTCTTCTTCC TGCTTTGTGG AAAACCCCAG AGCCACCGAC ACCCGGACCG

                                                ^end TM
 444  V  F  A   C  L  F  L   S  T  L   L  L  V   L  N  K  C   G  R  R
2461 CGTCTTTGCC TGCCTCTTCC TTTCTACGCT GCTCCTTGTG CTCAACAAAT GTGGACGGAG
     GCAGAAACGG ACGGAGAAGG AAAGATGCGA CGAGGAACAC GAGTTGTTTA CACCTGCCTC
```

FIG. 12D

SUBSTITUTE SHEET (RULE 26)

```
 464  N  K  F  G  I  N  R  P  A  V  L  A  P  E  D  G  L  A  M  S
2521 AAACAAGTTT GGGATCAACC GCCCGGCTGT GCTGGCTCCA GAGGATGGGC TGGCCATGTC
     TTTGTTCAAA CCCTAGTTGG CGGGCCGACA CGACCGAGGT CTCCTACCCG ACCGGTACAG

 484  L  H  F  M  T  L  G  G  S  S  L  S  P  T  E  G  K  G  S  G
2581 CCTGCATTTC ATGACATTGG GTGGCAGCTC CCTGTCCCCC ACCGAGGGCA AAGGCTCTGG
     GGACGTAAAG TACTGTAACC CACCGTCGAG GGACAGGGGG TGGCTCCCGT TTCCGAGACC

 504  L  Q  G  H  I  I  E  N  P  Q  Y  F  S  D  A  C  V  H  H  I
2641 GCTCCAAGGC CACATCATCG AGAACCCACA ATACTTCAGT GATGCCTGTG TTCACCACAT
     CGAGGTTCCG GTGTAGTAGC TCTTGGGTGT TATGAAGTCA CTACGGACAC AAGTGGTGTA

 524  K  R  R  D  I  V  L  K  W  E  L  G  E  G  A  F  G  K  V  F
2701 CAAGCGCCGG GACATCGTGC TCAAGTGGGA GCTGGGGGAG GGCGCCTTTG GGAAGGTCTT
     GTTCGCGGCC CTGTAGCACG AGTTCACCCT CGACCCCCTC CCGCGGAAAC CCTTCCAGAA

 544  L  A  E  C  H  N  L  L  P  E  Q  D  K  M  L  V  A  V  K  A
2761 CCTTGCTGAG TGCCACAACC TCCTGCCTGA GCAGGACAAG ATGCTGGTGG CTGTCAAGGC
     GGAACGACTC ACGGTGTTGG AGGACGGACT CGTCCTGTTC TACGACCACC GACAGTTCCG

 564  L  K  E  A  S  E  S  A  R  Q  D  F  Q  R  E  A  E  L  L  T
2821 ACTGAAGGAG GCGTCCGAGA GTGCTCGGCA GGACTTCCAA CGTGAGGCTG AGCTGCTCAC
     TGACTTCCTC CGCAGGCTCT CACGAGCCGT CCTGAAGGTT GCACTCCGAC TCGACGAGTG

 584  M  L  Q  H  Q  H  I  V  R  F  F  G  V  C  T  E  G  R  P  L
2881 CATGCTGCAG CACCAGCACA TCGTGCGCTT CTTCGGCGTC TGCACCGAGG GCCGCCCCCT
     GTACGACGTC GTGGTCGTGT AGCACGCGAA GAAGCCGCAG ACGTGGCTCC CGGCGGGGGA
```

FIG. 12E

SUBSTITUTE SHEET (RULE 26)

```
 604  L  M  V   F  E  Y  M   R  H  G   D  L  N   R  F  L  R   S  H  G
2941 GCTCATGGTC TTTGAGTATA TGCGGCACGG GGACCTCAAC CGCTTCCTCC GATCCCATGG
     CGAGTACCAG AAACTCATAT ACGCCGTGCC CCTGGAGTTG GCGAAGGAGG CTAGGGTACC

 624  P  D  A   K  L  L  A   G  G  E   D  V  A   P  G  P  L   G  L  G
3001 ACCTGATGCC AAGCTGCTGG CTGGTGGGGA GGATGTGGCT CCAGGCCCCC TGGGTCTGGG
     TGGACTACGG TTCGACGACC GACCACCCCT CCTACACCGA GGTCCGGGGG ACCCAGACCC

 644  Q  L  L   A  V  A  S   Q  V  A   A  G  M   V  Y  L  A   G  L  H
3061 GCAGCTGCTG GCCGTGGCTA GCCAGGTCGC TGCGGGGATG GTGTACCTGG CGGGTCTGCA
     CGTCGACGAC CGGCACCGAT CGGTCCAGCG ACGCCCCTAC CACATGGACC GCCCAGACGT

 664  F  V  H   R  D  L  A   T  R  N   C  L  V   G  Q  G  L   V  V  K
3121 TTTTGTGCAC CGGGACCTGG CCACACGCAA CTGTCTAGTG GGCCAGGGAC TGGTGGTCAA
     AAAACACGTG GCCCTGGACC GGTGTGCGTT GACAGATCAC CCGGTCCCTG ACCACCAGTT

 684  I  G  D   F  G  M  S   R  D  I   Y  S  T   D  Y  Y  R   V  G  G
3181 GATTGGTGAT TTTGGCATGA GCAGGGATAT CTACAGCACC GACTATTACC GTGTGGGAGG
     CTAACCACTA AAACCGTACT CGTCCCTATA GATGTCGTGG CTGATAATGG CACACCCTCC

 704  R  T  M   L  P  I  R   W  M  P   P  E  S   I  L  Y  R   K  F  T
3241 CCGCACCATG CTGCCCATTC GCTGGATGCC GCCCGAGAGC ATCCTGTACC GTAAGTTCAC
     GGCGTGGTAC GACGGGTAAG CGACCTACGG CGGGCTCTCG TAGGACATGG CATTCAAGTG

 724  T  E  S   D  V  W  S   F  G  V   V  L  W   E  I  F  T   Y  G  K
3301 CACCGAGAGC GACGTGTGGA GCTTCGGCGT GGTGCTCTGG GAGATCTTCA CCTACGGCAA
     GTGGCTCTCG CTGCACACCT CGAAGCCGCA CCACGAGACC CTCTAGAAGT GGATGCCGTT

 744  Q  P  W   Y  Q  L  S   N  T  E   A  I  D   C  I  T  Q   G  R  E
```

FIG. 12F

```
3361 GCAGCCCTGG TACCAGCTCT CCAACACGGA GGCAATCGAC TGCATCACGC AGGGACGTGA
     CGTCGGGACC ATGGTCGAGA GGTTGTGCCT CCGTTAGCTG ACGTAGTGCG TCCCTGCACT

 764  L  E  R   P  R  A  C   P  P  E   V  Y  A   I  M  R  G   C  W  Q
3421 GTTGGAGCGG CCACGTGCCT GCCCACCAGA GGTCTACGCC ATCATGCGGG GCTGCTGGCA
     CAACCTCGCC GGTGCACGGA CGGGTGGTCT CCAGATGCGG TAGTACGCCC CGACGACCGT

 784  R  E  P   Q  Q  R  H   S  I  K   D  V  H   A  R  L  Q   A  L  A
3481 GCGGGAGCCC CAGCAACGCC ACAGCATCAA GGATGTGCAC GCCCGGCTGC AAGCCCTGGC
     CGCCCTCGGG GTCGTTGCGG TGTCGTAGTT CCTACACGTG CGGGCCGACG TTCGGGACCG

            R1 site added with cloning primer^
              R1 site removed with cut and fill^
 804  Q  A  P   P  V  Y  L   D  V  L   G  O
3541 CCAGGCACCT CCTGTCTACC TGGATGTCCT GGGCTAGAAT TAATTCAATC GATGGCCGCC
     GGTCCGTGGA GGACAGATGG ACCTACAGGA CCCGATCTTA ATTAAGTTAG CTACCGGCGG

            ^sv40 early poly A
3601 ATGGCCCAAC TTGTTTATTG CAGCTTATAA TGGTTACAAA TAAAGCAATA GCATCACAAA
     TACCGGGTTG AACAAATAAC GTCGAATATT ACCAATGTTT ATTTCGTTAT CGTAGTGTTT
```

FIG. 12G

```
        ^sp6 RNA start
841 TATAGAATAA CATCCACTTT GCCTTTCTCT CCACAGGTGT CCACTCCCAG GTCCAACTGC
    ATATCTTATT GTAGGTGAAA CGGAAAGAGA GGTGTCCACA GGTGAGGGTC CAGGTTGACG

     ^cloning linker   ^begin gD from pchadII
901 ACCTCGGTTC TATCGATTGA ATTCCACTGC CTTCCACCAA GCTCTGCAGG ATCCCAGAGT
    TGGAGCCAAG ATAGCTAACT TAAGGTGACG GAAGGTGGTT CGAGACGTCC TAGGGTCTCA

961 CAGGGGTCTG TATCTTCCTG CTGGTGGCTC CAGTTCAGGA ACAGTAAACC CTGCTCCGAA
    GTCCCCAGAC ATAGAAGGAC GACCACCGAG GTCAAGTCCT TGTCATTTGG GACGAGGCTT

1021 TATTGCCTCT CACATCTCGT CAATCTCCGC GAGGACTGGG GACCCTGTGA CAAGCTTCAG
     ATAACGGAGA GTGTAGAGCA GTTAGAGGCG CTCCTGACCC CTGGGACACT GTTCGAAGTC

1081 CGCGAACGAC CAACTACCCC GATCATCAGT TATCCTTAAG GTCTCTTTTG TGTGGTGCGT
     GCGCTTGCTG GTTGATGGGG CTAGTAGTCA ATAGGAATTC CAGAGAAAAC ACACCACGCA

           ^Start gD
   1       M* G*  G* T* A*  A* R* L*  G* A* V* I*  L* F* V*  V* I* V*
1141 TCCGGTATGG GGGGGACTGC CGCCAGGTTG GGGGCCGTGA TTTTGTTTGT CGTCATAGTG
     AGGCCATACC CCCCCTGACG GCGGTCCAAC CCCCGGCACT AAAACAAACA GCAGTATCAC

  19 G* L* H* G*  V* R* G*  K* Y* A*  L* A  D  A  S  L  K  M  A  D
1201 GGCCTCCATG GGGTCCGCGG CAAATATGCC TTGGCGGATG CCTCTCTCAA GATGGCCGAC
     CCGGAGGTAC CCCAGGCGCC GTTTATACGG AACCGCCTAC GGAGAGAGTT CTACCGGCTG
```

FIG. 13A

xho and GTA mutated in^
start mature trkB^

```
 39 P  N  R  F   R  G  K   D  L  P   V  L  D  Q   L  L  E   V  C  P
1261 CCCAATCGAT TTCGCGGCAA AGACCTTCCG GTCCTGGACC AGCTGCTCGA GGTATGTCCC
     GGGTTAGCTA AAGCGCCGTT TCTGGAAGGC CAGGACCTGG TCGACGAGCT CCATACAGGG

 59 T  S  C  K   C  S  A   S  R  I   W  C  S  D   P  S  P   G  I  V
1321 ACGTCCTGCA AATGCAGTGC CTCTCGGATC TGGTGCAGCG ACCCTTCTCC TGGCATCGTG
     TGCAGGACGT TTACGTCACG GAGAGCCTAG ACCACGTCGC TGGGAAGAGG ACCGTAGCAC

 79 A  F  P  R   L  E  P   N  S  V   D  P  E  N   I  T  E   I  F  I
1381 GCATTTCCGA GATTGGAGCC TAACAGTGTA GATCCTGAGA ACATCACCGA AATTTTCATC
     CGTAAAGGCT CTAACCTCGG ATTGTCACAT CTAGGACTCT TGTAGTGGCT TTAAAAGTAG

 99 A  N  Q  K   R  L  E   I  I  N   E  D  D  V   E  A  Y   V  G  L
1441 GCAAACCAGA AAAGGTTAGA AATCATCAAC GAAGATGATG TTGAAGCTTA TGTGGGACTG
     CGTTTGGTCT TTTCCAATCT TTAGTAGTTG CTTCTACTAC AACTTCGAAT ACACCCTGAC

119 R  N  L  T   I  V  D   S  G  L   K  F  V  A   H  K  A   F  L  K
1501 AGAAATCTGA CAATTGTGGA TTCTGGATTA AAATTTGTGG CTCATAAAGC ATTTCTGAAA
     TCTTTAGACT GTTAACACCT AAGACCTAAT TTTAAACACC GAGTATTTCG TAAAGACTTT

139 N  S  N  L   Q  H  I   N  F  T   R  N  K  L   T  S  L   S  R  K
1561 AACAGCAACC TGCAGCACAT CAATTTTACC CGAAACAAAC TGACGAGTTT GTCTAGGAAA
     TTGTCGTTGG ACGTCGTGTA GTTAAAATGG GCTTTGTTTG ACTGCTCAAA CAGATCCTTT

159 H  F  R  H   L  D  L   S  E  L   I  L  V  G   N  P  F   T  C  S
1621 CATTTCCGTC ACCTTGACTT GTCTGAACTG ATCCTGGTGG GCAATCCATT TACATGCTCC
     GTAAAGGCAG TGGAACTGAA CAGACTTGAC TAGGACCACC CGTTAGGTAA ATGTACGAGG
```

FIG. 13B

```
 179 C  D  I  M    W  I  K    T  L  Q    E  A  K  S    S  P  D    T  Q  D
1681 TGTGACATTA TGTGGATCAA GACTCTCCAA GAGGCTAAAT CCAGTCCAGA CACTCAGGAT
     ACACTGTAAT ACACCTAGTT CTGAGAGGTT CTCCGATTTA GGTCAGGTCT GTGAGTCCTA

 199 L  Y  C  L    N  E  S    S  K  N    I  P  L  A    N  L  Q    I  P  N
1741 TTGTACTGCC TGAATGAAAG CAGCAAGAAT ATTCCCCTGG CAAACCTGCA GATACCCAAT
     AACATGACGG ACTTACTTTC GTCGTTCTTA TAAGGGGACC GTTTGGACGT CTATGGGTTA

 219 C  G  L  P    S  A  N    L  A  A    P  N  L  T    V  E  E    G  K  S
1801 TGTGGTTTGC CATCTGCAAA TCTGGCCGCA CCTAACCTCA CTGTGGAGGA AGGAAAGTCT
     ACACCAAACG GTAGACGTTT AGACCGGCGT GGATTGGAGT GACACCTCCT TCCTTTCAGA

 239 I  T  L  S    C  S  V    A  G  D    P  V  P  N    M  Y  W    D  V  G
1861 ATCACATTAT CCTGTAGTGT GGCAGGTGAT CCGGTTCCTA ATATGTATTG GGATGTTGGT
     TAGTGTAATA GGACATCACA CCGTCCACTA GGCCAAGGAT TATACATAAC CCTACAACCA

 259 N  L  V  S    K  H  M    N  E  T    S  H  T  Q    G  S  L    R  I  T
1921 AACCTGGTTT CCAAACATAT GAATGAAACA AGCCACACAC AGGGCTCCTT AAGGATAACT
     TTGGACCAAA GGTTTGTATA CTTACTTTGT TCGGTGTGTG TCCCGAGGAA TTCCTATTGA

 279 N  I  S  S    D  D  S    G  K  Q    I  S  C  V    A  E  N    L  V  G
1981 AACATTTCAT CCGATGACAG TGGGAAGCAG ATCTCTTGTG TGGCGGAAAA TCTTGTAGGA
     TTGTAAAGTA GGCTACTGTC ACCCTTCGTC TAGAGAACAC ACCGCCTTTT AGAACATCCT

