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# Cell receptor assays*

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**Abstract**: There are four general assay methods used to quantify a drug/biologic in a preparation, including: (1) *in vivo* bioassays; (2) *in vitro* bioassays; (3) immunoassays; and (4) receptor assays. The cell receptor assay is used to evaluate the first step in the molecular action of the drug/biologic, its interaction with a specific cellular receptor. Subsequently, the drug/biologic must initiate other events, such as internalisation, signal transduction, and/or alterations of one or more cellular constituents in order to elicit its biological effect. Major factors to consider in cell receptor assay development include: (1) establishment of a reference standard preparation; (2) labelling; purifying and characterisation of the biologic/drug; (3) cell receptor source; (4) methodology, e.g. separation of bound and free, and other factors affecting accuracy and reproducibility; (5) ligand specificity; and (6) correlation with bioactivity. It should be emphasised that cell receptor binding cannot be assumed to correlate with biological activity because of the requirement that subsequent steps must take place prior to achieving the final response. Chemically altered drugs/biologics may bind to a specific cell receptor without eliciting a biological activity. Thus, utilisation of a cell receptor assay requires careful evaluation at both the chemical and biological levels prior to its acceptance as a measure of potency.

**Keywords**: *Cell receptor assays; bioassays; human interferon-alpha 2; human monocytoid cells; Scatchard analysis; computer analysis.*

## Introduction

There are four general assay methods used to quantify a drug or biological agent in a product. As shown in Fig. 1, they include the bioassay, the structure assay, the immunoassay, and the receptor assay [1]. The bioassay represents an *in vitro* or *in vivo* assay which monitors a bioactivity that is shown to be related to the clinical-pharmacological activity of the product. The structure assay is based on the molecular structure of the active drug substance. The immunoassay quantitates the product based on the specific recognition of antigenic determinants present on the drug or biological agent. The receptor assay, which is the main focus of this report, reflects the ability of the drug or biological agent to interact with a specific cell receptor; this is the first step in the

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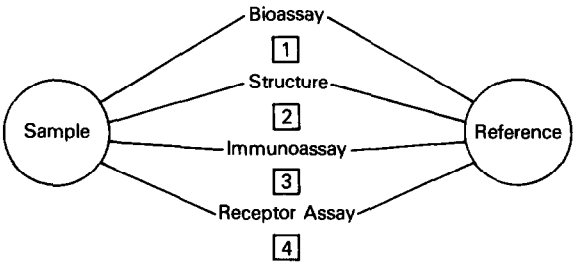


**Figure 1**
Four types of assay methods for biological agents or drugs.

biological action of the molecule. All four assay methods are dependent on the inclusion of a well characterised reference preparation. While all these systems can be used to quantitate a drug or biological agent, the results may differ because of differences in the molecular properties that the different assays measure. Therefore, the choice of an assay system for a biological agent or drug depends on the properties of the molecule itself, e.g. molecular structure and stability, the complexity of the assay system, the relevance of the assay system to the intended clinical use and the availability of a suitable reference preparation.

The action of a biological agent or drug is summarised in Fig. 2. First, the drug or biological agent (ligand) interacts with a specific cellular binding site or receptor. Additional steps following such receptor/ligand interactions can include signal transduction, internalisation, and/or alterations in one or more additional cellular constituents or regulatory pathways, all of which may ultimately contribute to the bioactivity. Thus, although the interaction with a specific cell surface receptor is a critical step in the molecule's action, it alone does not necessarily relate to bioactivity because of the multi-step process involved.

