## Isotopes used in Biology

Radioisotopes are used for various applications in Biology. The table below summarizes some generally useful information about some common isotopes.

| Property | $^{3}H$ | $^{14}C$ | $^{35}S$ | $^{32}P$ | $^{125}I$ | $^{131}I$ |
|---|---|---|---|---|---|---|
| Half-Live | 12.3 years | 5730 years | 87.4 days | 14.3 days | 60 days | 8.04 days |
| Decay Mode | $\beta$ | $\beta$ | $\beta$ | $\beta$ | $\gamma$(EC) | $\beta$ and $\gamma$ |
| E(max)/E(ave) (KeV) | 18.6/6 | 156/49 | 167/49 | 1709/690 | — | 806/108 |
| Biological Half-life | 12 days | 12 days | 44 days | 14 days | 42 days | 8 days |
| Critical Organ | whole body | whole body | whole body | bone | thyroid | thyroid |
| Shielding | none | Perspex (1cm) | Perspex (1cm) | Perspex (1cm) | lead (0.25 mm) | lead (13 mm) |

**Some Important Quantities to Consider in Biological Applications** :

**Specific Activity**:

Specific activity is the amount of activity per amount of substance. The units can be Ci/gram, milli-curies/milli-mole, CPM/pico-mole. The specific activity of a **pure isotope is** $A/N$, and is related to the half-life. As discussed previously, the activity $A$ and the number of radioactive nuclei $N$ are related by

$$A = \frac{Nln(2)}{\tau} \tag{1}$$

where $\tau$ is the half-life of the isotope. Solving for $A/N$ we have

$$\frac{A}{N} = \frac{ln(2)}{\tau} \tag{2}$$

This formula is useful in determining how many radioactive isotopes there are on a specific molecule. For example, the specific activity of pure $^{3}H$ (tritium) is 29 Ci/mmole. If the specific activity of your pure biological molecule (i.e. GABA) has a specific activity of 89 Ci/mmole, then 3 of the $H$ atoms in the molecule are $^{3}H$.

One usually does not do assays with pure isotopes, since this would be wasteful. Instead, one dilutes the radioactive molecules with non-radioactive ones. Thus, in practice the specific activity of the biological molecule used in your experiment is less than the sample from the supplier. We refer to this specific activity as the **experimental specific activity**. For example if we add 10 times the amount of non-radioactive GABA to the 89 Ci/mmole GABA, the experimental specific activity is reduced to 8.9 Ci/mmole of GABA in the experiment. In this case, only one

out of 10 GABA molecules is radioactive.

**Biological Half Life**:

In some instances, isotopes can leave the body before they have decayed away. In this case, the number of decays in the body is less than there would be if the isotope remained. The biological half-life is the time it takes for half the amount of the substance to leave the body. A substance that is not radioactive can also have a biological half-life. The total number of decays of an isotope in the body is therefore:

$$N = \frac{A\tau}{ln(2)} \tag{3}$$

where $\tau$ is the smaller of the biological half-life or the radioactive half-life. For example, in the chart above one can see that for iodine the biological half-life is around 42 days. In the case of $^{125}I$ the biological half-life is shorter than the radioactive one, which is 60 days, so 42 days should be used in the equation above. In the case of $^{131}I$ the radioactive half life is shorter (8 days), so one should use 8 days in the above equation.

**Shielding required**:

In the chart above, the required shielding is listed for the isotopes. Some comments are in order. Since the beta emitted by tritium ($^3H$) has a low energy, no shielding required **as long as the activity is low enough**. This is because the maximum energy of the emitted beta's is only 18.6KeV. If the tritium is outside your body and does not come in contact with your skin, even the beta's with the most energy ($E_{max} = 18.6$KeV) do not cause significant damage.

For the isotopes $^{14}C$, $^{35}S$ and $^{32}P$ Perspecx of thickness 1 cm is recommended. Perspecx is plexiglass. If high energy betas are emitted, lead should not be used as a shielding. This is because the highly positive lead nucleus ($Z = 82$) will cause the beta particles (electrons) to experience large acceleration. Charged particles that are accelerated radiate. In the case of the beta's, this acceleration is so great that x-rays are emitted. The production of x-rays in this manner is called **bremstrahlung radiation**. These secondary x-rays produced when the betas interact with the lead shielding can themselves cause radiation damage.

## Applications of Radioisotopes in Biology

Before using radioisotopes in biological or medical applications, one must first consider alternatives such as fluorescence techniques. However, often radioisotopes

are necessary due to thier sensitivity. Here we will discuss a few examples.

