CHAPTER 1

Performing Quantitative Experiments with Biomolecules

THE IMMEDIATE GOAL OF MANY QUANTITATIVE experiments is to answer the question "how much?" or "how fast?" What concentration of protein is required to bind to a specific DNA sequence? How fast does an RNA dissociate from its target protein? The ultimate goal is to use the answers to such questions to develop models for how biological molecules function in vitro and in cells. Scientists have developed terminology and equations to standardize the discussion and measurement of "how much?" and "how fast?" This book helps navigate the terminology and equations so that they can be readily applied to many biological binding reactions.

Topics covered:

- Quantitative versus qualitative studies
- Measuring the concentrations and activities of biomolecules
- Common techniques used to quantitatively assess binding
- Experimental considerations and data quantitation

Measuring "how much?" or "how fast?" requires performing quantitative experiments. Therefore, it is important to understand the difference between quantitative and qualitative experiments and when each is most appropriate. One is not superior to the other; each provides unique and potentially useful information. Finally, to perform quantitative experiments, it is essential to have accurate measurements of the total amount and activity of the biomolecules being studied before beginning the experiments. We have provided hypothetical illustrations throughout the chapter to help demonstrate the practical aspects of these topics. In addition, a computer simulation is provided on the Web site (http://kinetics.cshl.edu) that can be manipulated to help visualize concepts presented in the chapter.

TERMS AND PRINCIPLES

Quantitative is formally defined as relating to, concerning, or based on the amount or number of something. When applied to biological binding reactions, quantitative experiments typically seek to place a numerical value on the progress of a reaction or the rate at which it

Qualitative is formally defined as relating to or based on the quality or character of something, often as opposed to its size or quantity. When applied to biological binding reactions, qualitative experiments typically seek to make observations about a system that often do not involve quantitation of the results.

Concentration versus amount is an important distinction to make when designing quantitative experiments. The amount of a biomolecule is independent of the volume of a solution and can be expressed in units such as grams or moles. Concentration depends both on the amount of the biomolecule and on the volume of the solution and is defined as the amount of a biomolecule in a sample divided by the volume of the sample. The most useful expression of concentration is molar (M; moles/liter).

Standard curves are used to determine the amount of a molecule in a sample. Obtaining a standard curve typically requires titrating a known amount of a biomolecule and measuring the signal intensity for the assay being used. Data points are plotted with signal intensity on the *Y* axis and the amount of the biomolecule on the *X* axis. The points are then fit with a line, and the line is used to quantitate the amount of biomolecule in a sample of unknown concentration.

Linear range describes the region of a standard curve in which the signal intensity increases linearly as the sample amount increases. Amounts of sample that fall outside of the linear range for a given assay are not used in generating the standard curve.

Fractional activity refers to the portion of a sample that is functional. For example, a preparation of an RNA that binds a protein may contain some fraction of molecules that are improperly folded and cannot bind the target protein. The fractional activity of this sample would therefore be less than one, because not all of the RNA will have protein-binding activity.

QUANTITATIVE VERSUS QUALITATIVE

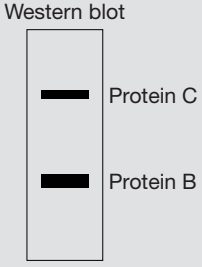
Typically, the ultimate goal of quantitative experiments is not simply to ascertain "how much?" or "how fast?" but to provide insight into how a reaction occurs. Determining quantitative parameters such as affinity between biomolecules and the rates of reaction steps, for example, can provide a model of how a reaction occurs, typically referred to as the mechanism of the reaction. Developing a model for the mechanism of a reaction is qualitative; therefore (ironically), quantitative experiments ultimately provide qualitative descriptions of reactions. For example, consider two proteins that bind overlapping DNA sequences, and both proteins cannot occupy the DNA simultaneously. Quantitative experiments reveal a tenfold difference in the affinities with which each protein binds its respective DNA sequence. Knowing that the concentrations of the two proteins are similar inside cells, one model to describe their function is that the protein with greater affinity typically occupies the DNA and the other does not. Once such a model is developed, further quantitative or qualitative experiments can be designed to test that model.

Determining whether a quantitative or a qualitative experiment is more appropriate depends on the nature of the information sought. If the goal is to determine whether or not a protein binds a specific DNA sequence, a qualitative experiment that simply detects the interaction will suffice. If the goal is to determine the affinity of the protein for a DNA sequence, or differences in the affinities for two different DNA sequences, a quantitative experiment will be required. Often, qualitative experiments provide initial observations that can then be investigated further using quantitative experiments. For example, a qualitative experiment may identify a molecule that inhibits a biological binding reaction. To then characterize the potency of inhibition and/or the mechanism by which the inhibitor functions, quantitative experiments would be required.