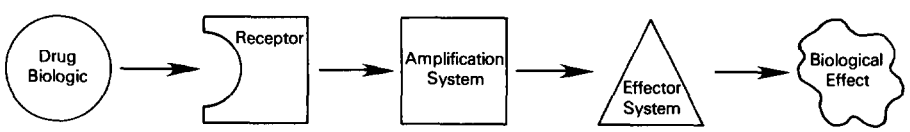


**Figure 2**
The action of a biological agent or drug.

## The development of a cell receptor assay

Table 1 lists the principal considerations for developing a cell receptor assay. The first involves the choice of a cell receptor source. The source may be cells derived from tissue

**Table 1**
Considerations for a cell receptor assay

| |
|---|
| Cell receptor source |
| Preparation, purification and stability of labeled biological agent or drug |
| Methodology |
| Analysis |
| Correlation with bioactivity |

culture, tissue homogenates or slices, or purified receptor preparations. Choice of the appropriate source is generally based on availability of the receptor source, the complexity of the preparation (including absence of cross-reacting receptors, as for example in insulin and insulin-like growth factors [2]), and number and affinity of cell receptors. In addition, cells and tissue should not secrete free receptor or the biological agent or drug in question.

*Labeling of drug or biological agent*

The preparation, purification and stability of the labeled drug or biological agent require careful consideration. Ligands labeled with radioisotopes and, to a lesser extent, fluorescent reporter groups have been used successfully for examining the interaction of many ligands with their respective receptors. A partial list of labels used is shown in Table 2. The biological agent or drug to be labeled should be pure and biologically active. Deleterious effects of the labeling procedure on the biological specific activity should be minimised.

Other factors affecting the choice of labeling procedure for the drug or biological agent include: the chemical and biological stability of the molecule; the required specific radioactivity of the ligand; and the complexity of the procedure itself. Care should be taken not to label the ligand too intensively, as that may damage the molecule. The

**Table 2**
Labeling of drug or biological agent

| Radionuclide | Procedure |
|---|---|
| $^{125}I$ | Bolton–Hunter reagent* |
| | Chloramine T† |
| | Lactoperoxidase/glucose oxidase‡ |
| | Iodo-Gen reagent§ |
| | Iodo-beads iodination reagent‖ |
| | 1,3,4,6-tetrachloro-3d,6α diphenyl glycoluril |
| $^{32}p$ | [γ-$^{32}$p]ATP/cAMP protein** kinase |
| $^{35}S$ | Methionine†† |
| $^{3}H$ | Reductive methylation‡‡ |

*A. E. Bolton and W. M. Hunter, *Biochem. J.* **133**, 529–538 (1973) and Ref. 3.

†F. C. Greenwood, W. M. Hunter and J. S. Glover, *Biochem. J.* **89**, 114–123.

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††H. Kung, in *Methods in Enzymology*, Vol. 119, *Interferons*, Part C (S. Pestka, Ed.), pp. 292–296. Harcourt Brace Jovanovich, New York (1986).

‡‡B. F. Tack, J. Dean, D. Eilat, P. E. Lorenz and A. N. Schechter, *J. biol. Chem.* **255**, 8842–8847 (1980).

choice of the purification scheme adopted for the labeled ligand should be designed to give: (a) efficient removal of excess labeling reagents, and (b) minimum loss of the labeled bioactive drug or biological agent. The procedures vary depending on the chemical properties of the labeled molecule. They can include gel filtration, dialysis, sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and high-performance liquid chromatography. The biological stability of the labeled drug or biological agent, and in the case of radioisotopes, the half-life is also important in evaluating an appropriate procedure for labeling the molecule. Storage conditions (e.g. pH, temperature, concentration of the labeled ligand and the addition of carrier protein) should be carefully evaluated to maximise the chemical and biological activity of the labeled ligand.

To exemplify this approach, Fig. 3 shows an autoradiogram of $^{125}$I-interferon alpha-2b. Human interferon alpha-2b (gift of Schering Corporation, Kenilworth, NJ, USA) was radiolabeled with $^{125}$I-Bolton–Hunter reagent [3]. The radiolabeled ligand was purified using gel filtration, dialysis and SDS-PAGE. Essentially no loss of anti viral activity was observed upon iodination and recovery from the procedure was approximately 40%. The radiospecific activity was in the range of 4–12 $\mu$Ci $\mu g^{-1}$ protein, with greater than 95% of the radiolabeled ligand being trichloroacetic acid precipitable. Bovine serum albumin (1 mg $ml^{-1}$) was used as a carrier protein, and the radiolabeled preparation was stored in liquid nitrogen. The ligand preparation could be used in binding experiments for one to two months.