**Urea Breath Test**

The Urea breath test is used to detect the presence of the bacteria Helicobacter pylori in the stomach. Helicobacter pylori is a bacteria that causes inflammation, ulcers and atrophy of the stomach. The way the test works is as follows: The patient is given a certain amount of urea with some of the carbon being $^{14}C$. If there is Helicobacter pylori in the stomach, then this bacteria will break down the urea and produce $C0_2$. The $CO_2$ will be exhaled by the patient and collected in a balloon. If some of the carbon in the exhaled $CO_2$ contains is $^{14}C$ then there must be some bacteria present in the stomach. The more $^{14}C$ exhaled, the more Helicobacter pylori present. The breath test can be repeated to determine the success of the treatment.

The urea breath test is a nice example we can use to determine the total dosage a patient receives. Suppose a $10\mu$Ci sample of $[^{14}C]$-urea is given to a 55 Kg patient. Let's determine the total dosage to her body.

To determine the total dosage, we need to calculate the total number of decays that will occur in her body. We can use the relationship $N = A\tau/ln(2)$. However, what should we use for the half-life $\tau$? The half-life of $^{14}C$ is 5730 years. The biological half-life of carbon is 12 days. We should use the shorter of the two, since most of the $^{14}C$ will decay outside of her body. Using 12 days, for $\tau$ we have:

$$N = \frac{(10)(37000)(12)(24)(3600)}{ln(2)} = 5.53 \times 10^{11} \tag{4}$$

To find dosage, we need to calculate the energy absorbed per mass. The endpoint energy of $^{14}C$ beta decay is 156KeV. Since the average energy is around 1/3 of this value, the average energy is around 52KeV. So the total energy/gram is given by:

$$\begin{aligned} (energy\ absorbed)/gram &= \frac{(5.53 \times 10^{11})(52000)(1.6 \times 10^{-12})}{55000} \\ &= 0.837 \frac{ergs}{gram} \end{aligned}$$

Since 100 ergs/gram is a REM, the total dosage is 0.00837 REM or 8.37 mREM.

In this example, we assumed that the urea (and hence the $^{14}C$) is uniformly distributed throughout the body. In this case, this is a fairly good assumption. However, in the case of $^{131}I$, iodine accumulates in the thyroid. Since the approximate mass of the thyroid is 20 grams, the dosage will be much higher. Instead of dividing by the total mass of 55000 grams, we would divide by just 20 grams in the equation above. The dosage would be nearly 3000 times more to the area of the thyroid. Auxillary

damage would also be done, since iodine also accumulates in salvatory glands.

**Transport Assay**

One aim of a transport assay is to measure the rate at which a molecule is transported across a membrane. It is perhaps easiest if we discuss transport assays by using a specific example, a study that we do here at Cal Poly. We measure the transport rate of the GABA molecule across the membrane in an expression system. The expression system we use are eggs from female African clawed frogs. The eggs, which are one cell, are rather large ($\approx$ 1 mm in diameter). Complimentary RNA (cRNA) is injected into the cell. The cRNA is folded into the membrane of the cell. Once in the membrane, the cRNA can be tested as to its transport properties.

The basic idea is as follows: frog eggs are put into a solution (analysis solution) containing GABA. Some of the GABA are "hot", that is some of the GABA contain $^3H$. After a specific time (around 30 minutes) the eggs are removed from the analysis solution. One of the eggs is placed in a scintillation cocktail, and the liquid scintillation detector measures the count rate in counts/min (CPM) of the $^3H$. This count rate will tell us how many GABA molecules entered the cell.

There are three solutions involved in the experiment. We will refer to GABA that contain $^3H$ as **hot GABA**, and non-radioactive GABA as **cold GABA**. The three solutions are:

1. **The Stock solution**: This is the solution that you have purchased from the supplier. All the GABA in the solution is hot. The supplier will indicate the specific activity of each molecule: 89.0 Ci/mmol. This tells us that each molecule has 3 radioactive $^3H$ atoms on it. The supplier will also indicate the **radioactive concentration**. In our case it is 1.0 mCi/ml. We will take a small amount of the stock solution and add it to the analysis solution. We will see that it is convenient to express the radioactive concentration in units of DPM/$\mu$l. So let's do it now: $(1.0\ mCi/ml)(3.7 \times 10^7\ DPS/mCi)(60\ sec/min)(1ml/1000\ \mu l) = 2.22 \times 10^6\ DPM/\mu l$. This was the activity when the isotope was purchased 6.28 years ago. The activity today is: $(2.2 \times 10^6)(1/2)^{6.28/12} = 1.54 \times 10^6 DPM/\mu l$.

