

The GraphPad

Guide to

Analyzing Radioligand Binding Data

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Contents

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Preface

A radioligand is a radioactively labeled drug that can associate with a receptor, transporter, enzyme, or any protein of interest. Measuring the rate and extent of binding provides information on the number of binding sites, and their affinity and accessibility for various drugs. Radioligand binding experiments are easy to perform, and provide useful data in many fields. But many find it difficult to analyze radioligand binding data. That's why I wrote this booklet.

If you want more detailed information beyond the scope of this booklet, consult one of these general references:

- LE Limbird, Cell surface receptors: A short course on theory and methods, Kluwer Academic Publishers, second edition, 1996.
- HI Yamamura, et. al. Methods in neurotransmitter receptor analysis, Raven Press, 1990.
- T Kenakin, Pharmacologic analysis of drug-receptor interaction, second edition, Raven Press, 1993.

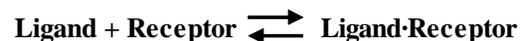
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The law of mass action

Most analyses of radioligand binding experiments are based on a simple model, called the law of mass action:



The model is based on these simple ideas:

- Binding occurs when ligand and receptor collide due to diffusion, and when the collision has the correct orientation and enough energy. The rate of association (number of binding events per unit of time) equals $[\text{Ligand}] \cdot [\text{Receptor}] \cdot k_{\text{on}}$, where k_{on} is the association rate constant in units of $\text{M}^{-1}\text{min}^{-1}$.
- Once binding has occurred, the ligand and receptor remain bound together for a random amount of time influenced by the affinity of the receptor and ligand for one another. The rate of dissociation (number of dissociation events per unit time) equals $[\text{ligand}\cdot\text{receptor}] \cdot k_{\text{off}}$, where k_{off} is the dissociation rate constant expressed in units of min^{-1} .
- After dissociation, the ligand and receptor are the same as they were before binding.
- Equilibrium is reached when the rate at which new ligand-receptor complexes are formed equals the rate at which the ligand-receptor complexes dissociate.

At equilibrium, ligand-receptor complexes form at the same rate that they dissociate:

$$[\text{Ligand}] \cdot [\text{Receptor}] \cdot k_{\text{on}} = [\text{Ligand}\cdot\text{Receptor}] \cdot k_{\text{off}}$$

Rearrange to define the equilibrium dissociation constant K_d .

$$\frac{[\text{Ligand}] \cdot [\text{Receptor}]}{[\text{Ligand}\cdot\text{Receptor}]} = \frac{k_{\text{off}}}{k_{\text{on}}} = K_d$$

The K_d , expressed in units of moles/liter or molar, is the concentration of ligand which occupies half of the receptors at equilibrium. A small K_d means that the receptor has a high affinity for the ligand. A large K_d means that the receptor has a low affinity for the ligand. Don't mix up K_d , the equilibrium dissociation constant, with k_{off} , the dissociation rate constant. They are not the same, and aren't even expressed in the same units.

The law of mass action predicts the fractional receptor occupancy at equilibrium as a function of ligand concentration. Fractional occupancy is the fraction of all receptors that are bound to ligand.

$$\text{Fractional Occupancy} = \frac{[\text{Ligand} \cdot \text{Receptor}]}{[\text{Receptor}]_{\text{total}}} = \frac{[\text{Ligand} \cdot \text{Receptor}]}{[\text{Receptor}] + [\text{Ligand} \cdot \text{Receptor}]}$$

A bit of algebra creates a useful equation. Multiply both numerator and denominator by [Ligand] and divide both by [Ligand · Receptor]. Then substitute the definition of K_d .

$$\text{Fractional occupancy} = \frac{[\text{Ligand}]}{[\text{Ligand}] + K_d}$$

When [Ligand]=0, the occupancy equals zero. When [Ligand] is very high (many times K_d), the fractional occupancy approaches 1.00. When [Ligand]= K_d , fractional occupancy is 0.50. The approach to saturation as [ligand] increases is slower than many people appreciate. When the ligand concentration equals four times its K_d , it will only occupy 80% of the receptors at equilibrium. The occupancy rises to 90% when the ligand concentration equals 9 times the K_d . It takes a concentration equal to 99 times the K_d to occupy 99% of the receptors at equilibrium.

Assumptions inherent in the law of mass action

Although termed a “law”, the law of mass action is simply a model based on these assumptions:

- All receptors are equally accessible to ligands.
- All receptors are either free or bound to ligand. The model ignores any states of partial binding.
- Neither ligand nor receptor are altered by binding.
- Binding is reversible.

If these assumptions are not met, you have two choices. One choice is to develop a more complicated model, beyond the scope of this booklet. The other choice is to analyze your data in the usual way, but interpret the results as an empirical description of the data without attributing rigorous thermodynamic meaning to the K_d values and rate constants.

Saturation binding experiments

Saturation binding experiments measure specific binding at equilibrium at various concentrations (often 6-12) of the radioligand to determine receptor number and affinity. Because this kind of experiment can be graphed as a Scatchard plot (more accurately attributed to Rosenthal), they are sometimes called “Scatchard experiments”.

The analyses depend on the assumption that the incubation has reached equilibrium. This can take anywhere from a few minutes to many hours, depending on the ligand, receptor, temperature, and other experimental conditions. Since lower concentrations of radioligand take longer to equilibrate, use a low concentration of radioligand (perhaps 10-20% of the K_d) when measuring how long it takes the incubation to reach equilibrium.

Nonspecific binding

In addition to binding to the receptors of physiological interest, radioligands bind to nonreceptor sites. When performing radioligand binding experiments, you need to measure both total and nonspecific binding, and calculate specific (receptor) binding as the difference.

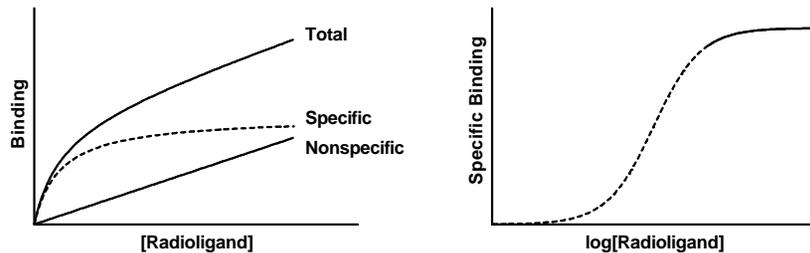
Assess nonspecific binding by measuring radioligand binding in the presence of a concentration of an unlabeled compound that binds to essentially all the receptors. Since all the receptors are occupied by the unlabeled drug, the radioligand only binds nonspecifically.

Which unlabeled drug should you use? The obvious answer is to use the same compound as the radioligand, but unlabeled. In many cases, this is necessary as no other drug is known to bind to the receptors. But most investigators avoid using the same compound as the hot and cold ligand for routine work, and prefer to define nonspecific binding with a standard drug that is known to bind to that particular receptor.