*Methodology*

Once a labeled ligand has been prepared and characterised, it is then appropriate to optimise binding conditions. This stage usually includes the selection of incubation buffer (with assessment of pH, ion dependency, e.g. $Ca^{2+}$, $Mg^{2+}$, carrier protein, and inhibitors of ligand degradation), temperature and duration of assay.

At temperatures greater than 0–4°C, internalisation (in the case of whole cells) and the action of protease can greatly affect the assessment of the binding parameters [4]. Other factors affecting the accuracy and precision of the procedure include: the preparation of the cells, tissue and receptors; the maintenance of constant temperature throughout; periodic mixing; and the method used to harvest the receptor bound radioactivity.

The separation of bound versus free radiolabeled ligand can be accomplished by a variety of techniques including centrifugation, dialysis, absorption, column chromatography, polyethylene glycol, filtration and in the case of adherent cells, aspiration. The choice should be based on recovery, background binding and dissociation rate of the ligand.

It is also important to establish the kinetics of binding for each set of incubation conditions. As shown in Fig. 4, maximum binding of $^{125}$I-interferon alpha-2b to the human monocytoid cell line, U-937, occurs at 1.5 h at 0–4°C in RPMI 1640 supplemented with 10% fetal calf serum (complete medium). Bound and free $^{125}$I-interferon alpha-2b were separated by centrifugation (microfuge, 15,000 rpm for 2 min and washed twice with complete medium).

Detection of the radiolabeled drug and biological agent can be accomplished using liquid scintillation ($^{3}$H, $^{32}$P, $^{35}$S) or solid scintillation (gamma) counting ($^{125}$I). It is also important to utilise or establish a standard receptor reference preparation whenever possible for use in the binding assay.

