FAQ: How can I determine my protein's concentration with Tycho?

Tycho™ NT.6 uses protein fluorescence as a readout to look at sample quality via thermal unfolding. In addition, the recorded fluorescence can also be used to investigate protein concentration. This FAQ will tell you everything you need to know about this topic.

Subquestions

I always thought determining protein concentration via fluorescence was impossible?

This is what most people remember from school, but the paradigm on this is changing. What is true is that fluorescence cannot be used in the same way that absorbance is used: to calculate protein concentration from one measurement without the need of a reference. As you probably know, the most common way to measure protein concentration is to measure its absorbance via UV/Vis spectroscopy. Then, using the exctinction coefficient ε (which can be calculated from the protein's amino acid sequence), the measured absorbance value can be converted into a concentration. For fluorescence, this approach is not feasible: fluorescence is a much more complex thing than absorbance, and a protein's fluorescence signal cannot be predicted from its sequence (yet). However, fluorescence can be used very nicely when a different approach is applied. We'll walk through this approch in this FAQ.

Why can't I predict my protein's fluorescence?

A protein's fluorescence signal depends on many different variables that interact in a very complex way. One major factor is of course the amino acid sequence and the numbers of fluorescent amino acids. For Tycho measurements, the relevant amino acids are Tryptophan and Tyrosine. Generally, the more Tryptophan and Tyrosine residues a protein has, the higher its fluorescence signal will be. However, all fluorophores, and Tryptophan especially, are very sensitive to its surroundings. (We make use of this phenomenon for many things: it's the reason why we can see protein unfolding in Tycho and Prometheus measurements, and it's also the reason why we can analyze molecular interactions via fluorescence in Monolith and Dianthus measurements, using MST and TRIC technologies.) The way a protein is folded will influence the fluorescence signal, because it will influence the immediate surroundings and neighboring molecular groups of the Tryptophan residues. To get an idea of how much the protein's structure influences fluorescence, check out our FAQ on the lower limit of detection on Tycho. It contains a list of proteins, how many Tryptophan and Tyrosine residues they contain, and how low of a concentration you can still use in Tycho. You'll see that even proteins with very similar numbers of fluorescent residues can have very different fluorescence signals, resulting in very different lower limits of detection.
Also, the buffer plays a big role, because Tryptophan is also very sensitive to its chemical environment.

Absorbance is also influenced by these factors, but to a much smaller degree. That's why it's commonly accepted to calculate a protein's extinction coefficient from its amino acid sequence. It's not very accurate, though, and many people aren't aware that an extinction coefficient based on pure calculation can be quite off.

If I can't predict my protein's fluorescence, that means I will need a known reference, right?

Correct. In order to determine protein concentration with Tycho, you will need a reference sample of the same protein, in the same buffer, of which you know the concentration. This somewhat limits the situations in which this approach can be used, but it's still very useful for example

- To compare protein concentration in routine workflows, for example from one prep to the next
- To re-check concentration after storage or dilution, or before an important assay where concentration is relevant

Keep in mind that for many other concentration determination techniques, you also need references: for colorimetric assays like Bradford and Lowry assays, for example. Here, it is common to use a different protein for a reference. Often, a reference curve is established using bovine serum albumin (BSA), for example. This approach has its advantages, of course, but it is often overlooked that many proteins behave quite differently from BSA and the concentrations determined with this approach aren't always very accurate.

So I'll need to establish a standard curve using a dilution series?

Actually, no! We found that one known concentration data point is enough. The standard "curve" is simply a straight line drawn through this one point and zero. More details on this follow.

If the buffer contains any fluorescent substances, a buffer blank can be measured and used instead of the zero.

That sounds pretty simple.

It is.

Do I need to re-measure my reference every time I want to determine a new sample's concentration?

No, this is not necessary. As long as you know that you're still working in the same buffer, you can pull up references measured in the past and use them in the calculation.

Won't the fluorescence signal be different across runs though? I know that the LED in Tycho always adjusts to how bright my samples are, so how can I compare?

The LED always adjusts to make sure the fluorescence intensity is within what the detector can see, that's correct. Excitation power and fluorescence intensity are linearly correlated (within certain conditions), meaning that if I double the excitation power, I will get double the fluorescence intensity. This means that by remembering and factoring in the excitation power used, Tycho can easily calculate how bright the sample "originally" is. That's why we call it "Sample Brightness" and not "Fluorescence Intensity" – it's a normalized value that's intrinsic to the sample, and we wanted to give it a different, new name. A sample with a Sample Brightness of X will always be measured to have a Sample Brightness X on Tycho, no matter what excitation power was used.

For the rest of this FAQ, we'll use the term "Sample Brightness" when talking about the value that Tycho outputs, and "fluorescence" when talking about the physical phenomenon. The concept of Sample Brightness is different from how you usually think about the fluorescence signal of a sample, and it's set up specifically to allow comparisons across runs, and even across Tychos.