Discerning the differences between qualitative and quantitative experiments can help to avoid erroneous conclusions caused by failing to appreciate the limitations of an experiment. In general, qualitative interpretations can be gleaned from quantitative experiments, but the converse is often not true. For example, an experiment that measures the affinity of a protein–protein interaction will provide a quantitative description of the binding energy for the interaction. Finding that two proteins interact with low affinity might also provide the qualitative prediction that other factors likely facilitate this interaction in cells. In contrast, from qualitative experiments, it is typically impossible to draw quantitative interpretations regarding parameters such as affinity, association rates, and kinetic stability. This point is emphasized in Illustration 1.1.

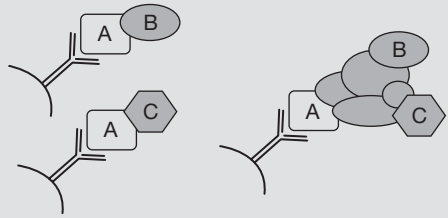
Illustration 1.1. Quantitative versus qualitative western analysis.

Protein A is immunoprecipitated from a cellular extract and associated proteins are detected by western blot with two antibodies: one that detects protein B and one that detects protein C. The band for protein B is much darker than that for protein C in the immunoprecipitated sample (see schematic of gel below). Is more protein B bound than protein C? Unfortunately, a quantitative interpretation cannot be made from the western data. The two antibodies are likely to recognize their epitopes with different affinities, making it impossible to correlate the amounts of proteins B and C in the immunoprecipitated sample with their relative signal strengths. When performed as described, this is a qualitative experiment that leads to the conclusion that both protein B and protein C are in a complex with protein A in the extract.



One way to make this experiment quantitative is to include on the western blot titrations of known amounts of proteins B and C in order to generate standard curves. The amounts of protein B and protein C that are coimmunoprecipitated can be calculated using the standard curves and the signal intensities for the experimental bands. This approach is explained in Illustration 1.5.

Even if the experimental result is quantitated, there are limitations on what can be concluded. For example, from the immunoprecipitation results described, it is not possible to conclude that proteins B and C bind directly to protein A; they could associate with a large macromolecular complex that contains A via interactions with other subunits, as illustrated in the schematic below. To measure relative affinities for the two interactions, purified proteins of known concentrations must be used, thereby allowing direct interactions to be studied quantitatively. Techniques to do so are described in Chapter 2.



MEASURING THE CONCENTRATIONS AND ACTIVITIES OF BIOMOLECULES

To correctly interpret data from quantitative experiments, it is imperative to know the purity, concentration, and activity of each biomolecule in the experiment. For example, a measurement of the affinity of an interaction between two biomolecules is only as good as the measurements of the concentrations of each of the interacting partners. In the following sections, we discuss general considerations for determining purities, concentrations, and activities of biomolecules.

Sample Purity

It is important to consider the purity of a sample before measuring the concentration of a biomolecule in the sample. For example, contaminating cellular nucleic acids in a plasmid prepared from bacterial cells will increase the UV absorbance of the sample, thereby artificially inflating the apparent concentration of the plasmid. If a sample is not pure, then either additional purification must be performed or a method must be used to determine the concentration of only the biomolecule of interest in the nonhomogeneous sample, usually by viewing that molecule directly, as opposed to quantitating the total amount of molecule(s) in the sample. For example, proteins and nucleic acids can be separated by gel electrophoresis, thereby allowing the desired biomolecule to be viewed independently of other contaminating biomolecules in the sample. Quantitative experiments can be performed with a nonhomogeneous sample if two conditions are met: (1) Techniques are employed to quantitate only the desired molecule and not contaminants when determining concentration and (2) the contaminants do not influence the reaction to be studied.

Measuring Concentrations of Biomolecules

Performing accurate quantitative experiments usually requires determining the concentrations of biomolecules in stock solutions; for example, a plasmid DNA preparation, a newly prepared recombinant protein, or a small molecule ligand. Techniques to measure concentration rely on the comparison of a sample of unknown concentration to a linear standard curve. For certain instruments, the researcher need not generate this curve because others have predetermined the linear range of detection. For example, using a spectrophotometer to determine the concentration of DNA in a solution does not require the generation of a standard curve because it has been determined that absorbance (A_{260}) measurements are

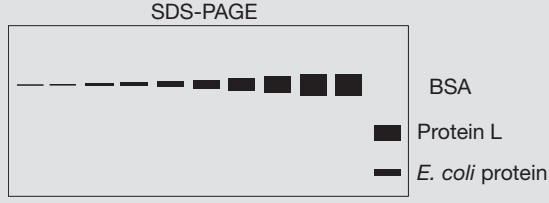
linear between 0.2 and 1.0. Typically, the DNA sample is diluted so that the A_{260} of the diluted sample is between 0.2 and 1.0 and the concentration of DNA is calculated from the A_{260}, the extinction coefficient for DNA (which is known), and the dilution factor. This same concept applies to determining the concentration of a pure protein; the extinction coefficient of the protein and the absorbance at 280 nm can be used to calculate the concentration. Alternatively, any of several commercially available reagents (e.g., a Bradford assay) can be used.