What concentration of unlabeled drug should you use? You want to use enough to block virtually all the specific radioligand binding, but not so much that you cause more general physical changes to the membrane that might alter specific binding. If you are studying a well-characterized receptor, a useful rule-of-thumb is to use the unlabeled compound at a concentration equal to 100 times its K_d for the receptors.

Ideally, you should get the same results defining nonspecific binding with a range of concentrations of several drugs.

Nonspecific binding is generally proportional to the concentration of radioligand (within the range it is used). The left figure shows total and nonspecific binding. The dotted curve shows the difference between total and nonspecific binding – the specific binding.



The right panel shows the specific binding again on a graph with a logarithmic X axis. Notice that the saturation binding curve plotted on a log axis looks like the familiar sigmoidal dose-response curve. The dotted curves in the two panels represent the same range of radioligand concentrations. The solid portion of the curve on the right shows binding at higher radioligand concentrations. These high concentrations are rarely used because radioligands are expensive and nonspecific binding would be too high a fraction of total binding.

Fitting a curve to determine B_{max} and K_d

Equilibrium specific binding at a particular radioligand concentration equals fractional occupancy times the total receptor number (B_{max}):

$$\text{Specific Binding} = \text{Fractional Occupancy} \cdot B_{\max} = \frac{B_{\max} \cdot [L]}{K_d + [L]}$$

This equation describes a rectangular hyperbola or a binding isotherm. [L] is the concentration of free radioligand, the value plotted on the X axis. B_{max} is the total number of receptors expressed in the same units as the Y values (i.e., cpm, sites/cell or fmol/mg protein) and K_d is the equilibrium dissociation constant (expressed in the same units as [L], usually nM). Typical values might be a B_{max} of 10-1000 fmol binding sites per milligram of protein and a K_d between 10 pM and 100 nM.

To determine the B_{max} and K_d, fit data to the equation using nonlinear regression.

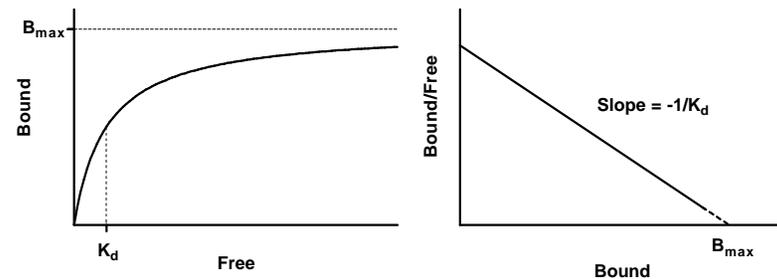
This analysis is based on these assumptions:

- Binding follows the law of mass action and has equilibrated.

- There is only one population of receptors.
- Only a small fraction of the radioligand binds so that the free concentration is essentially identical to the concentration added.
- There is no cooperativity. Binding of a ligand to one binding site does not alter the affinity of another binding site. In other words, the K_d is constant during the experiment.

Scatchard plots

Before nonlinear regression programs were widely available, scientists transformed data to make a linear graph, and then analyzed the transformed data with linear regression. There are several ways to linearize binding data, but Scatchard plots (more accurately attributed to Rose nthal) are used most often. In this plot, the X axis is specific binding (usually labeled "bound") and the Y axis is the ratio of specific binding to concentration of free radioligand (usually labeled "bound/free"). B_{max} is the X intercept; K_d is the negative reciprocal of the slope.



When making a Scatchard plot, you have to choose units for the Y axis. One choice is to express both free ligand and specific binding in cpm so the ratio bound/free is a unitless fraction. The advantage of this choice is that you can interpret Y values as the fraction of radioligand bound to receptors. If the highest Y value is large (greater than 0.10), then the free concentration will be substantially less than the added concentration of radioligand, and the standard analyses won't work. You should either revise your experimental protocol or use special analysis methods that deal with ligand depletion (see page 15). The disadvantage is that you cannot interpret the slope of the line without performing unit conversions.

An alternative is to express the Y axis as sites/cell/nM or fmol/mg/nM. While these values are hard to interpret, they simplify calculation of the

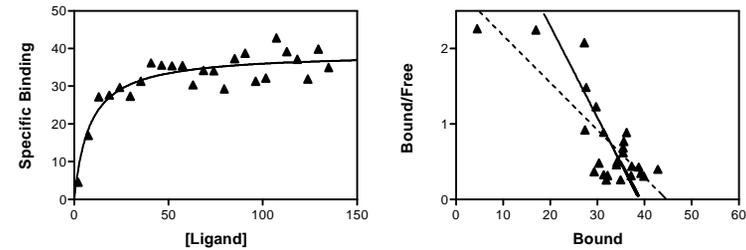
K_d which equals the reciprocal of the slope. The specific binding units cancel when you calculate the slope. The negative reciprocal of the slope is expressed in units of concentration (nM) which equals the K_d .

Why you shouldn't analyze data with Scatchard plots

While Scatchard plots are very useful for visualizing data, they are not the most accurate way to analyze data. The problem is that the linear transformation distorts the experimental error. Linear regression assumes that the scatter of points around the line follows a Gaussian distribution and that the standard deviation is the same at every value of X . These assumptions are not true with the transformed data. A second problem is that the Scatchard transformation alters the relationship between X and Y . The value of X (bound) is used to calculate Y (bound/free), and this violates the assumptions of linear regression.

Since the assumptions of linear regression are violated, the B_{max} and K_d you determine by linear regression of Scatchard transformed data are likely to be further from their true values than the B_{max} and K_d determined by nonlinear regression. Considering all the time and effort you put into collecting data, you want to use the best possible analysis technique. Nonlinear regression produces the most accurate results. Scatchard plots produce approximate results.

The figure below shows the problem of transforming data. The left panel shows data that follows a rectangular hyperbola (binding isotherm). The right panel is a Scatchard plot of the same data. The solid curve on the left was determined by nonlinear regression. The solid line on the right shows how that same curve would look after a Scatchard transformation. The dotted line shows the linear regression fit of the transformed data. The transformation amplified and distorted the scatter, and thus the linear regression fit does not yield the most accurate values for B_{max} and K_d . In this example, the B_{max} determined by the Scatchard plot is about 25% too large and the K_d determined by the Scatchard plot is too high. The errors could just as easily have gone in the other direction.



The experiment in the figure was designed to determine the B_{max} and the experimenter didn't care too much about the value of the K_d . So it was appropriate to obtain only a few data points at the beginning of the curve and many in the plateau region. Note however how the Scatchard transformation gives undo weight to the data point collected at the lowest concentration of radioligand (the lower left point in the left panel, the upper left point in the right panel). This point dominates the linear regression calculations on the Scatchard graph. It has "pulled" the regression line to become shallower, resulting in an overestimate of the B_{max} .

Although it is inappropriate to analyze data by performing linear regression on a Scatchard plot, it is often helpful to display data as a Scatchard plot. Many people find it easier to visually interpret Scatchard plots than binding curves, especially when comparing results from different experimental treatments.