 299 E  D  Q  D    S  V  N    L  T  V    H  F  A  P    T  I  T    F  L  E
2041 GAAGATCAAG ATTCTGTCAA CCTCACTGTG CATTTTGCAC CAACTATCAC ATTTCTCGAA
     CTTCTAGTTC TAAGACAGTT GGAGTGACAC GTAAAACGTG GTTGATAGTG TAAAGAGCTT
```

FIG. 13C

```
 319 S  P  T  S    D  H  H    W  C  I    P  F  T  V    K  G  N    P  K  P
2101 TCTCCAACCT CAGACCACCA CTGGTGCATT CCATTCACTG TGAAAGGCAA CCCAAAACCA
     AGAGGTTGGA GTCTGGTGGT GACCACGTAA GGTAAGTGAC ACTTTCCGTT GGGTTTTGGT

 339 A  L  Q  W    F  Y  N    G  A  I    L  N  E  S    K  Y  I    C  T  K
2161 GCGCTTCAGT GGTTCTATAA CGGGGCAATA TTGAATGAGT CCAAATACAT CTGTACTAAA
     CGCGAAGTCA CCAAGATATT GCCCCGTTAT AACTTACTCA GGTTTATGTA GACATGATTT

 359 I  H  V  T    N  H  T    E  Y  H    G  C  L  Q    L  D  N    P  T  H
2221 ATACATGTTA CCAATCACAC GGAGTACCAC GGCTGCCTCC AGCTGGATAA TCCCACTCAC
     TATGTACAAT GGTTAGTGTG CCTCATGGTG CCGACGGAGG TCGACCTATT AGGGTGAGTG

 379 M  N  N  G    D  Y  T    L  I  A    K  N  E  Y    G  K  D    E  K  Q
2281 ATGAACAATG GGGACTACAC TCTAATAGCC AAGAATGAGT ATGGGAAGGA TGAGAAACAG
     TACTTGTTAC CCCTGATGTG AGATTATCGG TTCTTACTCA TACCCTTCCT ACTCTTTGTC

 399 I  S  A  H    F  M  G    W  P  G    I  D  D  G    A  N  P    N  Y  P
2341 ATTTCTGCTC ACTTCATGGG CTGGCCTGGA ATTGACGATG GTGCAAACCC AAATTATCCT
     TAAAGACGAG TGAAGTACCC GACCGGACCT TAACTGCTAC CACGTTTGGG TTTAATAGGA

 419 D  V  I  Y    E  D  Y    G  T  A    A  N  D  I    G  D  T    T  N  R
2401 GATGTAATTT ATGAAGATTA TGGAACTGCA GCGAATGACA TCGGGGACAC CACGAACAGA
     CTACATTAAA TACTTCTAAT ACCTTGACGT CGCTTACTGT AGCCCCTGTG GTGCTTGTCT

 439 S  N  E  I    P  S  T    D  V  T    D  K  T  G    R  E  H    L  S  V
2461 AGTAATGAAA TCCCTTCCAC AGACGTCACT GATAAAACCG GTCGGGAACA TCTCTCGGTC
     TCATTACTTT AGGGAAGGTG TCTGCAGTGA CTATTTTGGC CAGCCCTTGT AGAGAGCCAG

 459 Y  A  V  V    V  I  A    S  V  V    G  F  C  L    L  V  M    L  F  L
2521 TATGCTGTGG TGGTGATTGC GTCTGTGGTG GGATTTTGCC TTTTGGTAAT GCTGTTTCTG
     ATACGACACC ACCACTAACG CAGACACCAC CCTAAAACGG AAAACCATTA CGACAAAGAC
```

FIG. 13D

```
 479 L  K  L  A    R  H  S    K  F  G    M  K  G  P    A  S  V    I  S  N
2581 CTTAAGTTGG CAAGACACTC CAAGTTTGGC ATGAAAGGCC CAGCCTCCGT TATCAGCAAT
     GAATTCAACC GTTCTGTGAG GTTCAAACCG TACTTTCCGG GTCGGAGGCA ATAGTCGTTA

 499 D  D  D  S    A  S  P    L  H  H    I  S  N  G    S  N  T    P  S  S
2641 GATGATGACT CTGCCAGCCC ACTCCATCAC ATCTCCAATG GGAGTAACAC TCCATCTTCT
     CTACTACTGA GACGGTCGGG TGAGGTAGTG TAGAGGTTAC CCTCATTGTG AGGTAGAAGA

 519 S  E  G  G    P  D  A    V  I  I    G  M  T  K    I  P  V    I  E  N
2701 TCGGAAGGTG GCCCAGATGC TGTCATTATT GGAATGACCA AGATCCCTGT CATTGAAAAT
     AGCCTTCCAC CGGGTCTACG ACAGTAATAA CCTTACTGGT TCTAGGGACA GTAACTTTTA

 539 P  Q  Y  F    G  I  T    N  S  Q    L  K  P  D    T  F  V    Q  H  I
2761 CCCCAGTACT TTGGCATCAC CAACAGTCAG CTCAAGCCAG ACACATTTGT TCAGCACATC
     GGGGTCATGA AACCGTAGTG GTTGTCAGTC GAGTTCGGTC TGTGTAAACA AGTCGTGTAG

 559 K  R  H  N    I  V  L    K  R  E    L  G  E  G    A  F  G    K  V  F
2821 AAGCGACATA ACATTGTTCT GAAAAGGGAG CTAGGCGAAG GAGCCTTTGG AAAAGTGTTC
     TTCGCTGTAT TGTAACAAGA CTTTTCCCTC GATCCGCTTC CTCGGAAACC TTTTCACAAG

 579 L  A  E  C    Y  N  L    C  P  E    Q  D  K  I    L  V  A    V  K  T
2881 CTAGCTGAAT GCTATAACCT CTGTCCTGAG CAGGACAAGA TCTTGGTGGC AGTGAAGACC
     GATCGACTTA CGATATTGGA GACAGGACTC GTCCTGTTCT AGAACCACCG TCACTTCTGG

 599 L  K  D  A    S  D  N    A  R  K    D  F  H  R    E  A  E    L  L  T
2941 CTGAAGGATG CCAGTGACAA TGCACGCAAG GACTTCCACC GTGAGGCCGA GCTCCTGACC
     GACTTCCTAC GGTCACTGTT ACGTGCGTTC CTGAAGGTGG CACTCCGGCT CGAGGACTGG
```

FIG. 13E

```
 619 N  L  Q  H    E  H  I    V  K  F    Y  G  V  C    V  E  G    D  P  L
3001 AACCTCCAGC ATGAGCACAT CGTCAAGTTC TATGGCGTCT GCGTGGAGGG CGACCCCCTC
     TTGGAGGTCG TACTCGTGTA GCAGTTCAAG ATACCGCAGA CGCACCTCCC GCTGGGGGAG

 639 I  M  V  F    E  Y  M    K  H  G    D  L  N  K    F  L  R    A  H  G
3061 ATCATGGTCT TTGAGTACAT GAAGCATGGG GACCTCAACA AGTTCCTCAG GGCACACGGC
     TAGTACCAGA AACTCATGTA CTTCGTACCC CTGGAGTTGT TCAAGGAGTC CCGTGTGCCG

 659 P  D  A  V    L  M  A    E  G  N    P  P  T  E    L  T  Q    S  Q  M
3121 CCTGATGCCG TGCTGATGGC TGAGGGCAAC CCGCCCACGG AACTGACGCA GTCGCAGATG
     GGACTACGGC ACGACTACCG ACTCCCGTTG GGCGGGTGCC TTGACTGCGT CAGCGTCTAC

 679 L  H  I  A    Q  Q  I    A  A  G    M  V  Y  L    A  S  Q    H  F  V
3181 CTGCATATAG CCCAGCAGAT CGCCGCGGGC ATGGTCTACC TGGCGTCCCA GCACTTCGTG
     GACGTATATC GGGTCGTCTA GCGGCGCCCG TACCAGATGG ACCGCAGGGT CGTGAAGCAC

 699 H  R  D  L    A  T  R    N  C  L    V  G  E  N    L  L  V    K  I  G
3241 CACCGCGATT TGGCCACCAG GAACTGCCTG GTCGGGGAGA ACTTGCTGGT GAAAATCGGG
     GTGGCGCTAA ACCGGTGGTC CTTGACGGAC CAGCCCCTCT TGAACGACCA CTTTTAGCCC

 719 D  F  G  M    S  R  D    V  Y  S    T  D  Y  Y    R  V  G    G  H  T
3301 GACTTTGGGA TGTCCCGGGA CGTGTACAGC ACTGACTACT ACAGGGTCGG TGGCCACACA
     CTGAAACCCT ACAGGGCCCT GCACATGTCG TGACTGATGA TGTCCCAGCC ACCGGTGTGT

 739 M  L  P  I    R  W  M    P  P  E    S  I  M  Y    R  K  F    T  T  E
3361 ATGCTGCCCA TTCGCTGGAT GCCTCCAGAG AGCATCATGT ACAGGAAATT CACGACGGAA
     TACGACGGGT AAGCGACCTA CGGAGGTCTC TCGTAGTACA TGTCCTTTAA GTGCTGCCTT

 759 S  D  V  W    S  L  G    V  V  L    W  E  I  F    T  Y  G    K  Q  P
3421 AGCGACGTCT GGAGCCTGGG GGTCGTGTTG TGGGAGATTT TCACCTATGG CAAACAGCCC
```

FIG. 13F

```
      TCGCTGCAGA CCTCGGACCC CCAGCACAAC ACCCTCTAAA AGTGGATACC GTTTGTCGGG

 779  W  Y  Q  L     S  N  N     E  V  I     E  C  I  T     Q  G  R     V  L  Q
3481  TGGTACCAGC TGTCAAACAA TGAGGTGATA GAGTGTATCA CTCAGGGCCG AGTCCTGCAG
      ACCATGGTCG ACAGTTTGTT ACTCCACTAT CTCACATAGT GAGTCCCGGC TCAGGACGTC

 799  R  P  R  T     C  P  Q     E  V  Y     E  L  M  L     G  C  W     Q  R  E
3541  CGACCCCGCA CGTGCCCCCA GGAGGTGTAT GAGCTGATGC TGGGGTGCTG GCAGCGAGAG
      GCTGGGGCGT GCACGGGGGT CCTCCACATA CTCGACTACG ACCCCACGAC CGTCGCTCTC

 819  P  H  M  R     K  N  I     K  G  I     H  T  L  L     Q  N  L     A  K  A
3601  CCCCACATGA GGAAGAACAT CAAGGGCATC CATACCCTCC TTCAGAACTT GGCCAAGGCA
      GGGGTGTACT CCTTCTTGTA GTTCCCGTAG GTATGGGAGG AAGTCTTGAA CCGGTTCCGT

 839  S  P  V  Y     L  D  I     L  G  O
3661  TCTCCGGTCT ACCTGGACAT TCTAGGCTAG GGCCCTTTTC CCCAGACCGA TCCTTCCCAA
      AGAGGCCAGA TGGACCTGTA AGATCCGATC CCGGGAAAAG GGGTCTGGCT AGGAAGGGTT

3721  CGTACTCCTC AGACGGGCTG AGAGGATGAA CATCTTTTAA CTGCCGCTGG AGGCCACCAA
      GCATGAGGAG TCTGCCCGAC TCTCCTACTT GTAGAAAATT GACGGCGACC TCCGGTGGTT

                              half Xho half Sal site from subcloning^
3781  GCTGCTCTCC TTCACTCTGA CAGTATTAAC ATCAAAGACT CCGAGAAGCT CTCGACCTGC
      CGACGAGAGG AAGTGAGACT GTCATAATTG TAGTTTCTGA GGCTCTTCGA GAGCTGGACG

                                    ^sv40 early poly A
3841  AGAAGCTTGG CCGCCATGGC CCAACTTGTT TATTGCAGCT TATAATGGTT ACAAATAAAG
      TCTTCGAACC GGCGGTACCG GGTTGAACAA ATAACGTCGA ATATTACCAA TGTTTATTTC
```

FIG. 13G

```
        ^sp6 RNA start
 841 TATAGAATAA CATCCACTTT GCCTTTCTCT CCACAGGTGT CCACTCCCAG GTCCAACTGC
     ATATCTTATT GTAGGTGAAA CGGAAAGAGA GGTGTCCACA GGTGAGGGTC CAGGTTGACG

      ^cloning linker
         ^RI site mutated in
         ^gD from pchadII
 901 ACCTGAATTC CACTGCCTTC CACCAAGCTC TGCAGGATCC CAGAGTCAGG GGTCTGTATC
     TGGACTTAAG GTGACGGAAG GTGGTTCGAG ACGTCCTAGG GTCTCAGTCC CCAGACATAG

 961 TTCCTGCTGG TGGCTCCAGT TCAGGAACAG TAAACCCTGC TCCGAATATT GCCTCTCACA
     AAGGACGACC ACCGAGGTCA AGTCCTTGTC ATTTGGGACG AGGCTTATAA CGGAGAGTGT

1021 TCTCGTCAAT CTCCGCGAGG ACTGGGGACC CTGTGACAAG CTTCAGCGCG AACGACCAAC
     AGAGCAGTTA GAGGCGCTCC TGACCCCTGG GACACTGTTC GAAGTCGCGC TTGCTGGTTG

                                                                ^Start
gD
   1                                                            M* G* G*
1081 TACCCCGATC ATCAGTTATC CTTAAGGTCT CTTTTGTGTG GTGCGTTCCG GTATGGGGGG
     ATGGGGCTAG TAGTCAATAG GAATTCCAGA GAAAACACAC CACGCAAGGC CATACCCCCC

   4  T* A* A*  R* L* G* A*  V* I* L*  F* V* V*  I* V* G* L*  H* G* V*
1141 GACTGCCGCC AGGTTGGGGG CCGTGATTTT GTTTGTCGTC ATAGTGGGCC TCCATGGGGT
     CTGACGGCGG TCCAACCCCC GGCACTAAAA CAAACAGCAG TATCACCCGG AGGTACCCCA

  24  R* G* K*  Y* A* L* A   D  A  S   L  K  M   A  D  P  N   R  F  R
1201 CCGCGGCAAA TATGCCTTGG CGGATGCCTC TCTCAAGATG GCCGACCCCA ATCGATTTCG
     GGCGCCGTTT ATACGGAACC GCCTACGGAG AGAGTTCTAC CGGCTGGGGT TAGCTAAAGC
```

FIG. 14A

```
                                        ^Xho site and GTA mutated in
                                                    ^begin mature trkC
  44  G  K  D    L  P  V  L    D  Q  L    L  E  V   C  P  A  N    C  V  C
1261  CGGCAAAGAC CTTCCGGTCC TGGACCAGCT GCTCGAGGTA TGCCCTGCAA ATTGTGTCTG
      GCCGTTTCTG GAAGGCCAGG ACCTGGTCGA CGAGCTCCAT ACGGGACGTT TAACACAGAC

  64  S  K  T    E  I  N  C    R  R  P    D  D  G   N  L  F  P    L  L  E
1321  CAGCAAGACT GAGATCAATT GCCGGCGGCC GGACGATGGG AACCTCTTCC CCCTCCTGGA
      GTCGTTCTGA CTCTAGTTAA CGGCCGCCGG CCTGCTACCC TTGGAGAAGG GGGAGGACCT