In contrast, many techniques to determine the concentrations of biomolecules will require generating a standard curve. For example, if a DNA or protein sample is too dilute, too impure, or in too small a volume to determine its concentration by measuring the absorbance, then a standard curve must be generated. Gel electrophoresis can be used to determine the concentration of the DNA or protein in the sample as follows. A sample of a known amount of DNA or protein is run on the appropriate gel with the unknown sample to generate a standard curve. The gel can be quantitated and the concentration of the unknown sample determined from the standard curve, as explained in Illustration 1.2.

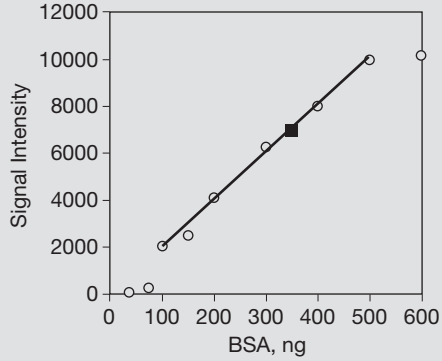
Illustration 1.2. Generating a standard curve and determining the concentration of a protein.

Protein K is a recombinant protein that has been expressed in *Escherichia coli* and purified by chromatography. A contaminating *E. coli* protein is present in the preparation of protein K as determined by SDS-PAGE (denaturing polyacrylamide gel electrophoresis). How should the concentration of protein K be determined? A technique such as absorbance at 280 nm or a Bradford assay cannot be used because the contaminating protein would contribute to the signal. Instead, protein K should be resolved from the contaminating protein by SDS-PAGE and then visualized and quantitated using a standard curve, as described below.

Protein K and a titration of known amounts of bovine serum albumin (BSA) (or another convenient protein) are subjected to electrophoresis through the same gel, as shown below.



The gel is stained with Coomassie, the intensity of each BSA band and protein K band is quantitated by densitometry, and the BSA results are used to generate a standard curve. A plot is generated with the signal intensity of the BSA bands on the *Y* axis and the amount of BSA on the *X* axis (circles in the plot). The points in the region of the curve that is linear are fit with a line to generate a standard curve that relates the amount of protein to a signal on the gel. (Fitting data with a line is discussed in Chapter 7.) Notice that some of the lowest and highest amounts of BSA are not included in the curve fit, because, for those amounts of protein, the signals are out of the linear range.



The solid square on the plot is the signal from the protein K band, which falls within the linear range of the standard curve. Importantly, if the signal intensity for this band were outside of the linear range, then this gel could not be used for quantitation of the amount of protein K. Another gel would have to be run, including known amounts of BSA to generate the standard curve and an amount of protein K that falls within the linear range. Using the equation for the line and the signal from protein K for the *Y* value, the amount of protein K that was loaded on the gel can be calculated.

Sample calculation to determine amount of protein K:

Equation that fits the standard curve: $Y = 20X - 84$

Signal intensity from protein K sample (filled square): 6921

Using this value as *Y,* solve for *X* to obtain 350 ng of protein K

The molecular mass of protein K is 50 kD; therefore, 7 pmoles of the protein was loaded on the gel. The volume of the protein sample loaded on the gel was 5 μl; therefore, the concentration of protein K in the sample is 1.4 μM.

Assessing the Activity of Biomolecules

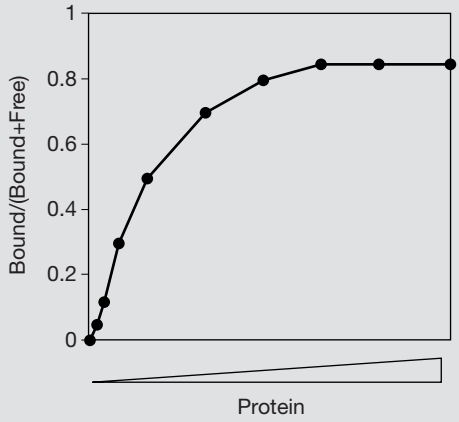
After the purity and the concentration of a sample are assessed, the activity of the biomolecule must be determined. Occasionally, preparations of biomolecules consist of mixtures of active and inactive forms, for reasons that may not be known. The portion of the total biomolecule that has activity is referred to as the fractional activity. It is important to measure the fractional activity of a sample before embarking on quantitative experiments for at least two reasons. First, if the majority of biomolecules in a sample are inactive, then the sample may need to be further purified or repurified to remove inactive molecules that could potentially inhibit the function of the active molecules. Often, it can be helpful to assess fractional activity at steps during the purification process. Second, knowing the fractional activity allows the concentration of the active species in solution (rather than the total concentration) to be used in quantitative studies. For example, a protein that has been purified to homogeneity may consist of some molecules that are active and some that are inactive (e.g., misfolded). If the total concentration of protein in the sample is used to design and perform quantitative experiments, then the measurements made will be incorrect. Clearly, the concentration of active protein in the sample should be used.