Competitive binding experiments

What is a competitive binding curve

Competitive binding experiments measure the binding of a single concentration of labeled ligand in the presence of various concentrations of unlabeled ligand.

Competitive binding experiments are used to:

- Validate an assay. Compete with drugs whose potencies are known from functional experiments. Demonstrating that these drugs bind with the expected potencies, or at least the expected order of potency, helps prove that your radioligand has identified the correct receptor. This kind of experiment is crucial, because there is usually no point studying a binding site unless it has physiological significance.
- Determine whether a drug binds to the receptor. You can screen thousands of compounds to find drugs that bind to the receptor. This can be faster and easier than other screening methods.
- Investigate the interaction of low affinity drugs with receptors. Binding assays are only useful when the radioligand has a high affinity ($K_d < 100$ nM or so). A radioligand with low affinity generally has a fast dissociation rate constant, and so won't stay bound to the receptor while you wash the filters. If you want to study the binding of a low affinity drug, use it as an unlabeled competitor.
- Determine receptor number and affinity by using the same compound as the labeled and unlabeled ligand. See "Homologous competitive binding curves" page 9.

Performing the experiment

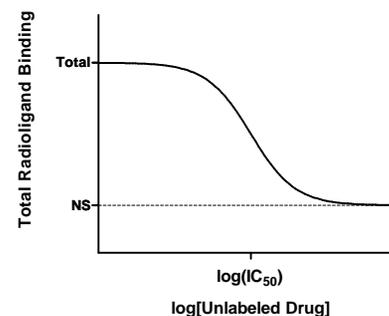
The experiment is done with a single concentration of radioligand. How much should you use? There is no clear answer. Higher concentrations of radioligand are more expensive and result in higher nonspecific binding, but also result in higher numbers of cpm bound and thus lower counting error. Lower concentrations save money and reduce nonspecific binding, but result in fewer counts of specific binding and thus more counting error. Many investigators choose a concentration

approximately equal to about the K_d of the radioligand for binding to the receptor, but this is not universal.

You need to let the incubation occur until equilibrium has been reached. How long does that take? Your first thought might be: "as long as it takes the radioligand to reach equilibrium in the absence of competitor." It turns out that this may not be long enough. You should incubate for 4-5 times the half-life for receptor dissociation as determined in an off-rate experiment (see page 10).

Typically, investigators use 12-24 concentrations of unlabeled compound spanning about six orders of magnitude.

Analyzing competitive binding data



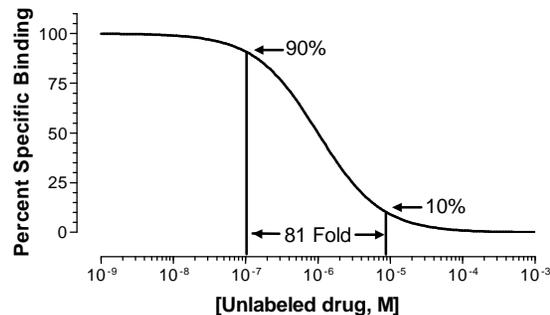
The top of the curve is a plateau at a value equal to radioligand binding in the absence of the competing unlabeled drug. This is total binding. The bottom of the curve is a plateau equal to nonspecific binding (NS). The difference between the top and bottom plateaus is the specific binding. Note that this is not the same as B_{max} . When you use a low concentration of radioligand (to save money and avoid nonspecific binding), you have not reached saturation so specific binding will be much lower than the B_{max} .

The Y axis can be expressed as cpm or converted to more useful units like fmol bound per milligram protein or number of binding sites per cell. Some investigators like to normalize the data from 100% (no competitor) to 0% (nonspecific binding at maximal concentrations of competitor).

The concentration of unlabeled drug that results in radioligand binding halfway between the upper and lower plateaus is called the IC_{50} (inhibitory concentration 50%) also called the EC_{50} (effective concentration 50%). The IC_{50} is the concentration of unlabeled drug that blocks

half the specific binding.

If the labeled and unlabeled ligand compete for a single binding site, the steepness of the competitive binding curve is determined by the law of mass action. The curve descends from 90% specific binding to 10% specific binding with an 81-fold increase in the concentration of the unlabeled drug. More simply, nearly the entire curve will cover two log units (100-fold change in concentration).



Competitive binding curves are described by this equation:

$$Y = \text{Nonspecific} + \frac{(\text{Total} - \text{Nonspecific})}{1 + 10^{\log[D] - \log(\text{IC}_{50})}}$$

Y is the total binding you measure in the presence of various concentrations of the unlabeled drug, and $\log[D]$ is the logarithm of the concentration of competitor plotted on the X axis. Nonspecific is binding in the presence of a saturating concentration of D, and Total is the binding in the absence of competitor. Y, Total and Nonspecific are all expressed in the same units, such as cpm, fmol/mg, or sites/cell.

Use nonlinear regression to fit your competitive binding curve to determine the $\log(\text{IC}_{50})$.

In order to determine the best-fit value of IC_{50} (the concentration of unlabeled drug that blocks 50% of the specific binding of the radioligand), the nonlinear regression problem must be able to determine the 100% (total) and 0% (nonspecific) plateaus. If you have collected data over wide range of concentrations of unlabeled drug, the curve will have clearly defined bottom and top plateaus and the program should have no trouble fitting all three values (both plateaus and the IC_{50}).

With some experiments, the competition data may not define a clear bottom plateau. If you fit the data the usual way, the program might stop with an error message. Or it might find a nonsense value for the

nonspecific plateau (it might even be negative). If the bottom plateau (0%) is incorrect, the IC_{50} will also be incorrect. To solve this problem, you should define the nonspecific binding from other data. All drugs that bind to the same receptor should compete for all specific radioligand binding and reach the same bottom plateau value. When running the curve fitting program, set the bottom plateau of the curve to a constant equal to binding in the presence of a standard drug known to block all specific binding.

Similarly, if the curve doesn't have a clear top plateau, you should set the total binding to be a constant equal to binding in the absence of any competitor.

Calculating the K_i from the IC_{50}

The value of the IC_{50} is determined by three factors:

- The K_i of the receptor for the competing drug. This is what you want to find out. It is the equilibrium dissociation constant for binding of the unlabeled drug – the concentration of the unlabeled drug that will bind to half the binding sites at equilibrium in the absence of radioligand or other competitors. The K_i is proportional to the IC_{50} . If the K_i is low (i.e. the affinity is high), the IC_{50} will also be low.
- The concentration of the radioligand. If you choose to use a higher concentration of radioligand, it will take a larger concentration of unlabeled drug to compete for the binding. So increasing the concentration of radioligand will increase the IC_{50} without changing the K_i .
- The affinity of the radioligand for the receptor (K_d). It takes more unlabeled drug to compete for a tightly bound radioligand (small K_d) than for a loosely bound radioligand (high K_d). Using a radioligand with a lower K_d (higher affinity) will increase the IC_{50} .