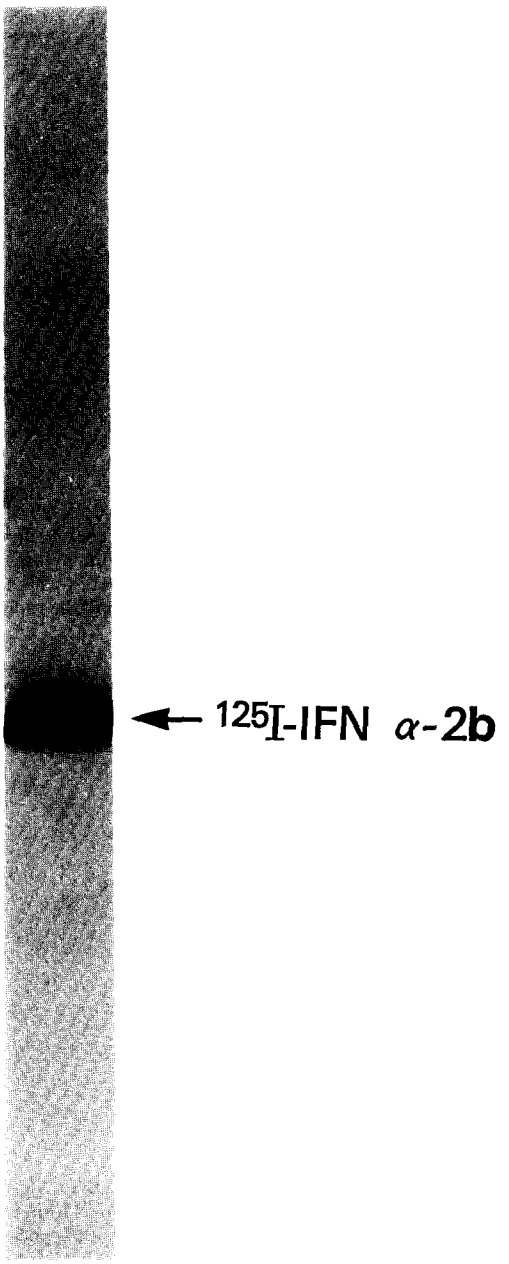


**Figure 3**
Autoradiogram of $^{125}$I-interferon alpha-2b.

*Quality control*

The receptor–ligand interaction should be characterised with respect to saturability and specificity. As shown in Fig. 5 using a fixed number of U-937 cells ($1 \times 10^7$ cells) per point, as one increases the concentration of radiolabeled ligand, one achieves saturation of binding of the radiolabeled interferon. Saturation occurs at 1.5 nM human $^{125}$I-interferon alpha-2b. Specificity can be ascertained by analysing the displacement of labeled ligand by unlabeled ligand. Specific binding is usually defined as total binding

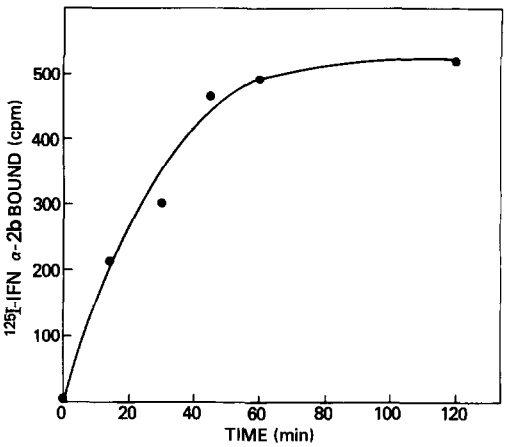


**Figure 4**
Kinetics of binding human $^{125}$I-interferon alpha-2b to the human monocytoid cell line, U-937. U-937 cells at $9 \times 10^6$ cells $ml^{-1}$ (24 well plate, 1 ml cells per well) were incubated at 0°C with 0.9 nM $^{125}$I-interferon alpha-2b in the presence and absence of 86 nM human interferon alpha-2b for the indicated times. The mixture was centrifuged 15,000 **g** at 4°C and the cells were washed twice with ice cold complete medium. The cells were then resuspended in 100 μl complete medium and the radioactivity was determined. Assays were performed in triplicate.

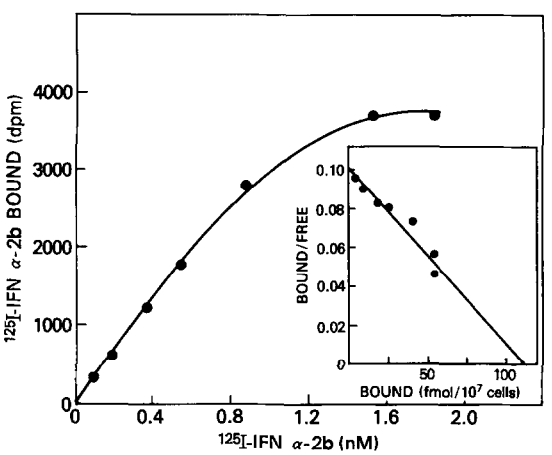


**Figure 5**
Specific binding of $^{125}$I-interferon alpha-2b to U-937 cells as a function of $^{125}$I-interferon alpha-2b concentration. Increasing concentrations of $^{125}$I-interferon alpha-2b were added to U-937 cells ($1 \times 10^7$ cells) in the presence and absence of a 100-fold molar excess of interferon alpha-2b. Samples were processed as described in Fig. 4. Assays were performed in triplicate. Inset: Scatchard plot of binding data.

minus non-saturable binding. However, specific binding and saturability cannot be equated with receptor identity, for the labeled drug or biological agent may bind to proteins or other constituents rather than to the receptor, and these may be effectively competed with by unlabeled ligand. Specificity can also be evaluated by: (a) competition with related ligands; (b) chemically cross-linking the radiolabeled ligand bound to its receptor and analysing the molecular weight of the complex by SDS-PAGE and autoradiography; or (c) by blocking ligand binding with an anti-receptor antibody. In