  84  G  Q  D    S  G  N  S    N  G  N    A  N  I   N  I  T  D    I  S  R
1381  AGGGCAGGAT TCAGGGAACA GCAATGGGAA CGCCAATATC AACATCACGG ACATCTCAAG
      TCCCGTCCTA AGTCCCTTGT CGTTACCCTT GCGGTTATAG TTGTAGTGCC TGTAGAGTTC

 104  N  I  T    S  I  H  I    E  N  W    R  S  L   H  T  L  N    A  V  D
1441  GAATATCACT TCCATACACA TAGAGAACTG GCGCAGTCTT CACACGCTCA ACGCCGTGGA
      CTTATAGTGA AGGTATGTGT ATCTCTTGAC CGCGTCAGAA GTGTGCGAGT TGCGGCACCT

 124  M  E  L    Y  T  G  L    Q  K  L    T  I  K   N  S  G  L    R  S  I
1501  CATGGAGCTC TACACCGGAC TTCAAAAGCT GACCATCAAG AACTCAGGAC TTCGGAGCAT
      GTACCTCGAG ATGTGGCCTG AAGTTTTCGA CTGGTAGTTC TTGAGTCCTG AAGCCTCGTA

 144  Q  P  R    A  F  A  K    N  P  H    L  R  Y   I  N  L  S    S  N  R
1561  TCAGCCCAGA GCCTTTGCCA AGAACCCCCA TTTGCGTTAT ATAAACCTGT CAAGTAACCG
      AGTCGGGTCT CGGAAACGGT TCTTGGGGGT AAACGCAATA TATTTGGACA GTTCATTGGC

 164  L  T  T    L  S  W  Q    L  F  Q    T  L  S   L  R  E  L    Q  L  E
1621  GCTCACCACA CTCTCGTGGC AGCTCTTCCA GACGCTGAGT CTTCGGGAAT TGCAGTTGGA
```

FIG. 14B

```
      CGAGTGGTGT GAGAGCACCG TCGAGAAGGT CTGCGACTCA GAAGCCCTTA ACGTCAACCT

 184  Q  N  F   F  N  C  S   C  D  I   R  W  M   Q  L  W  Q   E  Q  G
1681 GCAGAACTTT TTCAACTGCA GCTGTGACAT CCGCTGGATG CAGCTCTGGC AGGAGCAGGG
      CGTCTTGAAA AAGTTGACGT CGACACTGTA GGCGACCTAC GTCGAGACCG TCCTCGTCCC

 204  E  A  K   L  N  S  Q   N  L  Y   C  I  N   A  D  G  S   Q  L  P
1741 GGAGGCCAAG CTCAACAGCC AGAACCTCTA CTGCATCAAT GCTGATGGCT CCCAGCTTCC
      CCTCCGGTTC GAGTTGTCGG TCTTGGAGAT GACGTAGTTA CGACTACCGA GGGTCGAAGG

 224  L  F  R   M  N  I  S   Q  C  D   L  P  E   I  S  V  S   H  V  N
1801 TCTCTTCCGC ATGAACATCA GTCAGTGTGA CCTTCCTGAG ATCAGCGTGA GCCACGTCAA
      AGAGAAGGCG TACTTGTAGT CAGTCACACT GGAAGGACTC TAGTCGCACT CGGTGCAGTT

 244  L  T  V   R  E  G  D   N  A  V   I  T  C   N  G  S  G   S  P  L
1861 CCTGACCGTA CGAGAGGGTG ACAATGCTGT TATCACTTGC AATGGCTCTG GATCACCCCT
      GGACTGGCAT GCTCTCCCAC TGTTACGACA ATAGTGAACG TTACCGAGAC CTAGTGGGGA

 264  P  D  V   D  W  I  V   T  G  L   Q  S  I   N  T  H  Q   T  N  L
1921 TCCTGATGTG GACTGGATAG TCACTGGGCT GCAGTCCATC AACACTCACC AGACCAATCT
      AGGACTACAC CTGACCTATC AGTGACCCGA CGTCAGGTAG TTGTGAGTGG TCTGGTTAGA

 284  N  W  T   N  V  H  A   I  N  L   T  L  V   N  V  T  S   E  D  N
1981 GAACTGGACC AATGTTCATG CCATCAACTT GACGCTGGTG AATGTGACGA GTGAGGACAA
      CTTGACCTGG TTACAAGTAC GGTAGTTGAA CTGCGACCAC TTACACTGCT CACTCCTGTT

 304  G  F  T   L  T  C  I   A  E  N   V  V  G   M  S  N  A   S  V  A
2041 TGGCTTCACC CTGACGTGCA TTGCAGAGAA CGTGGTGGGC ATGAGCAATG CCAGTGTTGC
      ACCGAAGTGG GACTGCACGT AACGTCTCTT GCACCACCCG TACTCGTTAC GGTCACAACG
```

FIG. 14C

SUBSTITUTE SHEET (RULE 26)

```
 324  L  T  V   Y  Y  P  P   R  V  V   S  L  E   E  P  E  L   R  L  E
2101 CCTCACTGTC TACTATCCCC CACGTGTGGT GAGCCTGGAG GAGCCTGAGC TGCGCCTGGA
     GGAGTGACAG ATGATAGGGG GTGCACACCA CTCGGACCTC CTCGGACTCG ACGCGGACCT

 344  H  C  I   E  F  V  V   R  G  N   P  P  P   T  L  H  W   L  H  N
2161 GCACTGCATC GAGTTTGTGG TGCGTGGCAA CCCCCCACCA ACGCTGCACT GGCTGCACAA
     CGTGACGTAG CTCAAACACC ACGCACCGTT GGGGGGTGGT TGCGACGTGA CCGACGTGTT

 364  G  Q  P   L  R  E  S   K  I  I   H  V  E   Y  Y  Q  E   G  E  I
2221 TGGGCAGCCT CTGCGGGAGT CCAAGATCAT CCATGTGGAA TACTACCAAG AGGGAGAGAT
     ACCCGTCGGA GACGCCCTCA GGTTCTAGTA GGTACACCTT ATGATGGTTC TCCCTCTCTA

 384  S  E  G   C  L  L  F   N  K  P   T  H  Y   N  N  G  N   Y  T  L
2281 TTCCGAGGGC TGCCTGCTCT TCAACAAGCC CACCCACTAC AACAATGGCA ACTATACCCT
     AAGGCTCCCG ACGGACGAGA AGTTGTTCGG GTGGGTGATG TTGTTACCGT TGATATGGGA

 404  I  A  K   N  P  L  G   T  A  N   Q  T  I   N  G  H  F   L  K  E
2341 CATTGCCAAA AACCCACTGG GCACAGCCAA CCAGACCATC AATGGCCACT TCCTCAAGGA
     GTAACGGTTT TTGGGTGACC CGTGTCGGTT GGTCTGGTAG TTACCGGTGA AGGAGTTCCT

                ^begin ecd insert          ^end ecd insert
 424  P  F  P   E  S  T  D   N  F  I   L  F  D   E  V  S  P   T  P  P
2401 GCCCTTTCCA GAGAGCACGG ATAACTTTAT CTTGTTTGAC GAAGTGAGTC CCACACCTCC
     CGGGAAAGGT CTCTCGTGCC TATTGAAATA GAACAAACTG CTTCACTCAG GGTGTGGAGG

                                        ^begin TM
 444  I  T  V   T  H  K  P   E  E  D   T  F  G   V  S  I  A   V  G  L
2461 TATCACTGTG ACCCACAAAC CAGAAGAAGA CACTTTTGGG GTATCCATAG CAGTTGGACT
```

FIG. 14D

SUBSTITUTE SHEET (RULE 26)

```
      ATAGTGACAC TGGGTGTTTG GTCTTCTTCT GTGAAAACCC CATAGGTATC GTCAACCTGA

                                                          ^end TM
 464  A  A  F  A  C  V  L  L  V  V  L  F  V  M  I  N  K  Y  G  R
2521 TGCTGCTTTT GCCTGTGTCC TGTTGGTGGT TCTCTTCGTC ATGATCAACA AATATGGTCG
     ACGACGAAAA CGGACACAGG ACAACCACCA AGAGAAGCAG TACTAGTTGT TTATACCAGC

 484  R  S  K  F  G  M  K  G  P  V  A  V  I  S  G  E  E  D  S  A
2581 ACGGTCCAAA TTTGGAATGA AGGGTCCCGT GGCTGTCATC AGTGGTGAGG AGGACTCAGC
     TGCCAGGTTT AAACCTTACT TCCCAGGGCA CCGACAGTAG TCACCACTCC TCCTGAGTCG

 504  S  P  L  H  H  I  N  H  G  I  T  T  P  S  S  L  D  A  G  P
2641 CAGCCCACTG CACCACATCA ACCACGGCAT CACCACGCCC TCGTCACTGG ATGCCGGGCC
     GTCGGGTGAC GTGGTGTAGT TGGTGCCGTA GTGGTGCGGG AGCAGTGACC TACGGCCCGG

 524  D  T  V  V  I  G  M  T  R  I  P  V  I  E  N  P  Q  Y  F  R
2701 CGACACTGTG GTCATTGGCA TGACTCGCAT CCCTGTCATT GAGAACCCCC AGTACTTCCG
     GCTGTGACAC CAGTAACCGT ACTGAGCGTA GGGACAGTAA CTCTTGGGGG TCATGAAGGC

 544  Q  G  H  N  C  H  K  P  D  T  Y  V  Q  H  I  K  R  R  D  I
2761 TCAGGGACAC AACTGCCACA AGCCGGACAC GTATGTGCAG CACATTAAGA GGAGAGACAT
     AGTCCCTGTG TTGACGGTGT TCGGCCTGTG CATACACGTC GTGTAATTCT CCTCTCTGTA

                         ^begin TK
 564  V  L  K  R  E  L  G  E  G  A  F  G  K  V  F  L  A  E  C  Y
2821 CGTGCTGAAG CGAGAACTGG GTGAGGGAGC CTTTGGAAAG GTCTTCCTGG CCGAGTGCTA
     GCACGACTTC GCTCTTGACC CACTCCCTCG GAAACCTTTC CAGAAGGACC GGCTCACGAT
```

FIG. 14E

```
 584  N  L  S   P  T  K  D   K  M  L   V  A  V   K  A  L  K   D  P  T
2881 CAACCTCAGC CCGACCAAGG ACAAGATGCT TGTGGCTGTG AAGGCCCTGA AGGATCCCAC
     GTTGGAGTCG GGCTGGTTCC TGTTCTACGA ACACCGACAC TTCCGGGACT TCCTAGGGTG

 604  L  A  A   R  K  D  F   Q  R  E   A  E  L   L  T  N  L   Q  H  E
2941 CCTGGCTGCC CGGAAGGATT TCCAGAGGGA GGCCGAGCTG CTCACCAACC TGCAGCATGA
     GGACCGACGG GCCTTCCTAA AGGTCTCCCT CCGGCTCGAC GAGTGGTTGG ACGTCGTACT

 624  H  I  V   K  F  Y  G   V  C  G   D  G  D   P  L  I  M   V  F  E
3001 GCACATTGTC AAGTTCTATG GAGTGTGCGG CGATGGGGAC CCCCTCATCA TGGTCTTTGA
     CGTGTAACAG TTCAAGATAC CTCACACGCC GCTACCCCTG GGGGAGTAGT ACCAGAAACT

 644  Y  M  K   H  G  D  L   N  K  F   L  R  A   H  G  P  D   A  M  I
3061 ATACATGAAG CATGGAGACC TGAATAAGTT CCTCAGGGCC CATGGGCCAG ATGCAATGAT
     TATGTACTTC GTACCTCTGG ACTTATTCAA GGAGTCCCGG GTACCCGGTC TACGTTACTA

 664  L  V  D   G  Q  P  R   Q  A  K   G  E  L   G  L  S  Q   M  L  H
3121 CCTTGTGGAT GGACAGCCAC GCCAGGCCAA GGGTGAGCTG GGGCTCTCCC AAATGCTCCA
     GGAACACCTA CCTGTCGGTG CGGTCCGGTT CCCACTCGAC CCCGAGAGGG TTTACGAGGT

 684  I  A  S   Q  I  A  S   G  M  V   Y  L  A   S  Q  H  F   V  H  R
3181 CATTGCCAGT CAGATCGCCT CGGGTATGGT GTACCTGGCC TCCCAGCACT TTGTGCACCG
     GTAACGGTCA GTCTAGCGGA GCCCATACCA CATGGACCGG AGGGTCGTGA AACACGTGGC

 704  D  L  A   T  R  N  C   L  V  G   A  N  L   L  V  K  I   G  D  F
3241 AGACCTGGCC ACCAGGAACT GCCTGGTTGG AGCGAATCTG CTAGTGAAGA TTGGGGACTT
     TCTGGACCGG TGGTCCTTGA CGGACCAACC TCGCTTAGAC GATCACTTCT AACCCCTGAA

                                                 ^TK insert site
 724  G  M  S   R  D  V  Y   S  T  D   Y  Y  R   V  G  G  H   T  M  L
```

FIG. 14F

```
3301 CGGCATGTCC AGAGATGTCT ACAGCACGGA TTATTACAGG GTGGGAGGAC ACACCATGCT
     GCCGTACAGG TCTCTACAGA TGTCGTGCCT AATAATGTCC CACCCTCCTG TGTGGTACGA

 744  P  I  R  W  M  P  P  E  S  I  M  Y  R  K  F  T  T  E  S  D
3361 CCCCATTCGC TGGATGCCTC CTGAAAGCAT CATGTACCGG AAGTTCACTA CAGAGAGTGA
     GGGGTAAGCG ACCTACGGAG GACTTTCGTA GTACATGGCC TTCAAGTGAT GTCTCTCACT

 764  V  W  S  F  G  V  I  L  W  E  I  F  T  Y  G  K  Q  P  W  F
3421 TGTATGGAGC TTCGGGGTGA TCCTCTGGGA GATCTTCACC TATGGAAAGC AGCCATGGTT
     ACATACCTCG AAGCCCCACT AGGAGACCCT CTAGAAGTGG ATACCTTTCG TCGGTACCAA

 784  Q  L  S  N  T  E  V  I  E  C  I  T  Q  G  R  V  L  E  R  P
3481 CCAACTCTCA AACACGGAGG TCATTGAGTG CATTACCCAA GGTCGTGTTT TGGAGCGGCC
     GGTTGAGAGT TTGTGCCTCC AGTAACTCAC GTAATGGGTT CCAGCACAAA ACCTCGCCGG

 804  R  V  C  P  K  E  V  Y  D  V  M  L  G  C  W  Q  R  E  P  Q
3541 CCGAGTCTGC CCCAAAGAGG TGTACGATGT CATGCTGGGG TGCTGGCAGA GGGAACCACA
     GGCTCAGACG GGGTTTCTCC ACATGCTACA GTACGACCCC ACGACCGTCT CCCTTGGTGT

 824  Q  R  L  N  I  K  E  I  Y  K  I  L  H  A  L  G  K  A  T  P
3601 GCAGCGGTTG AACATCAAGG AGATCTACAA AATCCTCCAT GCTTTGGGGA AGGCCACCCC
     CGTCGCCAAC TTGTAGTTCC TCTAGATGTT TTAGGAGGTA CGAAACCCCT TCCGGTGGGG

                              ^stop
                   R1 site removed with cut and fill^
 844  I  Y  L  D  I  L  G  O
3661 AATCTACCTG GACATTCTTG GCTAGTGGTG GCTGGTGGTC ATGAATTAAT TCAATCGATG
     TTAGATGGAC CTGTAAGAAC CGATCACCAC CGACCACCAG TACTTAATTA AGTTAGCTAC