2. **The Analysis solution**: This is the solution in which the eggs will uptake GABA for a certain amount of time (around 30 minutes). We start with 5 mL of 1 mM GABA. "mM" stands for milli-molar, which is a concentration of 1 milli-mole per liter. Since the solution is only 5 milli-liters, we have a total of 5 micro-moles of GABA in the 5 mL analysis solution. It is easier to use pico-moles, so we have a total of $5 \times 10^6$ pico-moles of GABA in the analysis solution. All this GABA is cold.

3. **The scintillation cocktail solution**: This is where the frog egg is placed to count the radiation emitted in the liquid scintillation detector. We don't need to worry about this solution.

You need to add some of the stock solution to the analysis solution so that you will get an appropriate count rate in the experiment. From previous studies you estimate that you want to have around 1000 CPM when you put 1 $\mu$l of the analysis solution in the scintillation cocktail. Since the efficiency of the detector is 40%, this means that you need to have $1000/0.4 = 2500$ decays/min in each $\mu$l of the analysis solution. The analysis solution is 5ml, so the total DPM in the entire analysis solution is $(2500\ DPM/\mu l)(5000\mu l) = 12.5 \times 10^6\ DPM$.

Now, to find out how much stock solution to add is easy. We need a total of $12.5 \times 10^6$ DPM in the analysis solution. One $\mu l$ of stock has $1.54 \times 10^6$ DPM as derived above. So we need to add $(12.5 \times 10^6)/(1.54 \times 10^6) = 8.1\ \mu l$ of stock.

You might be wondering if we have added a lot of the hot GABA and the number of CPM/picomole has changed from our original estimate. Let's calculate the total number of hot GABA in the analysis solution by finding the number of pico-moles/$\mu l$ in the stock solution. $1\mu l$ has an activity of $1.54 \times 10^6$ DPM, which is 0.69 $\mu$Ci. 89 Ci/mmol corresponds to 89 $\mu$Ci/ for every 1000 pico-moles. So every $\mu l$ contains $(0.69/89)(1000) = 7.7$ pico-moles/$\mu l$. Since we added 8.1 $\mu l$ of stock, we only added $(8.1)(7.7) \approx 62$ pico moles of hot GABA to the analysis solution. This is small compared to the $5 \times 10^6$ pico-moles of cold GABA in the solution. That means that one out of every $80,000$ GABA is hot in the analysis solution.

Even after adding the stock to the analysis solution, the molarity is still approximately one mmole/liter. So 1 $\mu l$ of analysis solution contains 1000 pico-moles of GABA and will result in 1000 CPM when counted in the liquid scintillation detector. Thus, the **experimental specific activity** of the GABA is 1 CPM/pico-mole.

The hard part of the transport assay is done. Now we need to find the number of CPM that the cell produces and that will be the number of pico-moles that entered the cell. In our homework, since the count rate was 2221 CPM, 2221 pico-moles entered the cell. Since this occured in 30 minutes the GABA uptake is $2221/30 = 74$ pmoles/min.

In an actual experiment, we don't need to as many calculations. After we have decided how much of the stock to add, we need to calculate the GABA concentration in units of pico-Ci/$\mu l$. We will **measure the activity** of one $\mu l$ of the analysis

solution to determine the number of CPM/pmole. We must always determine this important conversion factor (CPM/pmole) from our experimental measurement of the CPM with our liquid scintillation detector. In our calculations we are not completely sure of the efficiency, or the amount of quenching, etc.

Let's do an example from our laboratory exercise. Suppose we place 10 $\mu l$ of stock into a 5 ml analysis solution which is 10 $\mu$M = $10^{-5}$ molar. Our stock has a radioactive concentration of 1 mCi/ml, and a specific activity of 89 Ci/mmole. We first need to determine the molarity of the GABA in the stock:

$$\frac{1\ mmol}{89\ Ci}\frac{1\ mole}{1000\ mmol}\frac{1\ Ci}{1000\ mCi}\frac{1\ mCi}{1ml}\frac{1000ml}{l} = 1.12 \times 10^{-5}\ Molar \qquad (5)$$

The stock is diluted when placed in the analysis solution. Since we put 10 $\mu l$ of stock into 5000 $\mu l$ of analysis solution, the stocks molarity is reduced by a factor of 500 to $2.247 \times 10^{-8}$ molar. Thus, the molarity of all the GABA (cold + hot) in the analysis solution is $10^{-5} + 2.247 \times 10^{-8} = 1.002247 \times 10^{-5}$ molar. Moles/liter can be converted to pmol/$\mu l$ by multiplying by the factor $10^6$. Thus, the concentration of total GABA in the analysis solution is $10.02247 \approx 10$ pmol/$\mu l$. When one $\mu l$ is placed in the scintillation cocktail and counted in the liquid scintillation detector, we **measure** 17482 CPM. So the CPM/pmole is 1748 CPM/pmole. This is the calibration factor that enables us to related CPM to pico-moles when we measure how much GABA entered the frog egg.