Calculate the K_i from the IC_{50} , using the equation of Cheng and Prusoff (Cheng Y., Prusoff W. H., *Biochem. Pharmacol.* 22: 3099-3108, 1973).

$$K_i = \frac{\text{IC}_{50} [\text{radioligand}]}{1 + \frac{[\text{radioligand}]}{K_d}}$$

In thinking about this equation, remember that K_i is a property of the receptor and unlabeled drug, while IC_{50} is a property of the experiment. By changing your experimental conditions (changing the radioligand

used or changing its concentration), you'll change the IC₅₀ without affecting the K_i.

This equation is based on these assumptions:

- Only a small fraction of both the labeled and unlabeled ligands has bound. This means that the free concentration is virtually the same as the added concentration. See "Ligand depletion" on page 15 to learn about methods that don't rely on this assumption.
- The receptors are homogeneous and all have the same affinity for the ligands.
- There is no cooperativity – binding to one binding site does not alter affinity at another site.
- The experiment has reached equilibrium.
- Binding is reversible and follows the law of mass action.
- You know the K_d of the radioligand from an experiment done under similar conditions.

Why determine log(IC₅₀) rather than IC₅₀?

The equation for a competitive binding curve (page 8) looks a bit strange since it combines logarithms and antilogarithms (10 to the power). A bit of algebra simplifies it :

$$Y = \text{Nonspecific} + \frac{(\text{Total} - \text{Nonspecific})}{1 + \frac{[\text{Drug}]}{\text{IC}_{50}}}$$

If you fit the data to this equation, you'll get the same curve and the same IC₅₀. Since the equation is simpler, why not use it? The difference appears only when you look at how nonlinear regression programs assess the accuracy of the fit as a confidence interval. Even after converting from a log scale to a linear scale, you'll end up with different confidence intervals for the IC₅₀.

Which confidence interval is correct? With nonlinear regression, the standard error of the fit variables are only approximately correct. Since the confidence intervals are calculated from the standard errors, they too are only approximately correct. The problem is that the real confidence interval may not be symmetrical around the best fit value. It may extend further in one direction than the other. However, nonlinear regression programs always calculate symmetrical confidence intervals (unless you use advanced techniques). When writing the equa-

tion for nonlinear regression, therefore, you want to arrange the variables so the uncertainty is as symmetrical as possible. Because data are collected at concentrations of D equally spaced on a log axis, the uncertainty is symmetrical when the equation is written in terms of the log of IC₅₀, but is not symmetrical when written in terms of IC₅₀. You'll get more accurate confidence intervals from fits of competitive binding data when the equation is written in terms of the log(IC₅₀).

Homologous competitive binding curves

A competitive binding experiment is termed homologous when the same compound is used as the hot and cold ligand. The term heterologous is used when the hot and cold ligands differ. Homologous competitive binding experiments can be used to determine the affinity of a ligand for the receptor and the receptor number. In other words, the experiment has the same goals as a saturation binding curve. Because homologous competitive binding experiments use a single concentration of radioligand (which can be low), they consume less radioligand and thus are more practical when radioligands are expensive or difficult to synthesize.

To analyze a homologous competitive binding curve, you need to accept these assumptions:

- The receptor has identical affinity for the labeled and unlabeled ligand. Since iodination often changes the binding properties of ligands, you may have to use an iodinated unlabeled compound (using nonradioactive iodine).
- There is no cooperativity.
- No ligand depletion. The methods in this section assume that only a small fraction of ligand binds. In other words the method assumes that free concentration equals the concentration you added.
- There is only one class of binding sites. It is difficult to detect a second class of binding sites unless the number of lower affinity sites vastly exceeds the number of higher affinity receptors (because the single low concentration of radioligand used in the experiment will bind to only a small fraction of low affinity receptors).

Analyze a homologous competitive binding curve using the same equation used for a one-site heterologous competitive binding to determine the top and bottom plateaus and the IC₅₀.

The Cheng and Prussoff equation lets you calculate the K_i from the IC₅₀

(see page 8). In the case of a homologous competitive binding experiment, you assume that the hot and cold ligand have identical affinities so that K_d and K_i are the same. Knowing that, simple algebra converts the equation to:

$$K_d = K_i = IC_{50} - [Radioligand]$$

You set the concentration of radioligand in the experimental design, and determine the IC_{50} from nonlinear regression. The difference between the two is the K_d of the ligand (assuming hot and cold ligands bind the same).

The difference between the top and bottom plateaus of the curve represents the specific binding of radioligand at the concentration you used. Depending on how much radioligand you used, this value may be close to the B_{max} or far from it. To determine the B_{max} , divide the specific binding by the fractional occupancy, calculated from the K_d and the concentration of radioligand.

$$B_{max} = \frac{\text{Top} - \text{Bottom}}{\text{Fractional Occupancy}} = \frac{\text{Top} - \text{Bottom}}{[Radioligand] / (K_d + [Radioligand])}$$

Kinetic binding experiments

What is a dissociation binding experiment?

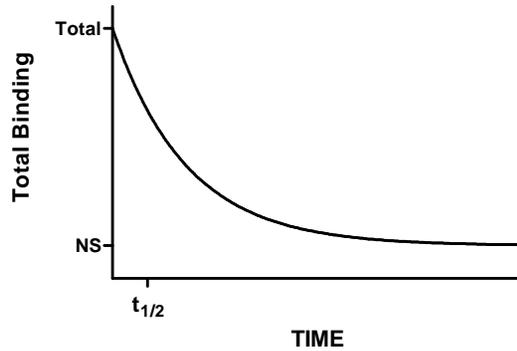
A dissociation binding experiment measures the “off rate” for radioligand dissociating from the receptor. Perform dissociation experiments to:

- Fully characterize the interaction of ligand and receptor.
- Confirm that the law of mass action applies.
- Help design your experimental protocol. If the dissociation is fast, you need to filter and wash your samples quickly so that negligible dissociation occurs. You may also need to lower the temperature of the buffer used to wash the filters, or switch to a centrifugation or dialysis assay. If the dissociation is slow, then you can filter the samples more leisurely, knowing that dissociation will be negligible during the wash.

To perform an off-rate experiment, first allow ligand and receptor to bind, perhaps to equilibrium. At that point, block further binding of radioligand to receptor using one of these methods:

- If the tissue is attached to a surface, remove the buffer containing radioligand and replace with fresh buffer without radioligand.
- Spin the suspension and resuspend in fresh buffer.
- Add a very high concentration of an unlabeled ligand. It will instantly bind to nearly all the unoccupied receptors and block binding of the radioligand.
- Dilute the incubation by a large factor, perhaps a 20-100 fold dilution. This will reduce the concentration of radioligand by that factor. At such a low concentration, new binding of radioligand will be negligible. This method is only practical when you use a fairly low radioligand concentration so its concentration after dilution is far below its K_d for binding.

After initiating dissociation, measure binding over time (typically 10-20 measurements) to determine how rapidly the ligand dissociates from the receptors.