                    ^sv40 early poly A
3721 GCCGCCATGG CCCAACTTGT TTATTGCAGC TTATAATGGT TACAAATAAA GCAATAGCAT
     CGGCGGTACC GGGTTGAACA AATAACGTCG AATATTACCA ATGTTTATTT CGTTATCGTA
```

FIG.14G

gD.TrkA

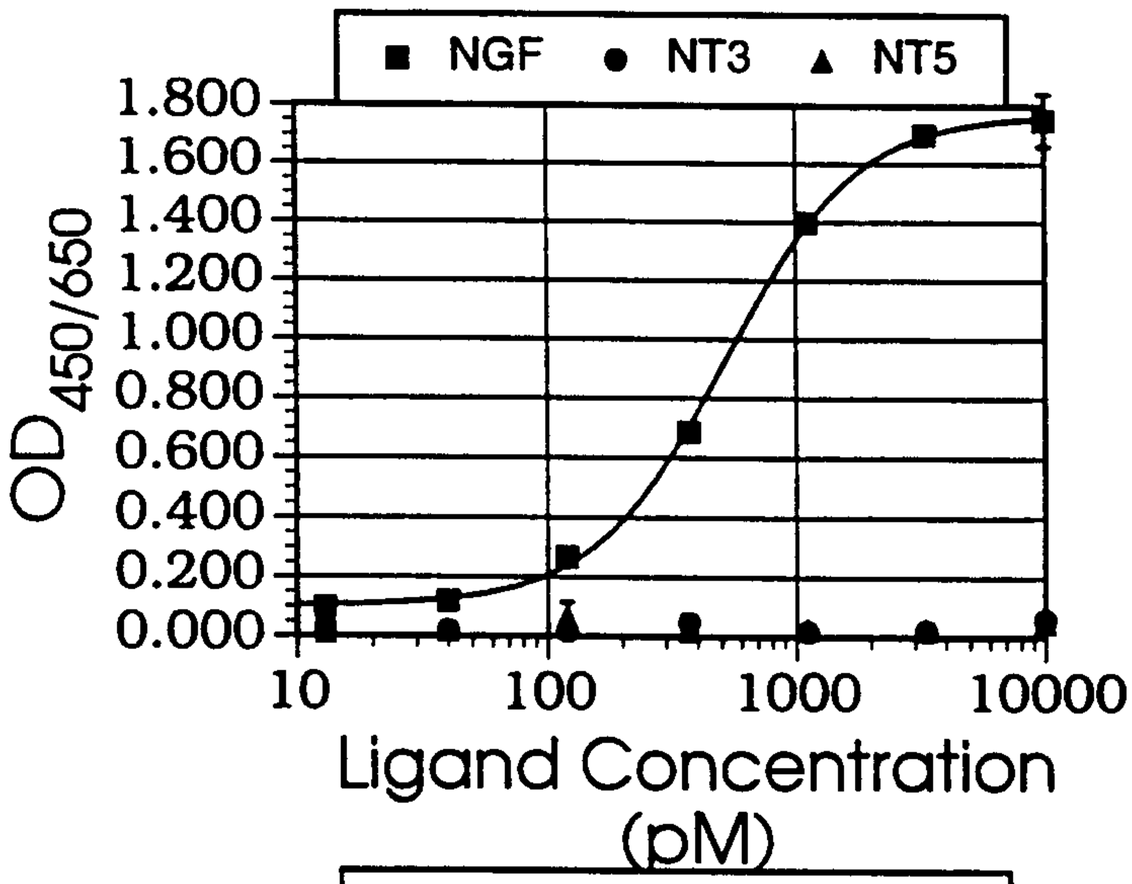


FIG. 15A

gD.TrkB

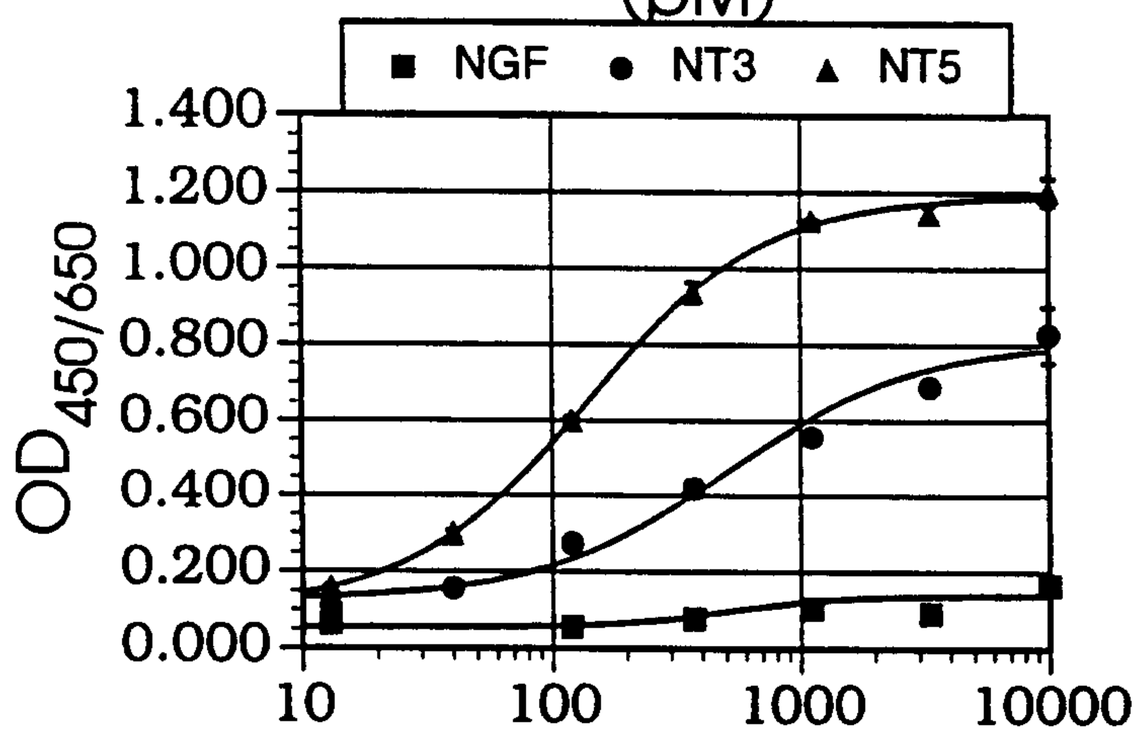


FIG. 15B

gD.TrkC

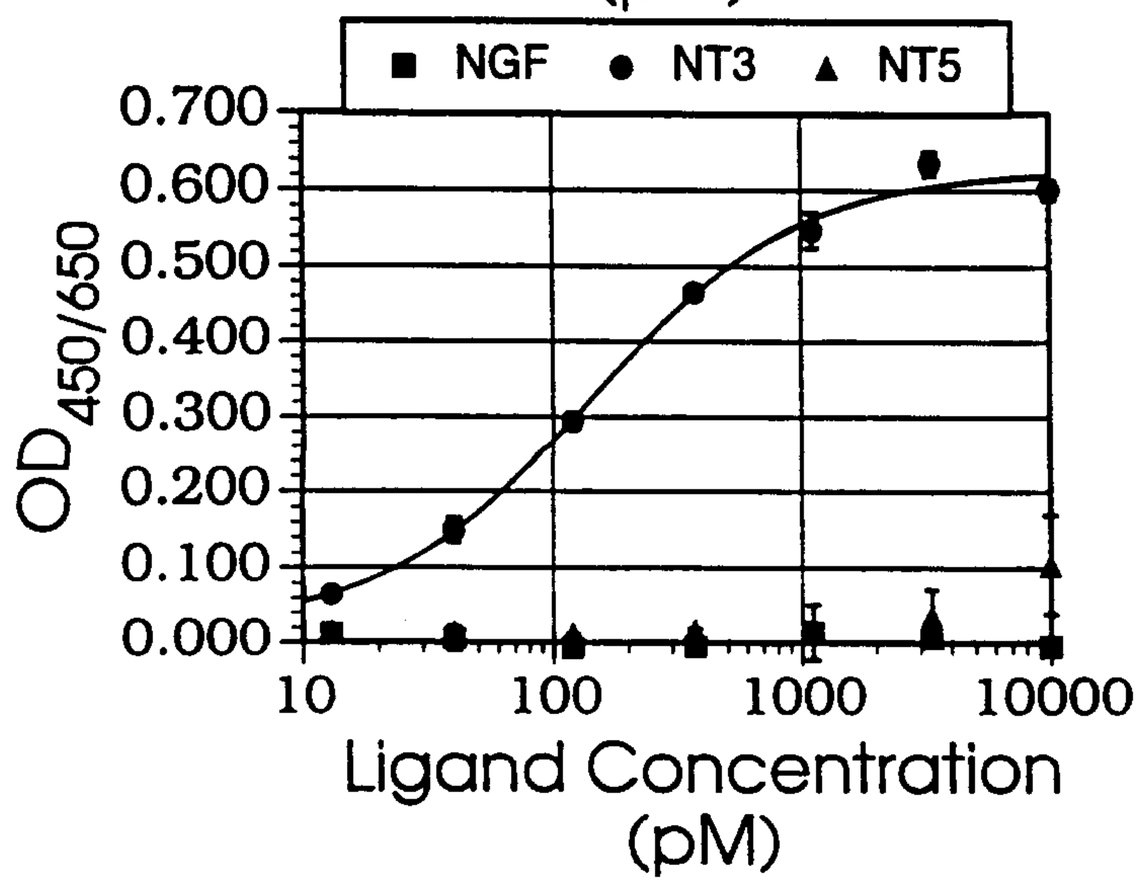


FIG. 15C

```
      aluI
     sstI
     sacI                                          aluI
     hgiJII                                  sau3AI pvuII
     hgiAI/aspHI                             mboI/ndeII[dam-]
     ecl136II                                dpnI[dam+]
     bsp1286                                pvuI/bspCI
     bsiHKAI                            pleI dpnII[dam-]
     bmyI                               hinfI  taqI[dam-]
     banII                         rmaI    mcrI    nspBII
   taqI                            maeI   taqI[dam-]
1 TTCGAGCTCG CCCGACATTG ATTATTGACT AGAGTCGATC GACAGCTGTG GAATGTGTGT CAGTTAGGGT
  AAGCTCGAGC GGGCTGTAAC TAATAACTGA TCTCAGCTAG CTGTCGACAC CTTACACACA GTCAATCCCA

                 nlaIV
              scrFI                                  sfaNI                 scrFI
              mvaI                                 ppu10I                  mvaI
              ecoRII                               nsiI/avaIII             ecoRII
              dsaV                                nlaIII                   dsaV
              bstNI                              sphI                       bstNI
              apyI[dcm+]                         nspI                       apyI
             bsaJI                               nspHI                     sexAI
71 GTGGAAAGTC CCCAGGCTCC CCAGCAGGCA GAAGTATGCA AAGCATGCAT CTCAATTAGT CAGCAACCAG
   CACCTTTCAG GGGTCCGAGG GGTCGTCCGT CTTCATACGT TTCGTACGTA GAGTTAATCA GTCGTTGGTC
```

FIG. 16A

```
                    nlaIV
          scrFI                          sfaNI
               mvaI                                ppul0I
          ecoRII                       nsiI/avaIII
          dsaV                        nlaIII
               bstNI                             sphI
[dcm+]         apyI[dcm+]                        nspI
              bsaJI                              nspHI
141 GTGTGGAAAG TCCCCAGGCT CCCCAGCAGG CAGAAGTATG CAAAGCATGC ATCTCAATTA GTCAGCAACC
    CACACCTTTC AGGGGTCCGA GGGGTCGTCC GTCTTCATAC GTTTCGTACG TAGAGTTAAT CAGTCGTTGG

                                                                        nlaIII
                                                                       styI
                                                                       ncoI
                                  aciI                              bslI dsaI
         aciI         aciI  fokI            aciI bsrI  aciI         aciI bsaJI
211 ATAGTCCCGC CCCTAACTCC GCCCATCCCG CCCCTAACTC CGCCCAGTTC CGCCCATTCT CCGCCCCATG
    TATCAGGGCG GGGATTGAGG CGGGTAGGGC GGGGATTGAG GCGGGTCAAG GCGGGTAAGA GGCGGGGTAC

                                        fnu4HI
                                        bglI
                                       sfiI
                                       haeIII/palI
                                     mnlI   mnlI      ddeI
                                haeIII/palI bsaJI  mnlI   aluI
                              mnlI bsaJI aciI    haeIII/palI                 mnlI
281 GCTGACTAAT TTTTTTTATT TATGCAGAGG CCGAGGCCGC CTCGGCCTCT GAGCTATTCC AGAAGTAGTG
    CGACTGATTA AAAAAAATAA ATACGTCTCC GGCTCCGGCG GAGCCGGAGA CTCGATAAGG TCTTCATCAC
```

FIG. 16B

```
                          rmaI
                         styI
                         bsaJI                                  haeIII/palI
                         blnI                                  mcrI
                         avrII                        aluI     eagI/xmaIII/eclXI
                        haeIII/palI                  rmaI      eaeI
                       stuI                          maeI      cfrI
      mnlI             haeI                         nheI      mspI
                      mnlI maeI                     aluI      hpaII
351 AGGAGGCTTT TTTGGAGGCC TAGGCTTTTG CAAAAAGCTA GCTTATCCGG
    TCCTCCGAAA AAACCTCCGG ATCCGAAAAC GTTTTTCGAT CGAATAGGCC

                              tfiI
    scrFI                     hinFI
    nciI                   aciI
    mspI                   thaI
    hpaII                  fnuDII/mvnI                        aciI
    dsaV                   bstUI                        maeII  rsaI
    cauII                  bsh1236I                   maeIII    csp6I  scfI
401 CCGGGAACGG TGCATTGGAA CGCGGATTCC CCGTGCCAAG AGTGACGTAA GTACCGCCTA TAGAGCGATA
    GGCCCTTGCC ACGTAACCTT GCGCCTAAGG GGCACGGTTC TCACTGCATT CATGGCGGAT ATCTCGCTAT
                                                  ^splice donor

                        fnu4HI
                        bbvI
                       nspBII                                                pflMI
     mnlI              aciI       nlaIII taqI          sfaNI                 bslI
471 AGAGGATTTT ATCCCCGCTG CCATCATGGT TCGACCATTG AACTGCATCG TCGCCGTGTC CCAAAATATG
    TCTCCTAAAA TAGGGGCGAC GGTAGTACCA AGCTGGTAAC TTGACGTAGC AGCGGCACAG GGTTTTATAC
                          DHFR ATG^
```

FIG. 16C

```
                                 haeIII/palI
                                haeI
                              scrFI
                              mvaI     bsrBI
                              ecoRII
                              dsaV
                              bstNI    aciI                    rsaI
                    bsmAI     apyI[dcm+]        xmnI          csp6I
                    bsaI     bsaJI  mnlI  ddeI  asp700       scaI
541 GGGATTGGCA AGAACGGAGA CCTACCCTGG CCTCCGCTCA GGAACGAGTT CAAGTACTTC CAAAGAATGA
    CCCTAACCGT TCTTGCCTCT GGATGGGACC GGAGGCGAGT CCTTGCTCAA GTTCATGAAG GTTTCTTACT