## Radioimmunoassay (RIA)

### Determination of Hormone Concentration in the Plasma

The concentration of most hormones in the blood is very low; from the low picomolar ($10^{-12}$) to the high nanomolar ($10^{-9}$ M) range. This very low concentration presented serious challenges to early investigators who were interested in the study of hormones. This is because traditional analytical means of measurement for steroids and proteins did not allow detection in the physiological range of hormone levels. Therefore, the initial tests of hormone presence or absence included **bioassays**. Bioassays involved the injection of tissue extracts into experimental animal models followed by observation of some specific anticipated phenotype. For example, long before sensitive laboratory pregnancy tests were developed, urine samples from suspected pregnant women were administered to female African clawed frogs (Xenopus laevis). Induction of egg laying by the animal was indicative of the presence of **human chorionic gonadotropin** (hCG). This is a hormone that is secreted by the placenta.

Today, however, except for a few in vitro assays, bioassays have become obsolete. In the late 1950s and early 1960s, a very clever method was developed by a woman scientist named Rosalyn Yalow and her colleague Solomon Berson, in which they tagged peptide hormones with radioisotopes. In addition, they raised specific antibodies against hormones. The two breakthroughs put together allowed for very accurate and sensitive measurement of hormone concentrations in plasma samples. The technique is called **radioimmunoassay** (RIA), so named because it combines the **high specificity** of immunological methods with the **high sensitivity** of radiotracer methods.

RIA analysis can allow sensitivity down to the femtomolar range (10-15 M)! Therefore, RIA is said to have very **high sensitivity** (i.e., detection at very low concentrations). In addition, because specific antibodies are used (they are carefully screened during antibody selection), these antibodies react only with the hormone of interest. Thus, RIA is said to have very **high specificity** (i.e., the antibody does not cross-react with other hormones).

For this work, Yalow received the Nobel Prize in Physiology or Medicine in 1977, thus, becoming the first woman to receive this prize. She was also the first woman to receive the Lasker Prize, which is the highest honor bestowed on a scientist in the U.S. This prize is often said to be the "American Nobel".

## Principles of RIA

RIA works based on **competition for binding** between non-labeled ("cold") and isotope labeled ("hot") hormone for its specific antibody. Typically, a fixed amount of the antibody is attached to the bottom of a tube. Then a fixed amount of hot hormone is added to the tube. The amount of hot hormone is typically high enough to saturate all of the antibody molecules attached to the bottom of the tube. Because the interaction between the antibody and the hormone involves very strong non-covalent forces, the contents of the tube can be discarded without disturbing the hormone molecules that are bound to the antibody molecules in the bottom of the tube. The tube can now be counted in a gamma counter to obtain a **counts per minute (CPM)**. This value represents the total count, and represents a situation when all of the antibody binding sites are occupied by the hot hormone. We give the label of $B_0$ to this CPM measurement.

This value alone, however, tells us nothing. In order to calibrate the measurements, known amounts of *cold hormone* are added to tubes that contain the same

fixed amount of antibody attached to the bottom, and in addition, the same fixed amount of hot hormone in the tube. Now in the tube, there is competition between the cold and hot hormone to bind to the antibody binding sites. The relative amount of cold or hot that binds to the antibody is strictly a function of their relative amounts in the tube. It is, of course, determined that the hot and the cold hormone bind to the antibody with the same affinity. The higher the concentration of the cold hormone, the better it can **compete** for the binding sites. Therefore, less hot hormone will bind, which will result in a smaller CPM ($B$) than $B_0$.