How to analyze dissociation experiments

Fit the data to this equation using nonlinear regression to determine the rate constant. This is called an exponential decay equation.

$$\text{Total Binding} = \text{NS} + (\text{Total} - \text{NS}) \cdot e^{-k_{\text{off}}t}$$

Total binding and nonspecific binding (NS) are expressed in cpm, fmol/mg protein, or sites/cell. Time (t) is usually expressed in minutes. The dissociation rate constant (k_{off}) is expressed in units of inverse time, usually min^{-1} . Since it is hard to think in those units, it helps to calculate the half-life for dissociation which equals $\ln(2)/k_{\text{off}}$ or $0.6931/k_{\text{off}}$. In one half-life, half the radioligand will have dissociated. In two half-lives, three quarters the radioligand will have dissociated, etc.

Typically the dissociation rate constant of useful radioligands is between 0.001 and 0.1 min^{-1} . If the dissociation rate constant is any faster, it would be difficult to perform radioligand binding experiments as the radioligand would dissociate from the receptors while you wash the filters.

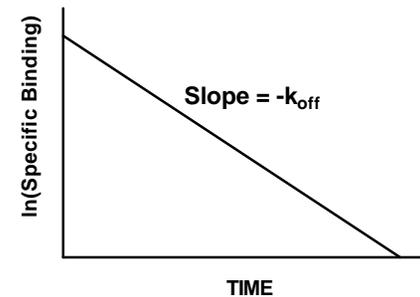
Using a dissociation experiment to confirm the law of mass action

This analysis assumes that the law of mass action applies to your experimental situation. Dissociation binding experiments also let you test that assumption. If the law of mass action applies to your system, the answer to all these questions is yes:

- Does all the specific binding dissociate? Is the binding truly reversible?
- Is the dissociation rate constant the same no matter how long you incubated the cells before initiating dissociation?
- Is the dissociation rate constant the same when you initiate dissociation by diluting or by adding unlabeled drug? If not, consider the possibility of cooperativity (binding sites are clustered, and binding of ligand to one binding site changes the affinity of the others).
- After dissociation, is the ligand chemically intact?

Displaying dissociation data on a log plot

If you plot $\ln(\text{specific binding})$ vs. time, the graph of a dissociation experiment will be linear if the system follows the law of mass action with a single affinity state. The slope of this line will equal $-k_{\text{off}}$.



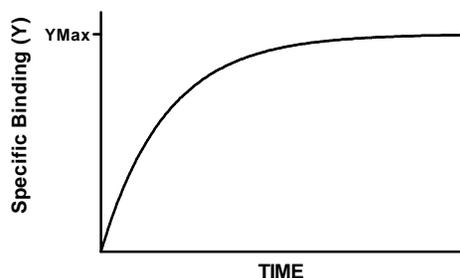
Notes:

- The log plot will only be linear if you take the logarithm of specific binding. A graph of $\log(\text{total binding})$ vs. time will not be linear.
- You must use the natural logarithm, not the log base ten in order for the slope to equal $-k_{\text{off}}$.
- Use the log plot to display data, not to analyze data. You'll get a more accurate rate constant by fitting the raw data using nonlinear regression.

Association rate experiments

Association binding experiments are used to determine the association rate constant. This is useful to characterize the interaction of the ligand with the receptor. It also is important as it lets you determine how long it takes to reach equilibrium in saturation and competition experiments.

You add radioligand and measure specific binding at various times thereafter.



Note that the maximum binding (Y_{\max}) is not the same as B_{\max} . The maximum binding achieved in an association experiment depends on the concentration of radioligand. Low to moderate concentrations of radioligand will bind to only a small fraction of all the receptors no matter how long you wait.

To analyze the data, use nonlinear regression to fit the specific binding data to the one phase exponential association equation.

$$Y = Y_{\max} \cdot (1 - e^{-k_{\text{ob}}t})$$

The observed rate constant, k_{ob} is expressed in units of inverse time, usually min^{-1} . It is a measure of how quickly the incubation reaches equilibrium, and is determined by three factors:

- The association rate constant, k_{on} . This is what you are trying to determine. If k_{on} is larger (faster), k_{ob} will be larger as well.
- The concentration of radioligand. If you use more radioligand, the system equilibrates faster and k_{ob} will be larger.
- The dissociation rate constant, k_{off} . Some people are surprised to see that the observed rate of association depends in part on the dissociation rate constant. This makes sense when you realize that an association experiment doesn't directly measure how long it takes radioligand to bind, but rather measures how long it

takes the binding to reach equilibrium. Equilibrium is reached when the rate of the forward binding reaction equals the rate of the reverse dissociation reaction. If the radioligand dissociates quickly from the receptor, equilibrium will be reached faster, but there will be less binding at equilibrium. If the radioligand dissociates slowly, equilibrium will be reached more slowly and there will be more binding at equilibrium.

To calculate the association rate constant usually expressed in units of $\text{Molar}^{-1} \text{min}^{-1}$, use the following equation. Typically ligands have association rate constants of about $10^8 \text{M}^{-1} \text{min}^{-1}$.

$$k_{\text{on}} = \frac{k_{\text{ob}} - k_{\text{off}}}{[\text{radioligand}]}$$

Analyses of association experiments assume:

- No ligand depletion. A small fraction of the radioligand binds to receptors, so the concentration of free radioligand equals the amount you added and does not change over time.
- No cooperativity. Binding to one site does not alter affinity at a n-other site.
- Homogeneous receptors.

Combining association and dissociation data

Once you have separately determined k_{on} and k_{off} , you can combine them to calculate the K_d of receptor binding:

$$K_d = \frac{k_{\text{off}}}{k_{\text{on}}}$$

The units are consistent: k_{off} is in units of min^{-1} ; k_{on} is in units of $\text{M}^{-1} \text{min}^{-1}$, so K_d is in units of M.

If binding follows the law of mass action, the K_d calculated this way should be the same as the K_d calculated from a saturation binding curve.

Two binding sites

Competitive binding with two sites

Competitive binding experiments are often used in systems where the tissue contains two classes of binding sites, perhaps two subtypes of a receptor. Analysis of these data are straightforward when you accept these assumptions:

- There are two distinct classes of receptors. For example, a tissue could contain a mixture of β_1 and β_2 adrenergic receptors.
- The unlabeled ligand has distinct affinities for the two sites.
- The labeled ligand has equal affinity for both sites.
- Binding has reached equilibrium.
- A small fraction of both labeled and unlabeled ligand bind. This means that the concentration of labeled ligand that you added is very close to the free concentration in all tubes.