                                                                scrFI
                                                                mvaI
                                                                ecoRII
                eco57I                                          dsaV
               mboII                tfiI                        bstNI
              earI/ksp632I          hinfI                       apyI[dcm+]
             mnlI                 alwNI    hphI                 sexAI            ddeI
611 CCACAACCTC TTCAGTGGAA GGTAAACAGA ATCTGGTGAT TATGGGTAGG AAAACCTGGT TCTCCATTCC
    GGTGTTGGAG AAGTCACCTT CCATTTGTCT TAGACCACTA ATACCCATCC TTTTGGACCA AGAGGTAAGG

             tfiI       tru9I         tru9I
             hinfI      mseI          mseI         ddeI
          mboII taqI   ahaIII/draI   aseI/asnI/vspI                      bslI   mnlI
681 TGAGAAGAAT CGACCTTTAA AGGACAGAAT TAATATAGTT CTCAGTAGAG AACTCAAAGA ACCACCACGA
    ACTCTTCTTA GCTGGAAATT TCCTGTCTTA ATTATATCAA GAGTCATCTC TTGAGTTTCT TGGTGGTGCT
```

FIG. 16D

```
     sstI
     sacI
     hgiJII
     hgiAI/aspHI
     ecl136II
     bsp1286
     bsiHKAI
     bmyI                                    tru9I                 mspI
     banII                                  aflII/bfrI             hpaII
      aluI            bstXI      fokI sfaNI mseI                  bsaWI
751 GGAGCTCATT TTCTTGCCAA AAGTTTGGAT GATGCCTTAA GACTTATTGA ACAACCGGAA TTGGCAAGTA
    CCTCGAGTAA AAGAACGGTT TTCAAACCTA CTACGGAATT CTGAATAACT TGTTGGCCTT AACCGTTCAT

                                                                      haeIII/palI
                                                                     haeI
                                                scrFI          scrFI
                                                mvaI                 mvaI
                                                ecoRII         ecoRII
                                                dsaV   tfiI    dsaV
                                                bstNI    nlaIII     bstNI     ddeI
      accI nlaIII              mnlI             apyI[dcm+]   hinfI  apyI[dcm+]
821 AAGTAGACAT GGTTTGGATA GTCGGAGGCA GTTCTGTTTA CCAGGAAGCC ATGAATCAAC CAGGCCACCT
    TTCATCTGTA CCAAACCTAT CAGCCTCCGT CAAGACAAAT GGTCCTTCGG TACTTAGTTG GTCCGGTGGA
```

FIG. 16E

```
                                  nlaIII
                               sau3AI
                               mboI/ndeII[dam-]
                               dpnI[dam+]                    maeII
      pleI                     dpnII[dam-]                 aflIII
      hinfI    maeIII alwI[dam-]  apoI          maeIII
891 TAGACTCTTT GTGACAAGGA TCATGCAGGA ATTTGAAAGT GACACGTTTT TCCCAGAAAT TGATTTGGGG
    ATCTGAGAAA CACTGTTCCT AGTACGTCCT TAAACTTTCA CTGTGCAAAA AGGGTCTTTA ACTAAACCCC

                                    hgaI
                                  hinlI/acyI
                                  ahaII/bsaHI
                             scrFI
                             mvaI      mnlI
                             ecoRII
                             dsaV
                             bstNI     ecoNI
                             apyI[dcm+]      mnlI
                mnlI         bsaJI     bslI ddeI
961 AAATATAAAC CTCTCCCAGA ATACCCAGGC GTCCTCTCTG
    TTTATATTTG GAGAGGGTCT TATGGGTCCG CAGGAGAGAC
```

FIG. 16F

```
          scrFI
          mvaI
          ecoRII
          dsaV
          bstNI
          apyI[dcm+]
       sau96I
       avaII                                                                sfaNI
       asuI   mnlI        sfaNI                    accI     mboII              mboII
1001 AGGTCCAGGA GGAAAAAGGC ATCAAGTATA AGTTTGAAGT CTACGAGAAG AAAGACTAAC AGGAAGATGC
     TCCAGGTCCT CCTTTTTCCG TAGTTCATAT TCAAACTTCA GATGCTCTTC TTTCTGATTG TCCTTCTACG
                                                                  ^END DHFR

                                                                 nlaIII
                                                                styI
                                                                ncoI
                                          ppul0I                dsaI
                          mnlI     aluI nsiI/avaIII            bsaJI
1071 TTTCAAGTTC TCTGCTCCCC TCCTAAAGCT ATGCATTTTT ATAAGACCAT GGGACTTTTG
     AAAGTTCAAG AGACGAGGGG AGGATTTCGA TACGTAAAAA TATTCTGGTA CCCTGAAAAC
```

FIG. 16G

```
                    styI
                    bsaJI
              sau3AI                    fnu4HI
              mboI/ndeII[dam-]          aciI
              dpnI[dam+]               thaI
              dpnII[dam-]              fnuDII/mvnI tru9I
              alwI[dam-]               bstUI       mseI
             bstYI/xhoII               bsh1236I   aseI/asnI/vspI
1131 CTGGCTTTAG ATCCCCTTGG CTTCGTTAGA ACGCGGCTAC AATTAATACA TAACCTTATG TATCATACAC
     GACCGAAATC TAGGGGAACC GAAGCAATCT TGCGCCGATG TTAATTATGT ATTGGAATAC ATAGTATGTG

                                                                          sau96I
                                                                          avaII
                                                                          asuI
                                                                       scrFI
                                                                       mvaI
                                                                       ecoRII
                                                                       dsaV
                                                                       bstNI
                                                                       apyI[dcm+]
                 maeIII
                hphI   scfI       fokI                           bslI  bsaJI
1201 ATACGATTTA GGTGACACTA TAGATAACAT CCACTTTGCC TTTCTCTCCA CAGGTGTCCA CTCCCAGGTC
     TATGCTAAAT CCACTGTGAT ATCTATTGTA GGTGAAACGG AAAGAGAGGT GTCCACAGGT GAGGGTCCAG
```

FIG. 16H


1271 CAACTGGGCCTGTCCGGCAACTCAAGCTGCTTAGGGAGGTCCCCGTTAATGATATGCTGGCGACGCAGGCCGCCGAAAATATAGGTGTTGACGCCGTGTTTCCGTTTGCGGCCTGTCGCTTACGTTAATATGCGTATGCGTATTGCGCAGTGGCGCAGCGCCAATCAGAATGGCGGAAGCGTCGAAGCCTGTAAAGCGGCGGTGCACAATCTTATCGAGCCATTTGATGTACCTGTAGCATCGCTCTTCTCCGCAAAGCTCGCCGATTTTTTGCGACACAATTTCGCACTGTGAGGGCCAAGAGTGCCGTTTAATGTGGCTTAACCTACGGCTACACTAGAAGAACAGTATTTGGTATCTGCGCTCTGCTGAAGCCAGTTACCTTCGGAAAAAGAGTTGGTAGCTCTTGATCCGGCAAACAAACCACCGCTGGTAGCGGTGGTTTTTTTGTTTGCAAGCAGCAGATTACGCGCAGAAAAAAAGGATCTCAAGAAGATCCTTTGATCTTTTCTACGGGGTCTGACGCTCAGTGGAACGAAAACTCACGTTAAGGGATTTTGGTCATGAGATTATCAAAAAGGATCTTCACCTAGATCCTTTTAAATTAAAAATGAAGTTTTAAATCAATCTAAAGTATATATGAGTAAACTTGGTCTGACAGTTACCAATGCTTAATCAGTGAGGCACCTATCTCAGCGATCTGTCTATTTCGTTCATCCATAGTTGCCTGACTCCCCGTCGTGTAGATAACTACGATACGGGAGGGCTTACCATCTGGCCCCAGTGCTGCAATGATACCGCGAGACCCACGCTCACCGGCTCCAGATTTATCAGCAATAAACCAGCCAGCCGGAAGGGCCGAGCGCAGAAGTGGTCCTGCAACTTTATCCGCCTCCATCCAGTCTATTAATTGTTGCCGGGAAGCTAGAGTAAGTAGTTCGCCAGTTAATAGTTTGCGCAACGTTGTTGCCATTGCTACAGGCATCGTGGTGTCACGCTCGTCGTTTGGTATGGCTTCATTCAGCTCCGGTTCCCAACGATCAAGGCGAGTTACATGATCCCCCATGTTGTGCAAAAAAGCGGTTAGCTCCTTCGGTCCTCCGATCGTTGTCAGAAGTAAGTTGGCCGCAGTGTTATCACTCATGGTTATGGCAGCACTGCATAATTCTCTTACTGTCATGCCATCCGTAAGATGCTTTTCTGTGACTGGTGAGTACTCAACCAAGTCATTCTGAGAATAGTGTATGCGGCGACCGAGTTGCTCTTGCCCGGCGTCAATACGGGATAATACCGCGCCACATAGCAGAACTTTAAAAGTGCTCATCATTGGAAAACGTTCTTCGGGGCGAAAACTCTCAAGGATCTTACCGCTGTTGAGATCCAGTTCGATGTAACCCACTCGTGCACCCAACTGATCTTCAGCATCTTTTACTTTCACCAGCGTTTCTGGGTGAGCAAAAACAGGAAGGCAAAATGCCGCAAAAAAGGGAATAAGGGCGACACGGAAATGTTGAATACTCATACTCTTCCTTTTTCAATATTATTGAAGCATTTATCAGGGTTATTGTCTCATGAGCGGATACATATTTGAATGTATTTAGAAAAATAAACAAATAGGGGTTCCGCGCACATTTCCCCGAAAAGTGCCACCTGACGTCTAAGAAACCATTATTATCATGACATTAACCTATAAAAATAGGCGTATCACGAGGCCCTTTCGTCTTCAAGAATTCGCCCTATAGTGAGTCGTATTACAATTCACTGGCCGTCGTTTTACAACGTCGTGACTGGGAAAACCCTGGCGTTACCCAACTTAATCGCCTTGCAGCACATCCCCCTTTCGCCAGCTGGCGTAATAGCGAAGAGGCCCGCACCGATCGCCCTTCCCAACAGTTGCGCAGCCTGAATGGCGAATGGCGCCTGATGCGGTATTTTCTCCTTACGCATCTGTGCGGTATTTCACACCGCATATGGTGCACTCTCAGTACAATCTGCTCTGATGCCGCATAGTTAAGCCAGCCCCGACACCCGCCAACACCCGCTGACGCGCCCTGACGGGCTTGTCTGCTCCCGGCATCCGCTTACAGACAAGCTGTGACCGTCTCCGGGAGCTGCATGTGTCAGAGGTTTTCACCGTCATCACCGAAACGCGCGAGGCAGCTGCGGTAAAGCTCATCAGCGTGGTCGTGAAGCGATTCACAGATGTCTGCCTGTTCATCCGCGTCCAGCTCGTTGAGTTTCTCCAGAAGCGTTAATGTCTGGCTTCTGATAAAGCGGGCCATGTTAAGGGCGGTTTTTTCCTGTTTGGTCACTGATGCCTCCGTGTAAGGGGGATTTCTGTTCATGGGGGTAATGATACCGATGAAACGAGAGAGGATGCTCACGATACGGGTTACTGATGATGAACATGCCCGGTTACTGGAACGTTGTGAGGGTAAACAACTGGCGGTATGGATGCGGCGGGACCAGAGAAAAATCACTCAGGGTCAATGCCAGCGCTTCGTTAATACAGATGTAGGTGTTCCACAGGGTAGCCAGCAGCATCCTGCGATGCAGATCCGGAACATAATGGTGCAGGGCGCTGACTTCCGCGTTTCCAGACTTTACGAAACACGGAAACCGAAGACCATTCATGTTGTTGCTCAGGTCGCAGACGTTTTGCAGCAGCAGTCGCTTCACGTTCGCTCGCGTATCGGTGATTCATTCTGCTAACCAGTAAGGCAACCCCGCCAGCCTAGCCGGGTCCTCAACGACAGGAGCACGATCATGCGCACCCGTGGCCAGGACCCAACGCTGCCCGAGATGCGCCGCGTGCGGCTGCTGGAGATGGCGGACGCGATGGATATGTTCTGCCAAGGGTTGGTTTGCGCATTCACAGTTCTCCGCAAGAATTGATTGGCTCCAATTCTTGGAGTGGTGAATCCGTTAGCGAGGTGCCGCCGGCTTCCATTCAGGTCGAGGTGGCCCGGCTCCATGCACCGCGACGCAACGCGGGGAGGCAGACAAGGTATAGGGCGGCGCCTACAATCCATGCCAACCCGTTCCATGTGCTCGCCGAGGCGGCATAAATCGCCGTGACGATCAGCGGTCCAGTGATCGAAGTTAGGCTGGTAAGAGCCGCGAGCGATCCTTGAAGCTGTCCCTGATGGTCGTCATCTACCTGCCTGGACAGCATGGCCTGCAACGCGGGCATCCCGATGCCGCCGGAAGCGAGAAGAATCATAATGGGGAAGGCCATCCAGCCTCGCGTCGCGAACGCCAGCAAGACGTAGCCCAGCGCGTCGGCCGCCATGCCGGCGATAATGGCCTGCTTCTCGCCGAAACGTTTGGTGGCGGGACCAGTGACGAAGGCTTGAGCGAGGGCGTGCAAGATTCCGAATACCGCAAGCGACAGGCCGATCATCGTCGCGCTCCAGCGAAAGCGGTCCTCGCCGAAAATGACCCAGAGCGCTGCCGGCACCTGTCCTACGAGTTGCATGATAAAGAAGACAGTCATAAGTGCGGCGACGATAGTCATGCCCCGCGCCCACCGGAAGGAGCTGACTGGGTTGAAGGCTCTCAAGGGCATCGGTCGAGATCCCGGTGCCTAATGAGTGAGCTAACTTACATTAATTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAGCTGCATTAATGAATCGGCCAACGCGCGGGGAGAGGCGGTTTGCGTATTGGGCGCTCTTCCGCTTCCTCGCTCACTGACTCGCTGCGCTCGGTCGTTCGGCTGCGGCGAGCGGTATCAGCTCACTCAAAGGCGGTAATACGGTTATCCACAGAATCAGGGGATAACGCAGGAAAGAACATGTGAGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAAAAGGCCGCGTTGCTGGCGTTTTTCCATAGGCTCCGCCCCCCTGACGAGCATCACAAAAATCGACGCTCAAGTCAGAGGTGGCGAAACCCGACAGGACTATAAAGATACCAGGCGTTTCCCCCTGGAAGCTCCCTCGTGCGCTCTCCTGTTCCGACCCTGCCGCTTACCGGATACCTGTCCGCCTTTCTCCCTTCGGGAAGCGTGGCGCTTTCTCATAGCTCACGCTGTAGGTATCTCAGTTCGGTGTAGGTCGTTCGCTCCAAGCTGGGCTGTGTGCACGAACCCCCCGTTCAGCCCGACCGCTGCGCCTTATCCGGTAACTATCGTCTTGAGTCCAACCCGGTAAGACACGACTTATCGCCACTGGCAGCAGCCACTGGTAACAGGATTAGCAGAGCGAGGTATGTAGGCGGTGCTACAGAGTTCTTGAAGTGGTGGCCTAACTACGGCTACACTAGAAGG

```
                                  sau96I
                        aciI      haeIII/palI
                       fnu4HI     asuI
                       bglI  nlaIII
                      sfiI  styI
                     eaeI   ncoI
                     cfrI   dsaI                          aluI
        ecoRI    taqI haeIII/palI                       fnu4HI
        apoI    claI/bsp106 bsaJI                      bbvI          maeIII
1321 GGGGAATTCA ATCGATGGCC GCCATGGCCC AACTTGTTTA TTGCAGCTTA TAATGGTTAC AAATAAAGCA
     CCCCTTAAGT TAGCTACCGG CGGTACCGGG TTGAACAAAT AACGTCGAAT ATTACCAATG TTTATTTCGT
                                      ^sv40