Determining the fractional activity of a preparation of a biomolecule requires using an activity assay for the reaction of interest. The simplest way to describe how to determine fractional activity is through Illustrations. In Illustrations 1.3 and 1.4, we discuss two approaches for determin-

Illustration 1.3. Measuring the fractional activity of an RNA that binds a protein.

A new preparation of a ^{32}P-labeled small noncoding RNA is made, and the fractional activity of the preparation must be determined. The protein known to bind this RNA is available, and the RNA–protein complex can be separated from free RNA using native gel electrophoresis. A series of binding reactions is set up with RNA held at a constant concentration and the amount of protein is titrated. The RNA–protein complex in each reaction is separated from free RNA using native gel electrophoresis, and phosphorimagery is used to quantitate the amount of bound RNA and free RNA in each case. The fraction of RNA in a complex with protein is calculated (bound/[bound + free]) and plotted versus the concentration of protein added to reactions, as shown in the plot on page 9 (the data

points are simply connected with lines). The fraction bound reaches a plateau at a value of 0.85; hence, the fractional activity of the RNA preparation is 0.85. In other words, 85% of the RNA is capable of binding the protein. The other 15% of the RNA cannot bind the protein; perhaps this RNA does not have the native secondary or tertiary structure. Therefore, the concentration of functional RNA, which is used in quantitative experiments, is 85% of the measured concentration of total RNA.



ing the fractional activity of a molecule. Because binding reactions are the primary focus of this book, these reactions are used as examples. First, we discuss how to determine fractional activity for a molecule that can readily be labeled and detected.

In some cases, it might not be possible or practical to directly visualize (e.g., label) the molecule for which fractional activity needs to be measured. This is often the case when the molecule of interest is a protein. It is possible, however, to measure the fractional activity of an unlabeled molecule by observing its interaction with a labeled binding partner, as explained in Illustration 1.4 (see also Simulation S1 on the Web site). Note that it is critical to know the stoichiometry of the molecules in the complex and to use the concentration of the functional oligomer. For example, if a protein binds a target as a dimer, the concentration of dimer (i.e., half of the concentration of monomer) should be used in reactions.

Illustration 1.4. Measuring fractional activity of a DNA-binding protein.

A new preparation of protein A, which binds DNA as a dimer, has been made and the fractional activity for binding its target DNA must be determined. Protein A cannot be easily labeled, so a DNA fragment containing the binding site for protein A is ^{32}P-labeled to allow monitoring of the DNA–protein A complex by filter binding. The approach to measure fractional activity requires two steps. First, the amount of DNA required to fully saturate a set amount of protein A is determined. (This step is required because in the second step, the concentrations of both the protein and the DNA need to be above the affinity constant that governs their interaction. For a detailed discussion of this concept, see Chapter 2.) A series of reactions is set up in which the concentration of protein A dimers is held constant at 10 nM, and the DNA is titrated from 10 nM to 800 nM. The DNA–protein A complex that forms is monitored by filter binding, followed by scintillation counting to determine the amount of complex retained on the filter. As the DNA is titrated up, the amount of complex plateaus when the protein A dimers are fully saturated. A concentration of 100 nM DNA is well within this plateau region, so this concentration of DNA is chosen for the second step.

A second series of reactions is set up in which the DNA is now held constant at the saturating amount (100 nM as determined in the first step), and protein A dimers are titrated from 10 nM to 500 nM. As in the first step, the complex is monitored using filter binding. Scintillation counts of complexes retained on the filters are plotted versus the concentration of protein A dimers added to reactions, as shown below. The plot indicates, given a DNA concentration of 100 nM, that a concentration of 140 nM protein A dimers is required to produce the maximum amount of complex. Dividing the concentration of DNA used in the reactions by the concentration of protein A dimers at saturation gives the fractional activity of the protein A preparation, which is calculated to be 0.7 (100 nM/140 nM). Thus, the concentration of functional dimers in subsequent experiments would always be equal to 70% of the total concentration of protein A.