In this fashion, a standard curve can be obtained by measuring counts from tubes, which have increasing known concentrations of the cold hormone. For any given cold hormone concentration, the fraction of the antibodies that has hot hormone bound to it (Fraction Bound$_{Hot}$), and the fraction that has cold hormone bound to it (Fraction Bound$_{Cold}$) can be determined by using:

$$\begin{aligned} Fraction\ Bound_{Hot} &= \frac{B}{B_0} \\ Fraction\ Bound_{Cold} &= \frac{B_0 - B}{B_0} = 1 - Fraction\ Bound_{Hot} \end{aligned}$$

Fraction Bound$_{Hot}$ represents the fraction of the antibody binding sites that is occupied by the *hot* hormone and, likewise, Fraction Bound$_{Cold}$ represents the fraction of the antibody binding sites that is occupied by the *cold* hormone. In the presence of the cold hormone, some of the binding sites are occupied by the cold hormone, which when counted for radioactivity, do not contribute to the radioactive signal. The radioactive signal (CPM), therefore, comes only from the bound hormone that is hot.

In addition, **non-specific binding** (NSB) must be determined. This is essentially the background counts. NSB refers to the amount of the hot hormone that would bind to the tube if the tube had no antibody attached to its bottom. Experimentally, this is accomplished by incubating the hot hormone in a tube that is in every respect identical to the tubes that contained attached antibodies. This tube, of course, lacks the attached antibody. The CPM obtained for NSB must be subtracted from all measurements. Therefore, for all samples, Fraction Bound$_{Hot}$ and Fraction Bound$_{Cold}$ are calculated as follows:

$$\begin{aligned} Fraction\ Bound_{Hot} &= \frac{B - NSB}{B_0 - NSB} \\ Fraction\ Bound_{Cold} &= 1 - Fraction\ Bound_{Hot} \end{aligned}$$

For the sample which contained no cold hormone, Fraction Bound$_{Hot}$ is obviously 1.00 and Fraction Bound$_{Cold}$ is zero (because $B = B_0$). To obtain a standard curve, Fraction BoundHot is plotted as a function of the known concentration of the cold hormone that was added to the experimental tubes. Typically a negative sigmoid plot is obtained. However, in practice, the values are transformed such that a straight standard curve is obtained as will be explained below after the summary. The standard curve can then be used to determine the hormone concentration in biological samples of interest.

### Summary of Requirements for RIA

1. Highly specific antibody against the hormone of interest. In modern commercial kits, these are covalently attached to the bottom of tube in a way that the antibody binding site is still available to bind hormone.

2. Radiolabeled hormone of interest. The hormone is typically labeled with 125I. Thankfully, this also comes in the commercial kit.

3. Known amounts of cold hormone in order to obtain a standard curve. These known standards are also provided with commercial kits.

4. Sample tubes that do not have antibody attached to their bottom. These are in every respect identical to the ones containing the antibody, but of course, lack the antibody. These will be used to determine non-specific binding of the hormone to the walls of the experimental tubes.

### Calibration and Experimental Data

In our experiment, a control blood sample was taken from an animal. A given amount of angiotensin II was then injected into the animal, and blood samples were collected at 2, 4, 6, and 8 hours after injection of angiotensin II. Angiotensin II stimulates cells of the zona glomerulosa of the adrenal cortex to secrete **aldosterone**. The blood samples were centrifuged to separate plasma. Plasma was then assayed for the **concentration of aldosterone** by using a commercially available aldosterone RIA kit (from Diagnostic Products Corporation, Los Angeles, California). The data that we will use for calibration are presented in the table below.

| Tube Number | Sample Information | CPM |
|---|---|---|
| 1 | Non-Specific Binding | 423 |
| 2 | Only Hot Aldosterone (no Cold) | 10231 |
| 3 | 25 pg/ml of Cold | 9750 |
| 4 | 50 pg/ml of Cold | 9392 |
| 5 | 100 pg/ml of Cold | 8173 |
| 6 | 200 pg/ml of Cold | 6390 |
| 7 | 600 pg/ml of Cold | 3754 |
| 8 | 1200 pg/ml of Cold | 2320 |

It is important to note that **in each of the tubes** the **same amount** of hot Aldosterone is added. The key idea in calculating the concentration of Aldosterone is the following:

$$\frac{Concentration\ of\ Cold}{Concentration\ of\ Hot} = \frac{Fraction\ Bound_{Cold}}{Fraction\ Bound_{Hot}} \tag{6}$$

The ratio on the right is easy to calculate. Let the Fraction Bound$_{Cold}$ be labeled FB(cold), and the Fraction Bound$_{Hot}$ be FB(hot). Then FB(cold)/FB(hot) = 1/FB(hot) - 1 = ($B_0$ - NSB)/($B$ - NSB) -1 = ($B_0 - B$)/($B$ - NSB). Thus, the above equation becomes:

$$\frac{Concentration\ of\ Cold}{Concentration\ of\ Hot} = \frac{B_0 - B}{B - NSB} \tag{7}$$