                                                          rmaI
        sfaNI   apoI                                  bsmI maeI
1391 ATAGCATCAC AAATTTCACA AATAAAGCAT TTTTTTCACT GCATTCTAGT TGTGGTTTGT CCAAACTCAT
     TATCGTAGTG TTTAAAGTGT TTATTTCGTA AAAAAAGTGA CGTAAGATCA ACACCAAACA GGTTTGAGTA
```

FIG. 16J

```
                                  sau3AI
                                  mboI/ndeII[dam-]
                                  dpnI[dam+]
                                  dpnII[dam-]
                                 pvuI/bspCI
                                 mcrI
                                taqI[dam-]  tru9I
                               claI/bsp106[dam-]
                              sau3AI        mseI
                              mboI/ndeII[dam-]
                              dpnI[dam+] xmnI
                              dpnII[dam-]  aseI/asnI/vspI
                     nlaIII  alwI[dam-]  asp700
1461 CAATGTATCT TATCATGTCT GGATCGATCG GGAATTAATT
     GTTACATAGA ATAGTACAGA CCTAGCTAGC CCTTAATTAA
                              sv40 origin^

                                                                rsaI
                      haeIII/palI                               csp6I
                     haeI                                     nlaIV
                  styI                                        kpnI
          fnu4HI ncoI                                         hgiCI
          bbvI   dsaI                                         banI
        hinPI    bsaJI                                        asp718     mnlI
        hhaI/cfoI nlaIII          mnlI      mnlI             acc65I   ddeI  aciI
1501 CGGCGCAGCA CCATGGCCTG AAATAACCTC TGAAAGAGGA ACTTGGTTAG GTACCTTCTG AGGCGGAAAG
     GCCGCGTCGT GGTACCGGAC TTTATTGGAG ACTTTCTCCT TGAACCAATC CATGGAAGAC TCCGCCTTTC
```

FIG. 16K

FIG. 16L

```
                                                                  nlaIV
                                                               scrFI
                                                               mvaI
                                                               ecoRII
                                                               dsaV
                                                               bstNI
          aluI                                                 apyI[dcm+]
         pvuII                                                bsaJI
         nspBII
1571 AACCAGCTGT GGAATGTGTG TCAGTTAGGG TGTGGAAAGT CCCCAGGCTC CCCAGCAGGC AGAAGTATGC
     TTGGTCGACA CCTTACACAC AGTCAATCCC ACACCTTTCA GGGGTCCGAG GGGTCGTCCG TCTTCATACG

                                                                  nlaIV
      sfaNI                  scrFI        scrFI
          ppu10I                   mvaI                  mvaI
    nsiI/avaIII              ecoRII       ecoRII
   nlaIII                    dsaV         dsaV
        sphI                       bstNI                 bstNI
        nspI                       apyI[dcm+]            apyI[dcm+]
        nspHI                     sexAI                 bsaJI
1641 AAAGCATGCA TCTCAATTAG TCAGCAACCA GGTGTGGAAA GTCCCCAGGC TCCCCAGCAG GCAGAAGTAT
     TTTCGTACGT AGAGTTAATC AGTCGTTGGT CCACACCTTT CAGGGGTCCG AGGGGTCGTC CGTCTTCATA

              sfaNI
            ppu10I
            nsiI/avaIII
           nlaIII
          sphI
          nspI                                                          aciI
          nspHI                                 aciI         aciI  fokI
1711 GCAAAGCATG CATCTCAATT AGTCAGCAAC CATAGTCCCG CCCCTAACTC CGCCCATCCC GCCCCTAACT
     CGTTTCGTAC GTAGAGTTAA TCAGTCGTTG GTATCAGGGC GGGGATTGAG GCGGGTAGGG CGGGGATTGA
```

```
                                    nlaIII
                                   styI
                                   ncoI
          bsrI                 bslI dsaI
      asiI       aciI          aciI bsaJI                                  mnlI
1781  CCGCCCAGTT CCGCCCATTC TCCGCCCCAT GGCTGACTAA TTTTTTTTAT TTATGCAGAG
      GGCGGGTCAA GGCGGGTAAG AGGCGGGGTA CCGACTGATT AAAAAAAATA AATACGTCTC

                                                                        styI
            fnu4HI                                                      bsaJI
            bglI                                                        blnI
           sfiI                                                         avrII
           haeIII/palI                                                 haeIII/palI
         mnlI    mnlI     ddeI                                        stuI
   haeIII/palI  bsaJI mnlI   aluI                   mnlI               haeI
      bsaJI aciI    haeIII/palI                   mnlI               mnlI
1841  GCCGAGGCCG CCTCGGCCTC TGAGCTATTC CAGAAGTAGT GAGGAGGCTT TTTTGGAGGC
      CGGCTCCGGC GGAGCCGGAG ACTCGATAAG GTCTTCATCA CTCCTCCGAA AAAACCTCCG
```

FIG. 16M

```
                                                  aciI                hinPI
                                                haeIII/palI           hhaI/cfoI
                                               mcrI                  thaI
                                               eagI/xmaIII/eclXI     fnuDII/mvnI
                                        taqI   eaeI                  bstUI                 bspMI
                                       xhoI   notI                  hinPI                scfI
                                       paeR7I cfrI    tru9I         hhaI/cfoI tru9I      pstI
                                       avaI   fnu4HI  pacI         ascI       ahaIII/draI
      rmaI                            mnlI   aciI    mseI tru9I bsh1236I mseI        bsgI
      maeI                 aluI maeIII  bsrBI fnu4HI    mseI bssHII  swaI        sse8387I
1901  CTAGGCTTTT GCAAAAAGCT GTTACCTCGA GCGGCCGCTT AATTAAGGCG CGCCATTTAA ATCCTGCAGG
      GATCCGAAAA CGTTTTTCGA CAATGGAGCT CGCCGGCGAA TTAATTCCGC GCGGTAAATT TAGGACGTCC
                        ^start pUC118
                                 ^linearization linker inserted into Hpa1 site

                                                                  scrFI
                                                                  mvaI
                                                                  ecoRII
                        haeIII/palI                               dsaV
                       eaeI                                       bstNI
   maeIII              cfrI                      maeIII           apyI[dcm+]          tru9I
         aluI      bsrI                   maeII   bsrI          bsaJI   maeIII      mseI
1971  TAACAGCTTG GCACTGGCCG TCGTTTTACA ACGTCGTGAC TGGGAAAACC CTGGCGTTAC CCAACTTAAT
      ATTGTCGAAC CGTGACCGGC AGCAAAATGT TGCAGCACTG ACCCTTTTGG GACCGCAATG GGTTGAATTA
```

FIG. 16N

```
                                                                  sau3AI
                                                            sau96I     mboI/ndeII[dam-]
                                                        haeIII/palI
                                                        asuI       dpnI[dam+]
                                    aluI                    mnlI         dpnII[dam-]
            fnu4HI                  pvuII                mboII    aciI  pvuI/bspCI
            bbvI   fokI             nspBII               earI/ksp632I   mcrI
2041 CGCCTTGCAG CACATCCCCC CTTCGCCAGC TGGCGTAATA GCGAAGAGGC CCGCACCGAT
     GCGGAACGTC GTGTAGGGGG GAAGCGGTCG ACCGCATTAT CGCTTCTCCG GGCGTGGCTA

                                                 hinPI
                                                 hhaI/cfoI
                                                nlaIV
                                                narI
                                                kasI
                                                hinlI/acyI
                                                hgiCI
                                                haeII      aciI
                                                banI    sfaNI
                                bglI            ahaII/bsaHI                    sfaNI
2101 CGCCCTTCCC AACAGTTGCG TAGCCTGAAT GGCGAATGGC GCCTGATGCG GTATTTTCTC CTTACGCATC
     GCGGGAAGGG TTGTCAACGC ATCGGACTTA CCGCTTACCG CGGACTACGC CATAAAAGAG GAATGCGTAG
```

FIG. 160

```
                                                                  hinPI
                                                                 thaI
                                                                 fnuDII/mvnI
                                                                 bstUI scfI     hinPI
                                                                 bsh1236I       hhaI/cfoI
                                                            rsaI hhaI/cfoI  fnu4HI
        aciI          aciI   maeII                          csp6I   bslI    aciI
2171 TGTGCGGTAT TTCACACCGC ATACGTCAAA GCAACCATAG TACGCGCCCT GTAGCGGCGC
     ACACGCCATA AAGTGTGGCG TATGCAGTTT CGTTGGTATC ATGCGCGGGA CATCGCCGCG

            fnu4HI                  fnu4HI
           thaI                   hinPI
           fnuDII/mvnI            hhaI/cfoI                                  hinPI
           bctUI                 thaI                                        hhaI/cfoI
          hinPI aciI             fnuDII/mvnI                            rmaI
          hhaI/cfoI              bstUI                            hinPI   haeII
      tru9I aciI                 bsh1236I      aciI               hhaI/cfoI    bsrBI
      mseI bsh1236I           maeIII bbvI  maeIII               haeII maeI    aciI
2231 ATTAAGCGCG GCGGGTGTGG TGGTTACGCG CAGCGTGACC GCTACACTTG CCAGCGCCCT AGCGCCCGCT
     TAATTCGCGC CGCCCACACC ACCAATGCGC GTCGCACTGG CGATGTGAAC GGTCGCGGGA TCGCGGGCGA

                                                                                nlaIV
                                                  mspI                         hgiJII
                                                  hpaII                        bsp1286
                                                 naeI                          bmyI
                mboII                   maeII cfr10I              aluI         banII
2301 CCTTTCGCTT TCTTCCCTTC CTTTCTCGCC ACGTTCGCCG GCTTTCCCCG TCAAGCTCTA AATCGGGGGC
     GGAAAGCGAA AGAAGGGAAG GAAAGAGCGG TGCAAGCGGC CGAAAGGGGC AGTTCGAGAT TTAGCCCCCG
```

FIG. 16P

```
                                                  mnlI
                                              nlaIV
                                              hgiCI
                nlaIV                         banI  taqI                        hphI
2371 TCCCTTTAGG GTTCCGATTT AGTGCTTTAC GGCACCTCGA CCCCAAAAAA CTTGATTTGG
     AGGGAAATCC CAAGGCTAAA TCACGAAATG CCGTGGAGCT GGGGTTTTTT GAACTAAACC

                 maeII   haeIII/palI
                draIII   sau96I                                   maeII pleI
                bsaAI    asuI                                    drdI   hinfI  maeII
2401 GTGATGGTTC ACGTAGTGGG CCATCGCCCT GATAGACGGT TTTTCGCCCT TTGACGTTGG AGTCCACGTT
     CACTACCAAG TGCATCACCC GGTAGCGGGA CTATCTGCCA AAAAGCGGGA AACTGCAACC TCAGGTGCAA

       tru9I     pleI                                  bslI
       mseI      hinfI          bsrI                   bslI  avaI
2501 CTTTAATAGT GGACTCTTGT TCCAAACTGG AACAACACTC AACCCTATCT CGGGCTATTC TTTTGATTTA
     GAAATTATCA CCTGAGAACA AGGTTTGACC TTGTTGTGAG TTGGGATAGA GCCCGATAAG AAAACTAAAT

                                                                        tru9I
                                          tru9I                tru9I      mseI
                              haeIII/palI mseI        aluI    mseI    apoI
2571 TAAGGGATTT TGCCGATTTC GGCCTATTGG  TTAAAAAATG AGCTGATTTA ACAAAAATTT
     ATTCCCTAAA ACGGCTAAAG CCGGATAACC  AATTTTTTAC TCGACTAAAT TGTTTTTAAA
```

FIG. 16Q

```
                                                    hgiAI/aspHI
      thaI                                          bsp1286
      fnuDII/mvnI              maeII                bsiHKAI
         apoI                 psp1406I              bmyI   ddeI
      bstUI    tru9I        tru9I                   apaLI/snoI  rsaI
      bsh1236I mseI   sspI mseI                     alw44I/snoI csp6I
2631 AACGCGAATT TTAACAAAAT ATTAACGTTT ACAATTTTAT GGTGCACTCT CAGTACAATC
     TTGCGCTTAA AATTGTTTTA TAATTGCAAA TGTTAAAATA CCACGTGAGA GTCATGTTAG

                                                       bsrI           hinPI
                                                    maeIII         fnu4HI
             fnu4HI     tru9I                      maeII        nlaIII hhaI/cfoI
          sfaNI aciI    mseI        aciI       bsaAI tth111I/aspI bbvI
2691 TGCTCTGATG  CCGCATAGTT AAGCCAACTC CGCTATCGCT ACGTGACTGG GTCATGGCTG CGCCCCGACA
     ACGAGACTAC  GGCGTATCAA TTCGGTTGAG GCGATAGCGA TGCACTGACC CAGTACCGAC GCGGGGCTGT

                        hinPI                          sfaNI
                        hhaI/cfoI                   mspI
                       thaI                         hpaII
                       fnuDII/mvnI                 scrFI
                       bstUI                       nciI
                nspBII bsh1236I                    dsaV fokI                 maeIII
      aciI      aciI hgaI       drdI               cauII    aciI          aluI
2761 CCCGCCAACA CCCGCTGACG CGCCCTGACG GGCTTGTCTG CTCCCGGCAT CCGCTTACAG ACAAGCTGTG
     GGGCGGTTGT GGGCGACTGC GCGGGACTGC CCGAACAGAC GAGGGCCGTA GGCGAATGTC TGTTCGACAC
```

FIG. 16R

```
                                                                                  thaI
                                                                                  fnuDII/mvnI
                scrFI                                                             bstUI
                nciI                                                              bsh1236I
                mspI                                                             hinPI
                hpaII      nspI                                                  hhaI/cfoI
                dsaV       nspHI                                                thaI mnlI
          esp3I        fnu4HI                                                   fnuDII/mvnI
           bsmAI       bbvI                                                     bstUI
         bslI  cauII aluI nlaIII    mnlI    hphI      hphI                       bsh1236I
2831 ACCGTCTCCG GGAGCTGCAT GTGTCAGAGG TTTTCACCGT CATCACCGAA ACGCGCGAGG CAGTATTCTT
     TGGCAGAGGC CCTCGACGTA CACAGTCTCC AAAAGTGGCA GTAGTGGCTT TGCGCGCTCC GTCATAAGAA

                     mnlI
                   haeIII/palI
     mboII       sau96I                                        nlaIII
     bpuAI       asuI                                  tru9I  rcaI
     bbsI      ecoO109I/draII                          mseI   bspHI
2901 GAAGACGAAA GGGCCTCGTG ATACGCCTAT TTTTATAGGT TAATGTCATG
     CTTCTGCTTT CCCGGAGCAC TATGCGGATA AAAATATCCA ATTACAGTAC

                                                                  aciI
                                                                 thaI
                                                                 fnuDII/mvnI
                         hinII/acyI                              bstUI
                         ahaII/bsaHI                             bsh1236I
                         aatII                                  hinPI
                     ddeI maeII                                 hhaI/cfoI
2951 ATAATAATGG TTTCTTAGAC GTCAGGTGGC ACTTTTCGGG GAAATGTGCG
     TATTATTACC AAAGAATCTG CAGTCCACCG TGAAAAGCCC CTTTACACGC
```

FIG. 16S

```
                                                                                bsmAI
                                                                            rcaI
                                                                        bsrBI nlaIII
      nlaIV                                                             aciI bspHI
3001 CGGAACCCCT ATTTGTTTAT TTTTCTAAAT ACATTCAAAT ATGTATCCGC TCATGAGACA ATAACCCTGA
     GCCTTGGGGA TAAACAAATA AAAAGATTTA TGTAAGTTTA TACATAGGCG AGTACTCTGT TATTGGGACT