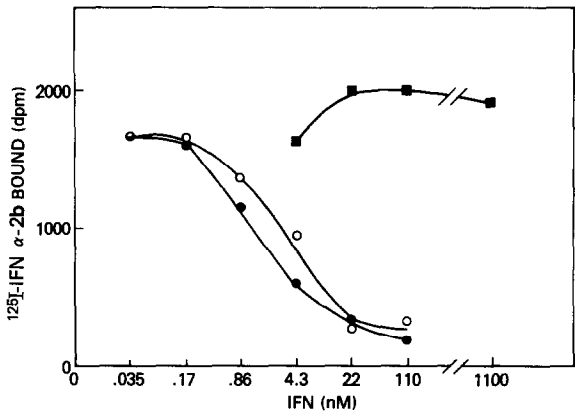


**Figure 6**
Specificity of the interaction of $^{125}$I-interferon alpha-2b with U-937 cells. $^{125}$I-interferon alpha-2b (80 pM) was added simultaneously with the indicated concentrations of human interferon alpha-2b(●), human interferon beta (Serono Laboratories, Rehovot, Israel)(○) and human interferon gamma (Genentech, Inc., S. San Francisco, CA)(■) to U-937 cells at 0°C. The incubation mixture was processed as described in Fig. 4. Assays were performed in triplicate.

Fig. 6, specificity of $^{125}$I-interferon alpha-2b with its binding site on U-937 cells is suggested by the demonstration that human interferons alpha-2b and beta, but not interferon-gamma inhibit the binding of the radiolabeled ligand to the cells.

### Analyses of receptor assay data

Receptor binding data can be evaluated by a number of graphical methods including the Scatchard Plot [5], the Hill Plot [6], and the Lineweaver–Burke Plot [7], and a number of other computer methods, e.g. LIGAND PROGRAM [8]. All these methods provide estimates of the key binding parameters, i.e. the dissociation constant(s) ($K_D$) and the number of receptors per cell. However, the computer analysis of ligand binding data provides greater flexibility and objectivity in establishing non-saturable binding, and in comparing the fit of the data in multiple model systems. An example of a Scatchard Analysis of $^{125}$I-interferon alpha-2b bound to U-937 cells is shown in Fig. 5. There appears to be a single class of high affinity binding sites ($K_D = 1.1 \times 10^{-10}$M) whose receptor number per cell is low, 6800 receptors per cell.

### Correlation of cell receptor assay with bioactivity

It is important to emphasise again that cell receptor binding cannot be assumed to correlate with biological activity, because of the multistep process involved leading to biological activity following the interaction of the drug and biological agent with the receptor. It is also important to point out that metabolism and distribution volumes are not accounted for in a receptor assay, as they are in an *in vivo* bioassay (e.g. pro-ACTH exhibits little or no receptor activity but does exhibit *in vivo* bioactivity because of conversion to ACTH). Therefore, to consider adopting a cell receptor assay in lieu of a bioassay, one must be concerned with establishing: the correlation of the cell receptor assay with bioactivity; the specificity of the assay; and the availability of a well

characterized reference preparation. With respect to the validation process, the relevance of the receptor assay system to the bioassay must be established. In addition, a correlation in the dose–response relationship and the stability of the drug or biological agent should be demonstrated between the cell receptor assay and the bioassay. Finally, any alterations induced in the material by heat, chemicals and/or enzymatic digestion should yield similar alterations of activity in both assay systems. The specificity of the receptor assay for the designated drug or biological agent should be verified. Extremely valuable for any assay system is again the availability and utilisation of a well-characterised reference preparation. It should be pointed out that it may not be possible to substitute a cell receptor assay for bioassay on a one-to-one basis. A combination of tests may be required, in addition to the cell receptor assay to establish a valid estimate of the amount of active drug or biological agent in a preparation. Finally, the amount of data to support the estimate of potency needs to be assessed on a case-by-case basis.

In conclusion, many factors affect the accuracy and precision of a cell receptor assay. A careful scientific approach is clearly important in the assessment of the ability of the cell receptor assay as a measure of the amount of active biological agent or drug.

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