Wait – even across Tychos? Will they be similar enough?

Yes. We purposely developed and calibrate Tycho in a way that allows such comparisons across systems.

How does Tycho record this Sample Brightness exactly?

As you know, Tycho records the fluorescence signal of the sample at 330 nm and at 350 nm throughout a run. At the beginning of the run, after the system has heated up to the starting temperature of 35 °C, it takes an extra-thorough reading. This is used to calculate the Sample Brightness. The Sample Brightness is the brightness at 330 nm **plus** the brightness at 350 nm, at 35 °C. It's expressed in relation to a standard sample that we use for Tycho calibration. So if a sample is twice as bright as our standard calibration sample, it will be output to have a Sample Brightness of 2.

OK. So how do I calculate my sample's concentration from its Sample Brightness?

It's actually really simple:

$$\frac{SB_R}{c_R} = \frac{SB_u}{c_u}$$

For a certain concentration range (more on that later), the Sample Brightness and the concentration of a protein sample show a linear correlation. Therefore, we know that the Sample Brightness of the reference SB_R will relate to its (known) concentration c_R just like the Sample Brightness of your unknown sample SB_u will relate to its unknown concentration c_u. Since we know SB_R, c_R and SB_u, we can easily calculate c_u by rearranging the equation to

$$c_u = \frac{SB_u c_R}{SB_R}$$

Essentially, what we do is this (Figure 1): We know one sample's concentration and Sample Brightness (blue datapoint). We draw a straight line through this datapoint and zero (gray line). We know the Sample Brightness of the sample where we don't know the concentration. Since we know that Sample Brightness and concentration correlate linearly, we can infer the concentration of this unknown sample from its Sample Brightness (green dashed line).

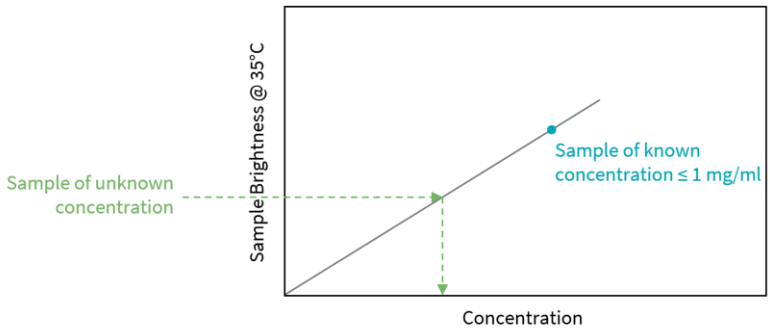

Figure 1: Inferring a sample's concentration from its Sample Brightness.

When we know the buffer to have a fluorescence signal of its own, we simply record its Sample Brightness too, and subtract this buffer blank from our other Sample Brightness values. We end up with a corrected Sample Brightness that allows us to conclude the unknown concentration again. Graphically, this looks like Figure 2:

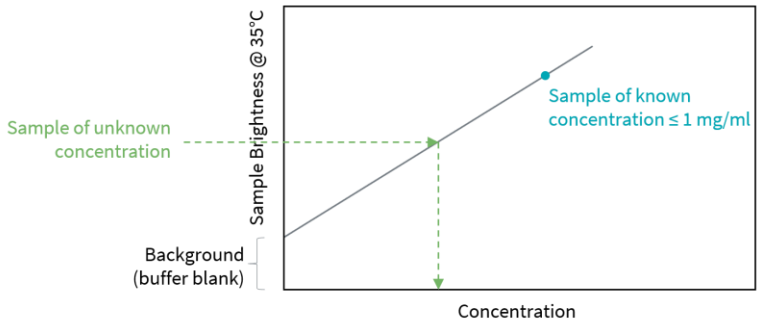

Figure 2: Inferring a sample's concentration from its Sample Brightness when the buffer is fluorescent.

We're working to integrate an easy workflow for this in our software. For now, you can either do the calculation yourself, or test out a software prototype that we're working on. Get in touch with us if you're interested.

What's the concentration range for this approach?

We've found that this approach works best for concentrations between 0.01 mg/mL and 1 mg/mL. The exact limits vary from protein to protein – as described above, fluorescence is very complex. Let's look at these limits in detail:

Lower limit

For most commonly used proteins – standard IgGs, and things like BSA, streptavidin, lysozyme – we find that 0.01 mg/mL still gives a good, clean signal for concentration measurements as well as for thermal unfolding. Some proteins have a very high intrinsic Brightness and can therefore be used at even lower concentrations (we'll talk a little about typical Sample Brightness values further below). We have also seen some proteins for which concentration determination works really well down to 0.001 mg/mL, even though the signal isn't good enough anymore to record a nice unfolding profile or to detect T_i. If you want to be sure how low you can go for your protein, measure some replicates and see how much they vary.