                                 mboII
                   sspI          earI/ksp632I
3071 TAAATGCTTC AATAATATTG AAAAAGGAAG AGTATGAGTA TTCAACATTT CCGTGTCGCC CTTATTCCCT
     ATTTACGAAG TTATTATAAC TTTTTCCTTC TCATACTCAT AAGTTGTAAA GGCACAGCGG GAATAAGGGA

          fnu4HI
          aciI                                hphI            hphI          sfaNI
3141 TTTTTGCGGC ATTTTGCCTT CCTGTTTTTG CTCACCCAGA AACGCTGGTG AAAGTAAAAG
     AAAAACGCCG TAAAACGGAA GGACAAAAAC GAGTGGGTCT TTGCGACCAC TTTCATTTTC

                          hgiAI/aspHI
                          bsp1286                                sau3AI
              sau3AI      bsiHKAI                                mboI/ndeII[dam-]
              mboI/ndeII[dam-]                                   dpnI[dam+]
              dpnI[dam+] bmyI                                    dpnII[dam-]
              dpnII[dam-]                                       bstYI/xhoII
          mboII[dam-]    apaLI/snoI          taqI    alwI[dam-]  aciI
        eco57I           alw44I/snoI   maeIII     bsrI          nspBII
3201 ATGCTGAAGA TCAGTTGGGT GCACGAGTGG GTTACATCGA ACTGGATCTC AACAGCGGTA
     TACGACTTCT AGTCAACCCA CGTGCTCACC CAATGTAGCT TGACCTAGAG TTGTCGCCAT
```

FIG. 16T

```
          sau3AI
          mboI/ndeII[dam-]                maeII
          dpnI[dam+]                     psp1406I          hgiAI/aspHI
          dpnII[dam-]                   xmnI               bsp1286  tru9I
          alwI[dam-]                    asp700             bsiHKAI  mseI
        bstYI/xhoII                   mboII               bmyI    ahaIII/draI
3261 AGATCCTTGA GAGTTTTCGC CCCGAAGAAC GTTTTCCAAT GATGAGCACT TTTAAAGTTC
     TCTAGGAACT CTCAAAAGCG GGGCTTCTTG CAAAAGGTTA CTACTCGTGA AAATTTCAAG

                                                 scrFI
                    aciI                         nciI
                  thaI                           mspI
                  fnuDII/mvnI                    hpaII
                  bstUI                          dsaV
                  bsh1236I                    hinlI/acyI
                hinPI                         hgaI cauII                    aciI
                hhaI/cfoI                     ahaII/bsaHI     bcgI  mcrI  fnu4HI
3321 TGCTATGTGG CGCGGTATTA TCCCGTGATG ACGCCGGGCA AGAGCAACTC GGTCGCCGCA
     ACGATACACC GCGCCATAAT AGGGCACTAC TGCGGCCCGT TCTCGTTGAG CCAGCGGCGT

                                      rsaI
                                      csp6I   bsrI
                ddeI                 scaI  hphI  maeIII      sfaNI     fokI
3381 TACACTATTC TCAGAATGAC TTGGTTGAGT ACTCACCAGT CACAGAAAAG CATCTTACGG
     ATGTGATAAG AGTCTTACTG AACCAACTCA TGAGTGGTCA GTGTCTTTTC GTAGAATGCC
```

FIG. 16U

```
                                                                                  haeIII/palI
                                                                                 eaeI
                                                                                 cfrI
                                          fnu4HI                                fnu4HI
          nlaIII                          bbvI          nlaIII                  aciI
3441 ATGGCATGAC AGTAAGAGAA TTATGCAGTG CTGCCATAAC CATGAGTGAT AACACTGCGG CCAACTTACT
     TACCGTACTG TCATTCTCTT AATACGTCAC GACGGTATTG GTACTCACTA TTGTGACGCC GGTTGAATGA

                        sau96I
                        avaII
                sau3AI  asuI                                                  nlaIII
                mboI/ndeII[dam-]                                            sau3AI maeIII
                dpnI[dam+]                                                  mboI/ndeII[dam-]
                dpnII[dam-]                                                 dpnI[dam+]
               pvuI/bspCI                                                   dpnII[dam-]
               mcrI   mnlI          aluI   aciI                 nlaIII alwI[dam-]
3511 TCTGACAACG ATCGGAGGAC CGAAGGAGCT AACCGCTTTT TTGCACAACA TGGGGGATCA TGTAACTCGC
     AGACTGTTGC TAGCCTCCTG GCTTCCTCGA TTGGCGAAAA AACGTGTTGT ACCCCCTAGT ACATTGAGCG

                       mspI
         sau3AI   nlaIV
         mboI/ndeII[dam-] aluI
         dpnI[dam+]   hpaII                                                      fnu4HI
         dpnII[dam-] bsaWI                                   maeIII     sfaNI   bbvI
3581 CTTGATCGTT GGGAACCGGA GCTGAATGAA GCCATACCAA ACGACGAGCG TGACACCACG ATGCCAGCAG
     GAACTAGCAA CCCTTGGCCT CGACTTACTT CGGTATGGTT TGCTGCTCGC ACTGTGGTGC TACGGTCGTC
```

FIG. 16V

```
                    hinPI                                          mspI
                    hhaI/cfoI                                      hpaII
                   mstI                                           scrFI
                   aviII/fspI     bsrI                     aluI   nciI
               maeII            tru9I                    rmaI    dsaV
              psp1406I          mseI                     maeI    cauII
3651 CAATGGCAAC AACGTTGCGC AAACTATTAA CTGGCGAACT ACTTACTCTA GCTTCCCGGC
     GTTACCGTTG TTGCAACGCG TTTGATAATT GACCGCTTGA TGAATGAGAT CGAAGGGCCG

                                                                bglI
                                                               sau96I
          tru9I      fokI                      sau96I          haeIII/palI
          mseI    bsrI        aciI             avaII      hinPI  asuI    mspI
         aseI/asnI/vspI    mnlI                asuI       hhaI/cfoI      hpaII
3711 AACAATTAAT AGACTGGATG GAGGCGGATA AAGTTGCAGG ACCACTTCTG CGCTCGGCCC TTCCGGCTGG
     TTGTTAATTA TCTGACCTAC CTCCGCCTAT TTCAACGTCC TGGTGAAGAC GCGAGCCGGG AAGGCCGACC

                                mspI                thaI
                                hpaII               fnuDII/mvnI
                               cfr10I               bstUI
                            nlaIV hphI         bsmAI aciI        fnu4HI
                          gsuI/bpmI           bsaI  bsh1236I     bbvI
3781 CTGGTTTATT GCTGATAAAT CTGGAGCCGG TGAGCGTGGG TCTCGCGGTA TCATTGCAGC
     GACCAAATAA CGACTATTTA GACCTCGGCC ACTCGCACCC AGAGCGCCAT AGTAACGTCG
```

FIG. 16W

FIG. 16X

```
          sau96I
          asuI                                                    pleI
         nlaIV                                                    hinfI
      bsrI haeIII/palI       mnlI                          eam1105I
3841  ACTGGGGCCA GATGGTAAGC CCTCCCGTAT CGTAGTTATC TACACGACGG GGAGTCAGGC
      TGACCCCGGT CTACCATTCG GGAGGGCATA GCATCAATAG ATGTGCTGCC CCTCAGTCCG

                                 ddeI
                            sau3AI        nlaIV
                            mboI/ndeII[dam-]
                            dpnI[dam+]    hgiCI          tru9I
           fokI             dpnII[dam-]   banI mnlI      mseI
3901  AACTATGGAT GAACGAAATA GACAGATCGC TGAGATAGGT GCCTCACTGA TTAAGCATTG
      TTGATACCTA CTTGCTTTAT CTGTCTAGCG ACTCTATCCA CGGAGTGACT AATTCGTAAC

                                                      tru9I
                                                      mseI            tru9I
      maeIII                                         ahaIII/draI      mseI
3961  GTAACTGTCA GACCAAGTTT ACTCATATAT ACTTTAGATT GATTTAAAAC TTCATTTTTA
      CATTGACAGT CTGGTTCAAA TGAGTATATA TGAAATCTAA CTAAATTTTG AAGTAAAAAT

                   rmaI       sau3AI
              sau3AI hphI     mboI/ndeII[dam-]
              mboI/ndeII[dam-]
              dpnI[dam+]      dpnI[dam+]
              dpnII[dam-]     dpnII[dam-]
        tru9I bstYI/xhoII     alwI[dam-]          nlaIII              maeII
        mseI  alwI[dam-]    bstYI/xhoII         rcaI               tru9I
       ahaIII/draI maeI   mboII[dam-]          bspHI              mseI
4021  ATTTAAAAGG ATCTAGGTGA AGATCCTTTT TGATAATCTC ATGACCAAAA TCCCTTAACG TGAGTTTTCG
      TAAATTTTCC TAGATCCACT TCTAGGAAAA ACTATTAGAG TACTGGTTTT AGGGAATTGC ACTCAAAAGC
```

FIG. 16Y

```
                                                  sau3AI
                                                  mboI/ndeII[dam-]
                                                  dpnI[dam+]  sau3AI
                                                  dpnII[dam-] mboI/ndeII[dam-]
                                                 bstYI/xhoII  dpnI[dam+]
                                       sau3AI  alwI[dam-]   dpnII[dam-]
                                       mboI/ndeII[dam-]     alwI[dam-]
                hgaI                   dpnI[dam+] mboII[dam-]
            ddeI                       dpnII[dam-]         bstYI/xhoII
4091 TTCCACTGAG CGTCAGACCC CGTAGAAAAG ATCAAAGGAT CTTCTTGAGA TCCTTTTTTT
     AAGGTGACTC GCAGTCTGGG GCATCTTTTC TAGTTTCCTA GAAGAACTCT AGGAAAAAAA

        thaI
        fnuDII/mvnI
        bstUI
        bsh1236I
       hinPI       fnu4HI                                aciI
       hhaI/cfoI   bbvI                        aciI    nspBII
4151 CTGCGCGTAA TCTGCTGCTT GCAAACAAAA AAACCACCGC TACCAGCGGT GGTTTGTTTG
     GACGCGCATT AGACGACGAA CGTTTGTTTT TTTGGTGGCG ATGGTCGCCA CCAAACAAAC

        sau3AI
        mboI/ndeII[dam-]
        dpnI[dam+]
        dpnII[dam-]
       alwI[dam-]
     mspI                                     bsrI             hinPI
     hpaII      aluI                       maeIII   eco57I     hhaI/cfoI
4211 CCGGATCAAG AGCTACCAAC TCTTTTTCCG AAGGTAACTG GCTTCAGCAG AGCGCAGATA CCAAATACTG
     GGCCTAGTTC TCGATGGTTG AGAAAAAGGC TTCCATTGAC CGAAGTCGTC TCGCGTCTAT GGTTTATGAC
```

```
          rmaI                 haeIII/palI
          maeI      bslI     haeI                    scfI     aciI        mnlI
4281 TCCTTCTAGT GTAGCCGTAG TTAGGCCACC ACTTCAAGAA CTCTGTAGCA CCGCCTACAT ACCTCGCTCT
     AGGAAGATCA CATCGGCATC AATCCGGTGG TGAAGTTCTT GAGACATCGT GGCGGATGTA TGGAGCGAGA

                                                            scrFI
                                                            nciI
                            fnu4HI                          mspI
                   alwNI    bbvI                            hpaII
                  bsrI  fnu4HI                              dsaV    pleI
             maeIII     bbvI    bsrI                        cauII   hinfI
4351 GCTAATCCTG TTACCAGTGG CTGCTGCCAG TGGCGATAAG TCGTGTCTTA CCGGGTTGGA CTCAAGACGA
     CGATTAGGAC AATGGTCACC GACGACGGTC ACCGCTATTC AGCACAGAAT GGCCCAACCT GAGTTCTGCT

                                                      hgiAI/aspHI
                        nspBII                        bsp1286
          mspI         fnu4HI                         bsiHKAI
          hpaII        bbvI mcrI                      bmyI
         bsaWI       hinPI aciI                       apaLI/snoI
      maeIII         hhaI/cfoI                        alw44I/snoI   aluI
4421 TAGTTACCGG ATAAGGCGCA GCGGTCGGGC TGAACGGGGG GTTCGTGCAC ACAGCCCAGC TTGGAGCGAA
     ATCAATGGCC TATTCCGCGT CGCCAGCCCG ACTTGCCCCC CAAGCACGTG TGTCGGGTCG AACCTCGCTT

                                                       hinPI
                                                       hhaI/cfoI
                   ddeI      scfI                     haeII
4491 CGACCTACAC CGAACTGAGA TACCTACAGC GTGAGCATTG AGAAAGCGCC ACGCTTCCCG AAGGGAGAAA
     GCTGGATGTG GCTTGACTCT ATGGATGTCG CACTCGTAAC TCTTTCGCGG TGCGAAGGGC TTCCCTCTTT
```

FIG. 16Z-1

```
                                                                                  scrFI
                                                                                  mvaI
                                                                                  ecoRII   mvaI
                    mspI                                                          dsaV     ecoRII
                    hpaII                                                         bstNI
                    bslI     fnu4HI                          hinPI mnlI           bsaJI
      aciI         bsaWI     aciI                            hhaI/cfoI   aluI apyI[dcm+]
4561 GGCGGACAGG TATCCGGTAA GCGGCAGGGT CGGAACAGGA GAGCGCACGA GGGAGCTTCC AGGGGGAAAC
     CCGCCTGTCC ATAGGCCATT CGCCGTCCCA GCCTTGTCCT CTCGCGTGCT CCCTCGAAGG TCCCCCTTTG

      scrFI


      dsaV
      bstNI                                                          taqI
      apyI[dcm+]                                  mnlI  drdI   hgaI              sfaNI
4631 GCCTGGTATC TTTATAGTCC TGTCGGGTTT CGCCACCTCT GACTTGAGCG TCGATTTTTG TGATGCTCGT
     CGGACCATAG AAATATCAGG ACAGCCCAAA GCGGTGGAGA CTGAACTCGC AGCTAAAAAC ACTACGAGCA

                                                 haeIII/palI
                                                fnu4HI
                                                aciI
                                               thaI bslI
                                               fnuDII/mvnI
                nlaIV                          bstUI
               aciI                            bsh1236I
4701 CAGGGGGGCG GAGCCTATGG AAAAACGCCA GCAACGCGGC
     GTCCCCCCGC CTCGGATACC TTTTTGCGGT CGTTGCGCCG
```

FIG. 16Z-2

SUBSTITUTE SHEET (RULE 26)

```
                        haeIII/palI
                    scrFI
                    mvaI bslI
                    ecoRII
                    dsaV                        nlaIII
                    bstNI                           nspI
                    apyI[dcm+]      haeIII/palI     nspHI                          tfiI
                nlaIV  haeI             haeI        aflIII                         hinfI
4741 CTTTTTACGG TTCCTGGCCT TTTGCTGGCC TTTTGCTCAC ATGTTCTTTC CTGCGTTATC CCCTGATTCT
     GAAAAATGCC AAGGACCGGA AAACGACCGG AAAACGAGTG TACAAGAAAG GACGCAATAG GGGACTAAGA

                                                            fnu4HI
                                                            bbvI
                                                    bsrBI   aciI
                        aciI            aluI        aciI    fnu4HI          mcrI
4811 GTGGATAACC GTATTACCGC CTTTGAGTGA GCTGATACCG CTCGCCGCAG CCGAACGACC
     CACCTATTGG CATAATGGCG GAAACTCACT CGACTATGGC GAGCGGCGTC GGCTTGCTGG