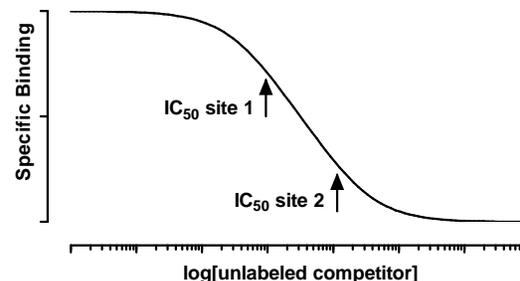
If you accept those assumptions, binding follows this equation:

$$Y = NS + (Total - NS) \left[\frac{F}{1 + 10^{\log[D] - \log(IC_{50_1})}} + \frac{1 - F}{1 + 10^{\log[D] - \log(IC_{50_2})}} \right]$$

This equation has five variables: the total and nonspecific binding (the top and bottom binding plateaus), the fraction of binding to receptors of the first type of receptor (F), and the IC_{50} of the unlabeled ligand for each type of receptors. If you know the K_d of the labeled ligand and its concentration, you can convert the IC_{50} values to K_i values (see page 8).

Since there are two different kinds of receptors with different affinities, you might expect to see a biphasic competitive binding curve. In fact, you will only see a biphasic curve only in unusual cases where the affinities are extremely different. More often you will see a shallow curve with the two components blurred together. For example, the following graph shows competition for two equally abundant sites with a ten fold (one log unit) difference in IC_{50} . If you look carefully, you can see that the curve is shallow (it takes more than two log units

to go from 90% to 10% competition), but you cannot see two distinct components.

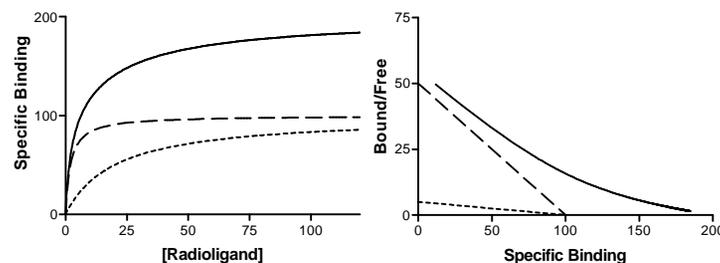


Analyzing saturation binding experiments with two sites

When the radioligand binds to two classes of receptors, analyze the data by using this equation.

$$\text{Specific Binding} = Y = \frac{B_{\max 1} \cdot [L]}{K_{d1} + [L]} + \frac{B_{\max 2} \cdot [L]}{K_{d2} + [L]}$$

The left panel of the figure below shows specific binding to two classes of receptors present in equal quantities, whose K_d values differ tenfold. The right panel shows the transformation to a Scatchard plot. In both graphs the dotted and dashed lines show binding to the two individual receptors that sum to the solid curves.



Note the following:

- The graph of specific binding is not obviously biphasic. It is very hard to see the presence of two binding affinities by eye. The best way to detect the second site is to fit data to one- and two-sites, and let the nonlinear regression program compare the two fits.

- The curvature of the Scatchard plot is not dramatic. Although it is pretty obvious in this idealized figure, the curvature would be easy to miss in an experiment with only a dozen or so data points and a fair amount of scatter.
- You might be surprised by the location of the solid and dashed line in the Scatchard plot. The two components of a biphasic Scatchard are not the asymptotes of the curve.

Comparing one- and two-site models

A two-site model almost always fits your data better than a one-site model. A three-site model fits even better, and a four-site model better yet! As you add more variables (sites) to the equation, the curve becomes “more flexible” and gets closer to the points. You need to use statistical calculations to see if the improvement in fit between two-site and one-site models is more than you’d expect to see by chance.

Before thinking about statistical comparisons, you should look at whether the results make sense. Sometimes the two-site fit gives results that are clearly nonsense. Disregard a two-site fit when:

- The two IC_{50} or K_d values are almost identical.
- One of the IC_{50} or K_d values is outside the range of your data.
- One of the sites has a very small fraction of the receptors. If there are so few sites, the IC_{50} or K_d cannot be determined reliably.
- The best-fit values for the bottom and top plateaus are far from the range of Y values observed in your experiment (applies to competitive binding curves only).

If the two-site fit seems reasonable, then you should test whether the improvement is statistically significant.

Even if the simpler one-site model is correct, you expect it to fit worse (have the higher sum-of-squares) because it has fewer inflection points (more degrees of freedom). In fact, statisticians have proven that the relative increase in the sum of squares (SS) is expected to equal the relative increase in degrees of freedom (DF). In other words, if the one-site model is correct you expect that:

$$(SS1 - SS2) / SS2 \approx (DF1 - DF2) / DF2$$

If the more complicated two-site model is correct, then you expect the relative increase in sum-of-squares (going from two-sites to one-site) to be greater than the relative increase in degrees of freedom:

$$(SS1 - SS2) / SS2 > (DF1 - DF2) / DF2$$

The F ratio quantifies the relationship between the relative increase in sum-of-squares and the relative increase in degrees of freedom.

$$F = \frac{(SS1 - SS2) / SS2}{(DF1 - DF2) / DF2}$$

If the one-site model is correct you expect to get an F ratio near 1.0. If the ratio is much greater than 1.0, there are two possibilities:

- The two-site model is correct.
- The one-site model is correct, but random scatter led the two-site model to fit better by chance. The P value tells you how rarely this coincidence would occur.

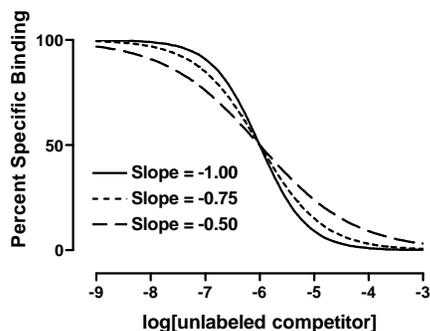
Many programs calculate the P value for you. If not, you can find it using a table of F statistics. You need to know that the numerator has DF1-DF2 degrees of freedom and the denominator has DF2 degrees of freedom. To use statistical tables you also have to remember that a larger value of F corresponds to a lower P value.

The P value answers this question: If the one-site model is really correct, what is the chance that you’d randomly obtain data that fits the two-site model so much better? If the P value is small, you conclude that the two-site model is significantly better than the one-site model. Most scientists reach this conclusion when $P < 0.05$, but this threshold P value is arbitrary.

Advanced topics

The slope factor or Hill slope

Many competitive binding curves are shallower than predicted by the law of mass action for binding to a single site. The steepness of a binding curve can be quantified with a slope factor, often called a Hill slope. A one-site competitive binding curve that follows the law of mass action has a slope of -1.0. If the curve is more shallow, the slope factor will be a negative fraction (i.e. -0.85 or -0.60). The slope factor is negative because the curve goes downhill.



To quantify the steepness of a competitive binding curve (or a dose-response curve), fit the data to this equation:

$$Y = \text{Nospecific} + \frac{(\text{Total} - \text{Nonspecific})}{1 + 10^{(\log(\text{IC50}) - \log[D])\text{SlopeFactor}}}$$

The slope factor is a number that describes the steepness of the curve. In most situations, there is no way to interpret the value in terms of chemistry or biology. If the slope factor differs significantly from 1.0, then the binding does not follow the law of mass action with a single site.