Upper limit and the inner filter effect

The upper limit that we run into is due to a phenomenon called the inner filter effect. In order for fluorescence to happen, excitation light gets partly absorbed by the fluorophore molecules and re-emitted as fluorescence. If the concentration of fluorophores is very high, many such molecules are present, and much of the excitation light is absorbed by the molecules closest to the light source, while the molecules farther away from the light source may not receive much light:

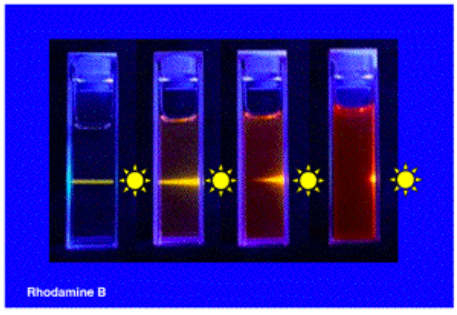

Figure 3: Image taken from https://www.mrc-lmb.cam.ac.uk/rlw/text/tutorials/inner_filter_correction.htm, where the source is cited as Jameson et. Al., Methods in Enzymology (2002), 360:1.

For these farther-away molecules, less light is available and they won't fluoresce (much), meaning that the overall fluorescence signal of the sample will be lower than expected. As a result, concentration and fluorescence correlate in a linear way for low concentrations, but not for high concentrations. For an example, here's the Sample Brightness of BSA from a concentration of 122 nM (0.008 mg/mL) up to 250 µM (16.6 mg/mL). At low concentrations, the curve starts out straight, but then it levels out. The red data point represents 15.6 µM (1 mg/mL), which can be considered the last data point in the linear range.

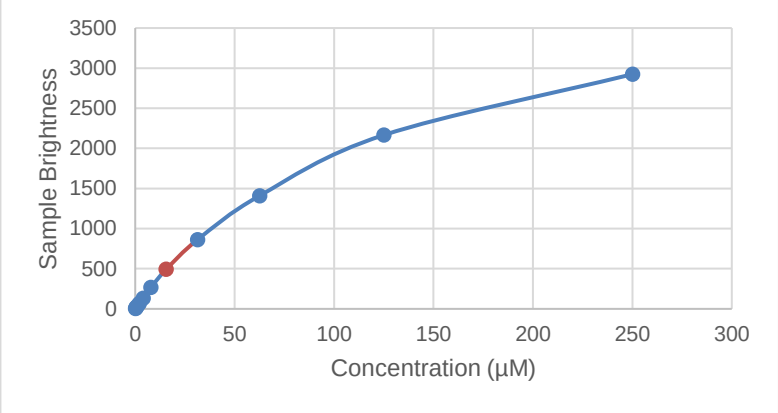

Figure 4: Serial dilution of BSA from a concentration of 250 µM (16.6 mg/mL) down to 122 nM (0.008 mg/mL). The red data point represents 15.6 µM (1 mg/mL), which can be considered the last data point in the linear range.

Now, since we have all of this information for BSA, we could theoretically use Sample Brightness to determine pretty much any concentration of BSA up to at least 250 µM. But this necessitates recording this whole standard curve for many concentration points. To simplify the process, we recommend to stay within the linear range (up to around 1 mg/mL, see red datapoint in Figure 4), where one known data point is enough to determine the concentration of an unknown sample (because you can draw a line instead of a complex curve). Where exactly this range lies for your protein will vary. We found 1 mg/mL to be safe, but if you're curious whether your protein's linear range extends to higher concentrations, just try it out!

But I thought Tycho could handle high concentrations (up to and above 200 mg/mL) too?

Yes, it can – and we did that for the BSA standard curve above. High concentrations are routinely measured on Tycho for looking at protein quality. Tycho is set up in a way that allows investigation of very high concentrations and Sample Brightnesses, because Tycho can simply turn down the LED to reduce the fluorescence signal and avoid oversaturation of the detector. However, for concentration determination we recommend to stay within the linear range described above, simply to make the whole process easier and more convenient. The inner filter effect is a limitation imposed on this approach by sheer physics – it's not a technical limitation of Tycho or Tycho technology.

Can you tell me what Sample Brightness values I should be expecting for my concentration determination assay?

We can tell you about our experience, but again, it's hard to generalize because this is going to vary for each protein. For example, BSA is pretty bright, but not extreme. For BSA, in the conditions and buffer that we used for the dilution series measurements shown in Figure 4, the linear correlation extends to

about 1 mg/mL, which shows a Sample Brightness of around 500. The lowest BSA concentration that we measured was 0.008 mg/mL, which showed a Sample Brightness of 4.6.

We've noticed that for Sample Brightness values below 4, we get more noise, so if you go below that, you might want to measure some replicates to counteract that.

Concerning the upper limit, again, it will be set by the specific protein and when it starts to exhibit the inner filter effect.