                                                        hinPI
          fnu4HI                                        haeII
          bbvI pleI                                 sapI hhaI/cfoI
        hinPI  hinfI                                mboII                      mnlI
        hhaI/cfoI                   mnlI  aciI      earI/ksp632I             aciI
4871 GAGCGCAGCG AGTCAGTGAG CGAGGAAGCG  GAAGAGCGCC CAATACGCAA ACCGCCTCTC
     CTCGCGTCGC TCAGTCACTC GCTCCTTCGC  CTTCTCGCGG GTTATGCGTT TGGCGGAGAG
```

FIG. 16Z-3

```
          thaI
          fnuDII/mvnI
          bstUI
          bsh1236I
         hinPI
         hhaI/cfoI
        thaI
        fnuDII/mvnI
        bstUI                  tru9I  aluI
        bsh1236I haeIII/palI       pvuII
       bslI    eaeI  tfiI aseI/asnI/vspI
       aciI    cfrI  hinfI  mseI  nspBII                              bsrI     aciI
4931 CCCGCGCGTT GGCCGATTCA TTAATCCAGC TGGCACGACA GGTTTCCCGA CTGGAAAGCG
     GGGCGCGCAA CCGGCTAAGT AATTAGGTCG ACCGTGCTGT CCAAAGGGCT GACCTTTCGC

                                                          scrFI
                                                          mvaI
                                                          ecoRII
                                                          dsaV
                          tru9I                     nlaIV  bstNI
                 hinPI    mseI      maeIII          hgiCI  apyI[dcm+]
                 hhaI/cfoI aseI/asnI/vspI  mnlI     banI bsaJI
4991 GGCAGTGAGC GCAACGCAAT TAATGTGAGT TACCTCACTC ATTAGGCACC CCAGGCTTTA CACTTTATGC
     CCGTCACTCG CGTTGCGTTA ATTACACTCA ATGGAGTGAG TAATCCGTGG GGTCCGAAAT GTGAAATACG
```

FIG. 16Z-4

```
        mspI                                  aciI
        hpaII                             bsrBI                                  aluI      nlaIII
5061 TTCCGGCTCG TATGTTGTGT GGAATTGTGA GCGGATAACA ATTTCACACA GGAAACAGCT ATGACCATGA
     AAGGCCGAGC ATACAACACA CCTTAACACT CGCCTATTGT TAAAGTGTGT CCTTTGTCGA TACTGGTACT

            tru9I
            mseI
           aseI/asnI/vspI
         xmnI
         asp700
5131 TTACGAATTA A
     AATGCTTAAT T

>length: 5141
```

FIG. 16Z-5

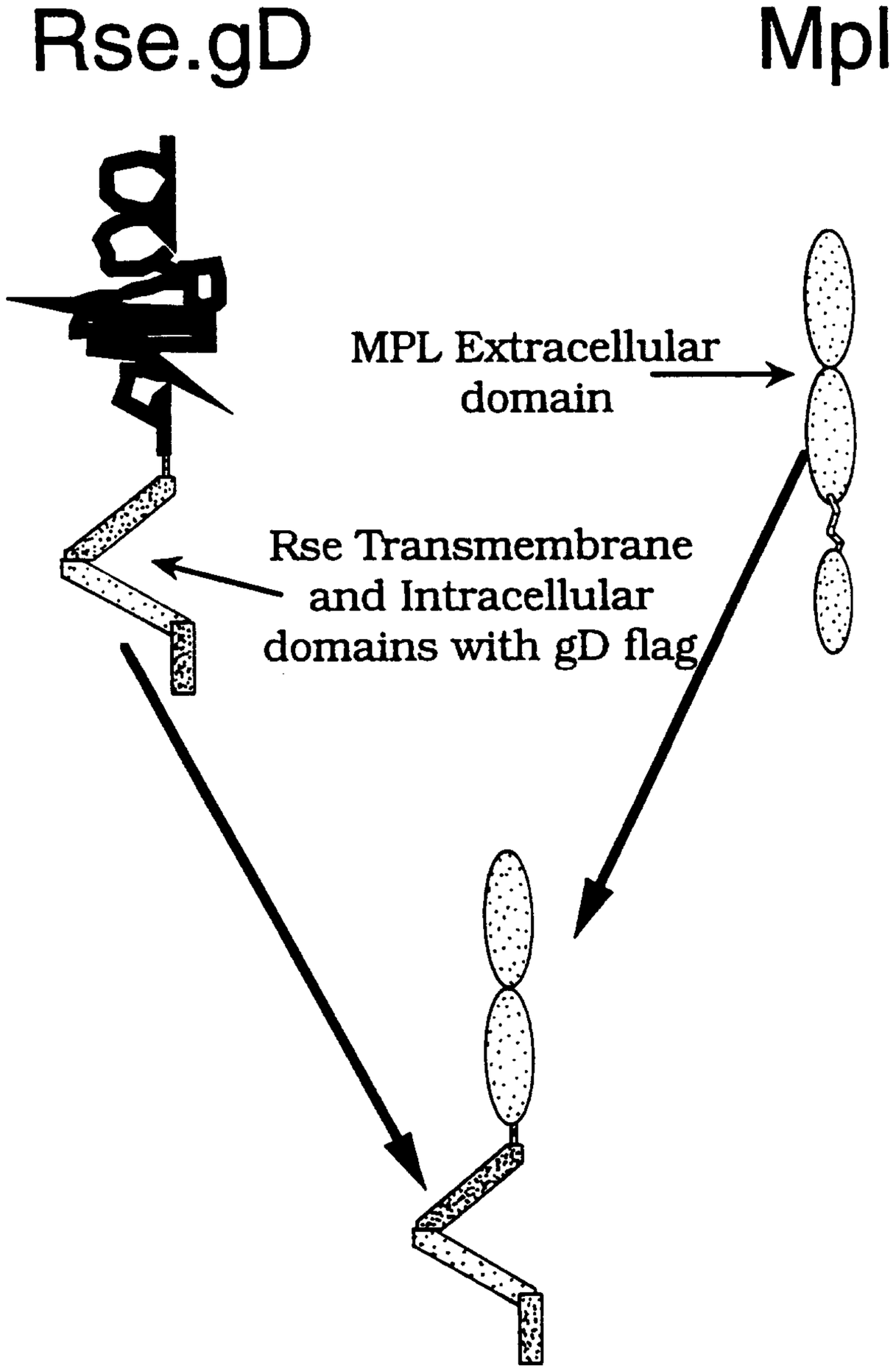

Rse.gD
Mpl
MPL Extracellular domain
Rse Transmembrane and Intracellular domains with gD flag

FIG. 17

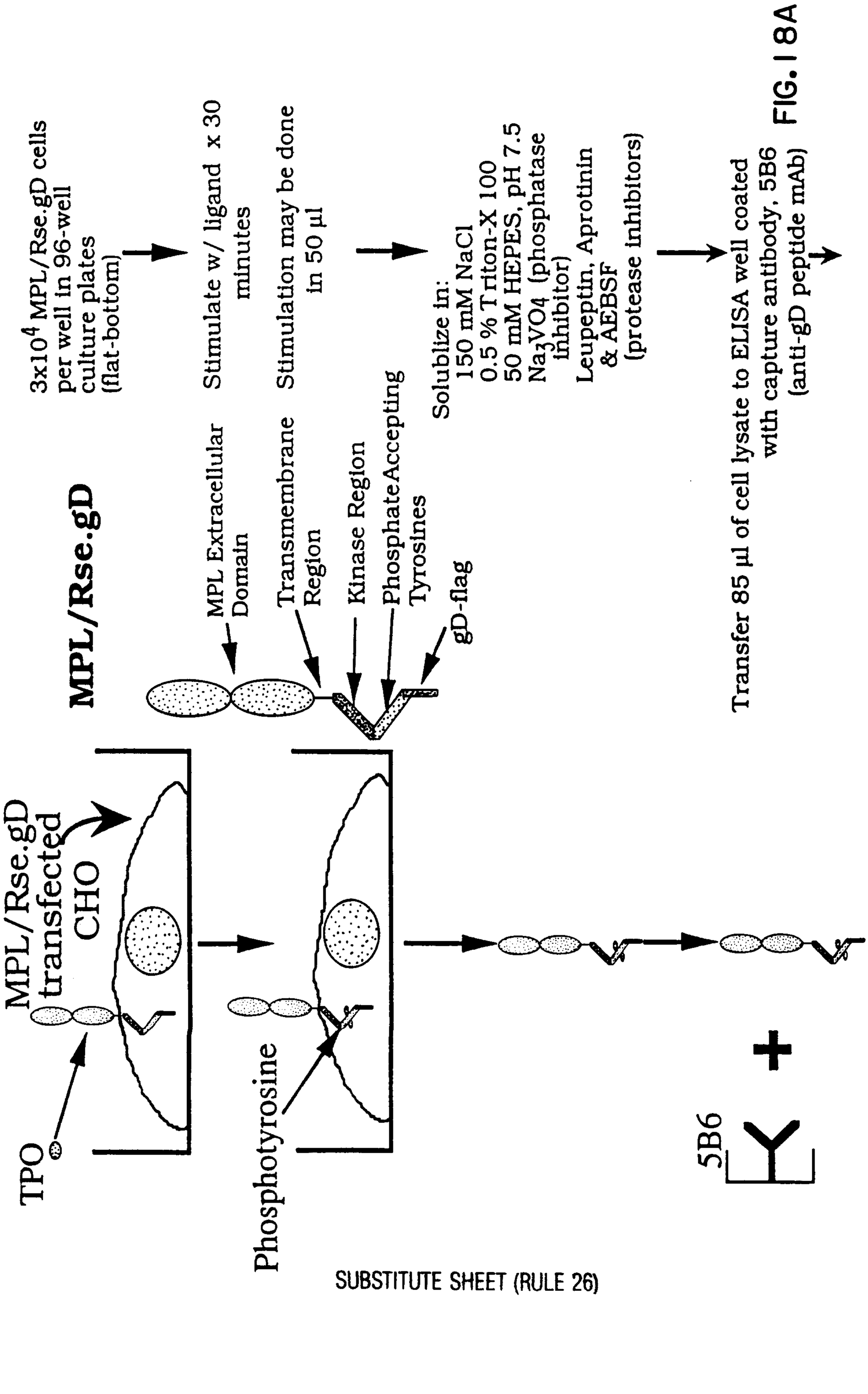

MPL/Rse.gD
MPL/Rse.gD transfected CHO
TPO
Phosphotyrosine
MPL Extracellular Domain
Transmembrane Region
Kinase Region
Phosphate Accepting Tyrosines
gD-flag
3x10^4 MPL/Rse.gD cells per well in 96-well culture plates (flat-bottom)
Stimulate w/ ligand x minutes
Stimulation may be done in 50 µl
Solublize in:
150 mM NaCl
0.5 % Triton-X 100
50 mM HEPES, pH 7.5
Na3VO4 (phosphatase inhibitor)
Leupeptin, Aprotinin & AEBSF
(protease inhibitors)
Transfer 85 µl of cell lysate to ELISA well coated with capture antibody, 5B6 (anti-gD peptide mAb)
5B6
+

FIG. 18A

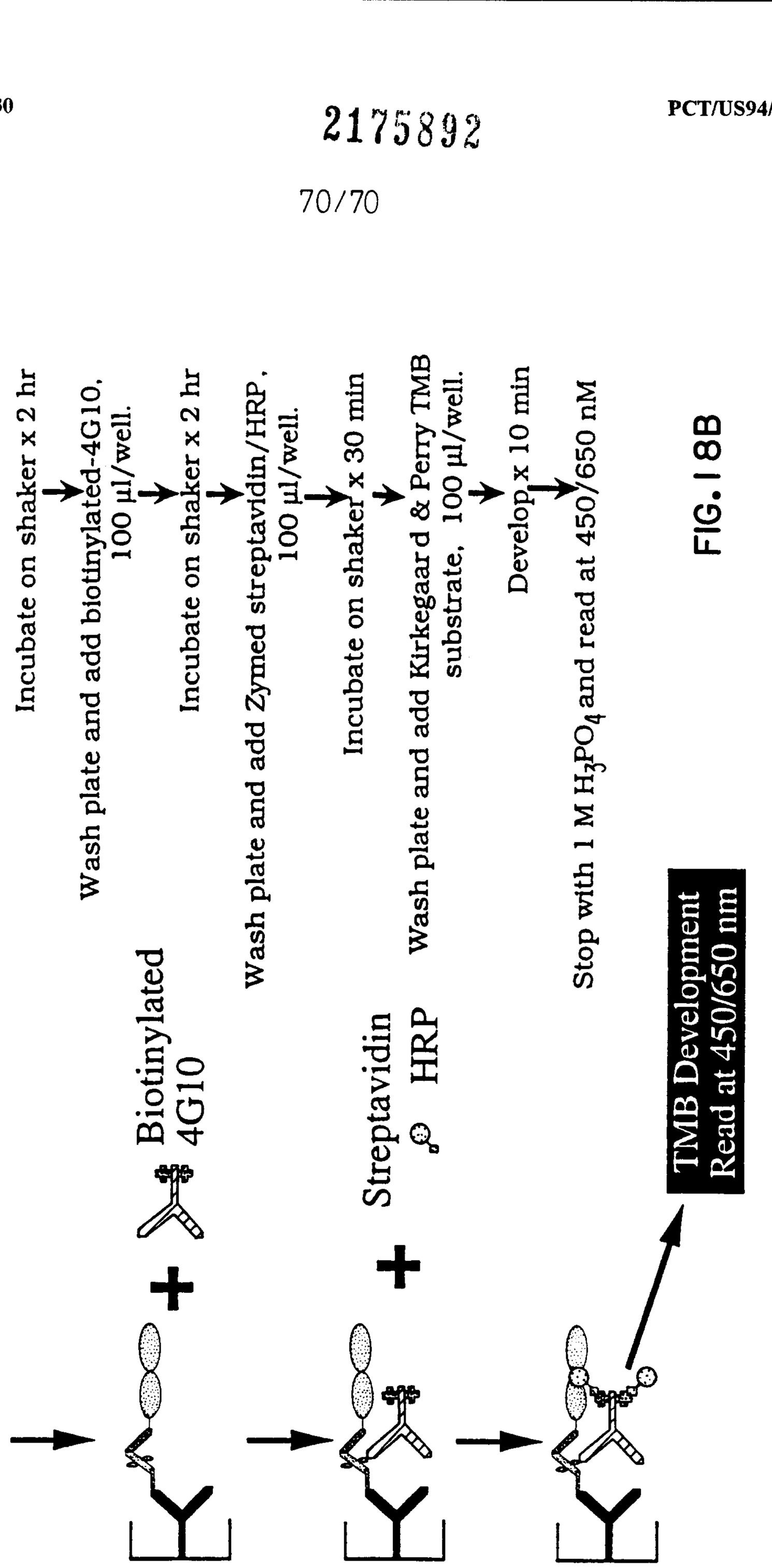

Incubate on shaker x 2 hr
Wash plate and add biotinylated-4G10, 100 µl/well.
Incubate on shaker x 2 hr
Wash plate and add Zymed streptavidin/HRP, 100 µl/well.
Incubate on shaker x 30 min
Wash plate and add Kirkegaard & Perry TMB substrate, 100 µl/well.
Develop x 10 min
Stop with 1 M H_3PO_4 and read at 450/650 nm
Biotinylated 4G10
Streptavidin
HRP
TMB Development
Read at 450/650 nm

FIG. 18B