Explanations for shallow binding curves include:

- Heterogeneous receptors. The receptors do not all bind the unlabeled drug with the same affinity. This can be due to the presence of different receptor subtypes, or due to heterogeneity in receptor coupling to other molecules such as G proteins. In the example on page 13, the slope factor equals -0.78.

- Negative cooperativity. Binding sites are clustered (perhaps several binding sites per molecule) and binding of the unlabeled ligand to one site causes the remaining site(s) to bind the unlabeled ligand with lower affinity.
- Curve fitting problems. If the top and bottom plateaus are not correct, then the slope factor is not meaningful. Don't try to interpret the slope factor unless the curve has clear top and bottom plateaus.

Agonist binding

All of the analyses presented in this booklet are based on the law of mass action. This assumes that the receptor and ligand reversibly bind, but nothing else happens. The law of mass action may not be true when the ligand or competitor is an agonist. By definition, something happens when an agonist binds to the receptor. For example, the agonist may alter the interaction of the receptor with a G protein, which then changes the affinity of the receptor for the agonist. These complexities mean that the law of mass action may be too simple when agonists are involved, and that the results of analyses based on the law of mass action can't be rigorously interpreted.

Some investigators have attempted to fit data to more complicated models, but these analyses are beyond the scope of this booklet. Despite the theoretical complexities, agonist binding curves often turn out to fit rectangular hyperbolas or one- or two-site competitive binding curves. Most investigators analyze their data using these standard analyses. The best-fit curves often fit the data nicely, and provide K_d or K_i values that can be compared between conditions. It is important to realize that these analyses are based on a model that is too simplistic. Treat the best-fit values of K_d or K_i as empirical descriptions of the data, and are not as true equilibrium dissociation constants.

Ligand depletion

The equations that describe the law of mass action include the variable [Ligand] which is the free concentration of ligand. All the analyses presented so far depend on the assumption that a very small fraction of the ligand binds to receptors (or to nonspecific sites) so you can assume that the free concentration of ligand is approximately equal to the concentration added. This is sometimes called "zone A".

In some experimental situations, the receptors are present in high concentration and have a high affinity for the ligand, so that assumption is not valid. A large fraction of the radioligand binds to receptors so the

free concentration of radioligand is quite a bit lower than the concentration added. The system is not in "zone A". The discrepancy is not the same in all tubes or at all times.

Many investigators use this rule of thumb. If less than 10% of the ligand binds, don't worry about ligand depletion.

If possible you should design your experimental protocol to avoid situations where more than 10% of the ligand binds. You can do this by using less tissue in your assays. The problem is that this will also decrease the number of counts. An alternative is to increase the volume of the assay without changing the amount of tissue. The problem with this approach is that you'll need more radioligand.

If you can't avoid radioligand depletion, you need to account for the depletion in your analyses. You may use several approaches.

- Measure the free concentration of ligand in every tube.
- Calculate the free concentration in each tube by subtracting the number of cpm (counts per minute) of total binding from the cpm of added ligand. This can then be converted to the free concentration in molar. This method works only for saturation binding experiments, and cannot be extended to analysis of competition or kinetic experiments. One problem with this approach is that experimental error in determining specific binding also affects the calculated value of free ligand concentration. When you fit curves, both X and Y would include experimental error, and the errors will be related. This violates the assumptions of nonlinear regression. Using simulated data, Swillens (see reference below) has shown that this can be a substantial problem. Another problem is that the free concentration of radioligand will not be the same in the tubes used for determining total and nonspecific binding. Therefore you cannot calculate specific binding as the difference between the total binding and nonspecific binding.
- Swillens (Molecular Pharmacology, 47: 1197-1203, 1995) developed an alternative approach – fit total binding as a function of added ligand using an equation that accounts both for nonspecific binding and for ligand depletion. By analyzing simulated experiments, that paper shows that fitting total binding gives more reliable results than you would get by calculating free ligand by subtraction.

Calculations with Radioactivity

Efficiency of detecting radioactivity

Efficiency is the fraction of the radioactive disintegration that are detected by the counter. Determine efficiency by counting a standard sample under conditions identical to those used in your experiment.

With ^{125}I , the efficiency is usually greater than 90%, depending on the geometry of the counter. The detector doesn't entirely surround the tube, so a few gamma rays (photons) miss the detector.

With ^3H , the efficiency of counting is much lower, often about 40%. The low efficiency is mostly a consequence of the physics of decay, and can not be improved by better instrumentation or better scintillation fluid. When a tritium atom decays, a neutron converts to a proton and the reaction shoots off an electron and neutrino. The energy released is always the same, but it is randomly partitioned between the neutrino (not detected) and an electron (that we try to detect). When the electron has sufficient energy, it will travel far enough to encounter a fluor molecule in the scintillation fluid. This fluid amplifies the signal and gives of a flash of light detected by the scintillation counter. The intensity of the flash (number of photons) is proportional to the energy of the electron. If the electron has insufficient energy, it is not captured by the fluor and is not detected. If it has low energy, it is captured but the light flash has few photons and is not detected by the instrument. Since the decay of many tritium atoms does not lead to a detectable number of photons, the efficiency of counting is much less than 100%.

Efficiency of counting ^3H is reduced by the presence of any color in the counting tubes, if the mixture of water and scintillation fluid is not homogeneous, or if the radioactivity is trapped in tissue chunks (so emitted electrons don't travel into the scintillation fluid).

Specific radioactivity

When you buy radioligands, the packaging usually states the specific radioactivity as Curies per millimole (Ci/mmol). Since you measure counts per minute (cpm), the specific radioactivity is more useful when stated in terms of cpm. Often the specific radioactivity is expressed as cpm/fmol (1 fmol = 10^{-15} mole).

To convert from Ci/mmol to cpm/fmol, you need to know that 1 Ci

equals 2.22×10^{12} disintegrations per minute. Use this equation to convert Z Ci/mole to Y cpm/fmol when the counter has an efficiency (expressed as a fraction) equal to E.

$$Y \frac{\text{cpm}}{\text{fmol}} = Z \frac{\text{Ci}}{\text{mmole}} \cdot 2.22 \times 10^{12} \frac{\text{dpm}}{\text{Ci}} \cdot 10^{-12} \frac{\text{mmole}}{\text{fmole}} \cdot E \frac{\text{cpm}}{\text{dpm}} = Z \cdot 2.22 \cdot E$$

$$Y \frac{\text{cpm}}{\text{fmol}} = Z \frac{\text{Ci}}{\text{mmole}} \cdot 2.22 \cdot E$$

You may also encounter the unit Becquerel, which equals one radioactive disintegration per second.