Thus, we have:

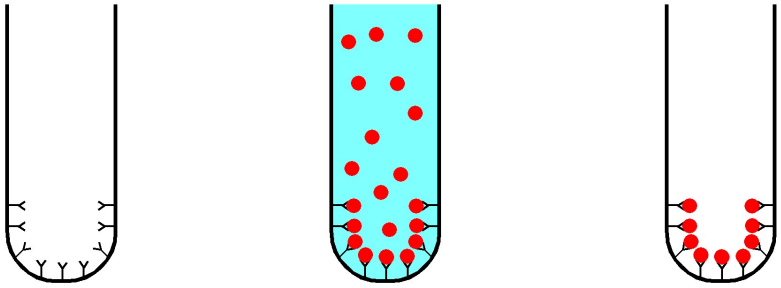
$$Concentration\ of\ Cold = (Concentration\ of\ Hot)\frac{B_0 - B}{B - NSB} \tag{8}$$

This equation can be used to calibrate our experiment. For all the cases, $B_0 = 10231$ and NSB = 432. The values of $B$ are in the last column on the right, and the Concentration of Cold is the middle column in the table. If we let $x \equiv \frac{B_0 - B}{B - NSB}$, a graph of (Concentration of Cold) versus $x$ should yield a straight line with a slope of (Concentration of Hot). This will be our calibration graph. Using the data in the table, we will make the calibration graph and apply it to the plasma sample data of an animal. Suppose for example, a plasma sample recorded 6641 CPM after measuring the tube. This is the value of $B = 6641$. From the above values of $B_0 = 10231$ and NSB = 423, $x = (10231 - 6641)/(6641 - 423) = 0.577$. We can use this value of $x$ in the calibration equation to find the Cold Concentration in the plasma sample.

One nice feature of this kind of assay is that one doesn't need to know the concentration of the Hot hormone. As long as the "Concentration of Hot" is the same in each tube throughout the experiment then the technique will work.

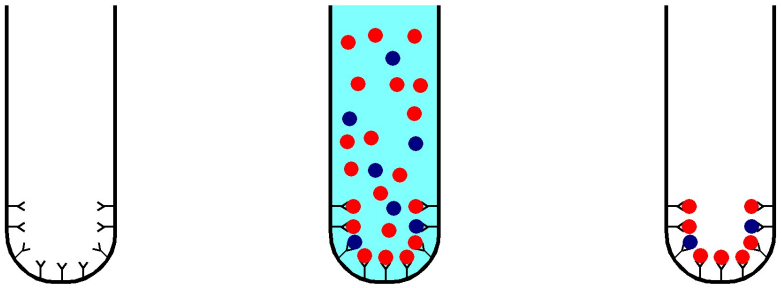
- **Hot hormone (fixed amount)**
- **Cold hormone (variable amount)**
- Hormone antibody attached to tube (fixed amount)

## A. Total Count ($B_0$)



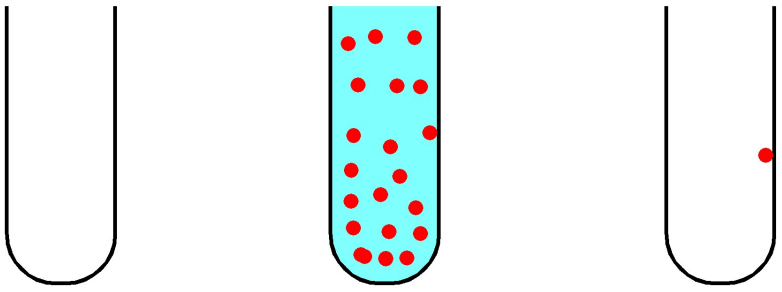
1. Tube with antibody attached
2. Hot hormone added to tube. Incubate.
3. Discard unbound hot hormone. Count in a gamma counter ($B_0$).

## B. Sample Counts ($B$)



1. Tube with antibody attached
2. Hot hormone added to tube. Cold hormone added (known amount or unknown sample). Incubate.
3. Discard unbound hormone. Count in a gamma counter ($B$). $B < B_0$

## C. Non-Specific Binding



1. Tube without antibody.
2. Hot hormone added to tube. Incubate.
3. Discard unbound hormone. Count in a gamma counter ($NSB$). $NSB <<<< B_0$