Calculating the concentration of the radioligand

Rather than trust your dilutions, you can accurately calculate the concentration of radioligand in a stock solution. Measure the number of counts per minute in a small volume of solution and use this equation. C is cpm counted, V is volume of the solution you counted in ml, and Y is the specific activity of the radioligand in cpm/fmol (calculated in the previous section).

$$\text{Concentration in pM} = \frac{C \text{ cpm} / Y \text{ cpm} / \text{fmol}}{V \text{ ml}} \cdot \frac{0.001 \text{ pmol} / \text{fmol}}{0.001 \text{ liter} / \text{ml}} = \frac{C}{Y} \cdot \frac{1}{V}$$

Radioactive decay

Radioactive decay is entirely random. A particular atom has no idea how old it is, and can decay at any time. The probability of decay at any particular interval is the same as the probability of decay during any other interval. If you start with N_0 radioactive atoms, the number remaining at time t is:

$$N_t = N_0 \cdot e^{-k_{\text{decay}} \cdot t}$$

k_{decay} is the rate constant of decay expressed in units of inverse time. Each radioactive isotope has a different value of k_{decay} .

The half-life ($t_{1/2}$) is the time it takes for half the isotope to decay. Half-life and decay rate constant are related by this equation:

$$t_{1/2} = \frac{\ln(2)}{k_{\text{decay}}} = \frac{0.693}{k_{\text{decay}}}$$

This table shows the half-lives and rate constants for commonly used radioisotopes. The table also shows the specific activity assuming that each molecule is labeled with one radioactive atom. (This is often the case with ^{125}I and ^{32}P . Tritiated molecules often incorporate two or three tritium atoms, which increases the specific radioactivity.)

Isotope	Half life	k_{decay}	Specific Radioactivity
^3H	12.43 years	0.056 year^{-1}	28.7 Ci/mmol
^{125}I	59.6 days	0.0116 day^{-1}	2190 Ci/mmol
^{32}P	14.3 days	0.0485 day^{-1}	9128 Ci/mmol
^{35}S	87.4 days	0.0079 day^{-1}	1493 Ci/mmol

You can calculate radioactive decay from a date where you knew the concentration and specific radioactivity using this equation.

$$\text{Fraction Remaining} = e^{-k_{\text{decay}} \cdot \text{Time}}$$

For example, after ^{125}I decays for 20 days, the fraction remaining equals 79.5%. Although data appear to be scanty, most scientists assume that the energy released during decay destroys the ligand so it no longer binds to receptors. Therefore the specific radioactivity does not change over time. What changes is the concentration of ligand. After 20 days, therefore, the concentration of the iodinated ligand is 79.5% of what it was originally, but the specific radioactivity remains 2190 Ci/mmol. This approach assumes that the unlabeled decay product is not able to bind to receptors and has no effect on the binding. Rather than trust this assumption, you should always try to use newly synthesized or repurified radioligand for key experiments. This approach also assumes that your radioligand is "carrier free", which means that you don't have a mixture of labeled and unlabeled ligands.

Counting error and the Poisson distribution

The decay of a population of radioactive atoms is random, and therefore subject to a sampling error. For example, the radioactive atoms in a tube containing 1000 cpm of radioactivity won't give off exactly 1000 counts in every minute. There will be more counts in some minutes and fewer in others, with the distribution of counts following a Poisson distribution. This variability is intrinsic to radioactive decay and cannot be reduced by more careful experimental controls.

After counting a certain number of counts in your tube, you want to know the "real" number of counts. Obviously, there is no way to know

that. But you can calculate a range of counts that is 95% certain to contain the true average value. So long as the number of counts, C , is greater than about 50 you can calculate the confidence interval using this approximate equation:

$$95\% \text{ Confidence Interval: } (C - 1.96\sqrt{C}) \text{ to } (C + 1.96\sqrt{C})$$

Computer programs can calculate a more exact P value (these calculations necessary when C is less than about 100) For example, if you measure 100 radioactive counts in an interval, you can be 95% sure that the true average number of counts ranges approximately between 80 and 120 (using the equation here) or more exactly between 81.37 and 121.61 (using the more exact equation).

When calculating the confidence interval, you must set C equal to the total number of counts you measured experimentally, not the number of counts per minute.

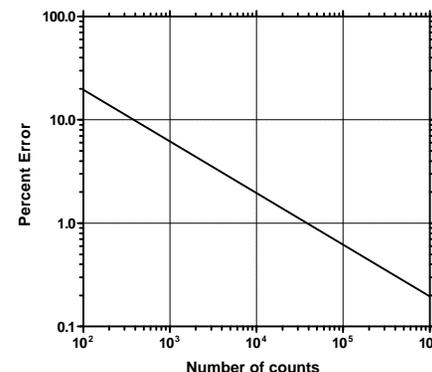
Example: You placed a radioactive sample into a scintillation counter and counted for 10 minutes. The counter tells you that there were 225 counts per minute. What is the 95% confidence interval? Since you counted for 10 minutes, the instrument must have detected 2250 radioactive disintegrations. The 95% confidence interval of this number extends from 2157 to 2343. This is the confidence interval for the number of counts in 10 minutes, so the 95% confidence interval for the average number of counts per minute extends from 216 to 234. If you had attempted to calculate the confidence interval using the number 225 (counts per minute) rather than 2250 (counts detected), you would have calculated a wider (incorrect) interval.

The Poisson distribution explains why it is helpful to count your samples longer when the number of counts is small. For example, this table shows the confidence interval for 100 cpm counted for various times. When you count for longer times, the confidence interval will be narrower.

	1 minute	10 minutes	100 minutes
Counts per minute (cpm)	100	100	100
Total counts	100	1000	10000
95% CI of counts	81.4 to 121.6	938 to 1062	9804 to 10196
95% CI of cpm	81.4 to 121.6	93.8 to 106.2	98.0 to 102.0

This graph shows percent error as a function of number of counts (C). Percent error is defined from the width of the confidence interval divided by the number of counts:

$$\text{Percent Error} = 100 \cdot \frac{1.96 \cdot \sqrt{C}}{C}$$



GraphPad Prism

All the graphs in this booklet were created using GraphPad Prism, a general-purpose curve fitting and scientific graphics program for Windows.

Although GraphPad Prism was designed to be a general purpose program, it is particularly well-suited for analyses of radioligand binding data.

- Prism provides a menu of commonly-used equations (including equations similar to all those mentioned in this booklet). To fit a curve, all you have to do is pick the right equation. Prism does all the rest automatically, from picking initial values to graphing the curve.
- Prism can automatically compare one- and two-site models.
- Prism automatically calculates K_i from IC_{50} .
- Prism's manual and help screens explain the principles of curve fitting with nonlinear regression and help you interpret the results. You don't have to be a statistics expert to use Prism.

Please visit our web site at <http://www.graphpad.com>. You can read about the Prism and download a free demo. Or contact GraphPad Software to request a brochure and demo disk by phone (619-457-3909), fax (619-457-8141) or email (sales@graphpad.com). The demo is not a slide show – it is a functional version of Prism with no limitations in data analysis. Try it out with your own data, and see for yourself why Prism is the best solution for analyzing and graphing scientific data.