

FAQ for a standard curve

From Ainslie Lab @ UNC ainslielab.web.unc.edu

Standard curves are needed for many analyses in the lab including (but not limited to): release profiles, encapsulation efficiency calculations, Greiss assays, and ELISAs. There is a standard way to make up these curves and here are some FAQs.

Question: When should I run a standard curve?

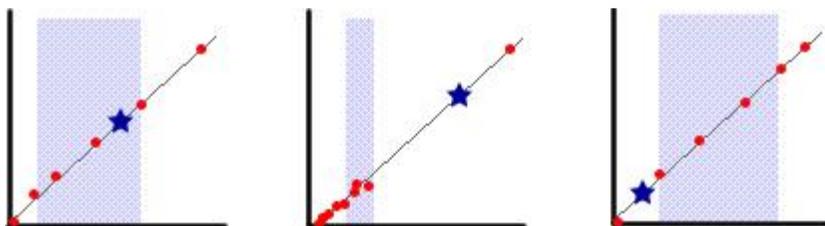
A standard curve should be run for every plate or sample run. What this means is that if you are running your analysis in a 96-well plate that each plate should have a standard curve. Exceptions to each plate can be afforded if the drug or agent used for the standard is of limited supply or very costly. But at a minimum you should run one standard curve with each set of plates for that day. If you are running analysis on a LCMS or HPLC each daily run should have a standard curve.

Question: Why does a standard curve need to be run so often?

Variances can occur in many things that comprise the standard curve, including bulb intensity of the plate reader, solution concentrations if a new solution is made every time, plate thickness from batch to batch or manufacturer to manufacturer, degradation of components in solution, variation in pipette volumes etc. These differences can be noted in the observed in the background (empty wells) or even wells containing control solutions (e.g. PBS, media). Sometimes the variance can be significant, and sometimes it is not. If you are analysing in a fluorescence or luminescent spectrum something else that can significantly affect your readings is whether the plate reader or fluoro-/lumino-meter is set to automatically change the reading values based on the maximum fluorescent intensity observed in the plate. This is often the default value for many plate readers and can adjusted in the reader program.

Question: What concentration range should I run my standard curve?

The concentration range is sometimes set if you are running standard assays from a kit like an ELISA or a Greiss Assay. If you have to establish your own standard curve you should perform some calculations to find the actual upper limit you should use. If it is to calculate an encapsulation efficiency, then it should be one or two times the upper limit you would have if 100% of your drug is loaded in your system. Similarly with concentration release, if 100% of your drug is released use that value times one or two. You should then have dilutions from that range to zero. Zero and the solution your standards are made in should always be the same solution (e.g. PBS, media, serum) your samples are ran. Your concentration range should be well distributed with two or more samples above and below your estimated sample concentration. For example, in the figure below the red dots represent the points on the standard curve and the blue star the sample point. The graph of the left is correct, and the other two graphs are wrong. The middle is wrong 1) because the points are not well distributed and 2) there is only one point (an outlier point at that) that is above that value. The right graph is wrong because the only point below the sample point is the last or zero point and there should be at least two points, since two points make a line. For each of the graphs, the valid range for that graph is given in the light blue box. Generally, a standard curve is 5 points or more. Sometimes in a tight sample range it can be less but should not be less than 3.



Question: Should I use 10-fold dilutions or half-dilutions?

The answer to this question all depends on what you expect your sample size to be. Do you expect low controls that are near zero and samples that are in a much higher range? 10-fold dilutions are samples that are diluted 1:1, 1:10, 1:100 and so on. Half-dilutions are 1:1, 1:2, 1:4, 1:8 and so on. I have previously done a range of ten-fold dilutions in combination with half-dilutions to achieve good point distribution within my sample sets. Sometime this question can not be answered without running samples.

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Question: What can I do if my samples end up being outside the range of my standard curve?

There is something you can do that might help in not having to trash all of your work. If you have extra solution available (e.g. PBS, media, serum) to dilute your samples, you can do half or 10-fold dilutions of your sample to get it within a range. You can then run your samples again immediately (in the same plate is possible) to determine your sample readings. If you are concerned that your sample will have degraded or such due to exposure to light or something else, just remember all of your samples were exposed equally, and it will probably be fine. Of course, this would not work with something as precise as fluorescence lifetime.

Question: What should I do if my standard curve has a R-squared value that is very low (<0.7)?

In this situation your experiments are more likely a wash. If your standard curve is poor, then your analysis will be poor. If only parts of your curve are bad and not where your samples are, then perhaps it will be okay, assuming there are enough good points below or above. Without ethical proof that one point is poor due to things like volume error or splashing, a point should not be omitted, that is cherry picking data and just bad science. To limit this error, one can always practice a standard curve first, if feasible.

Question: What can I do to make sure my standard curve is as accurate as possible?

There are several things:

- Volume is of utmost importance with many light-based readings (see Beer's law), having irregular volumes from well to well can significantly affect your readings. Some things to avoid volume errors:
 - Use the same pipettor and use it properly. **Do not** push the pipettor fully down when taking up liquid and **do** push it fully down when expelling liquid. If possible, use the same pipettor for standards and samples.
 - When expelling from the pipettor, minimize splashing
 - Do not shake or disturb the plate very much
 - When mixing, do not pipet up the entire volume and expel the volume under the liquid level (if possible) to avoid accumulation of foam or bubbles
 - Avoid bubbles. In extreme instances a syringe needle can be used to pop bubbles, however, be aware of cross contamination of standard if you are going from well to well
- Use good laboratory practices like writing down how much was weighed. If you weigh 1.1 mg and dilute it in 1 mL, then make your sample concentration 1.1 mg/1 mL not 1 mg/mL. By writing it down you can go back to it later if you have errors.
- Be careful not to get things that would interfere with the signal on the bottom of the plate. Also leaving the lid on the plate when you run it, considering the plate lid can interfere, particularly if it has condensate on it.
- Depending on the accuracy of some plate readers, having wells nearby that are extremely high or extremely low can throw off your readings. By minimizing these extreme neighbors, it might help.
- Running your standards in the same solution as the samples will help as well. Things like media and serum have very high backgrounds and absence of using such solutions can drastically change your data.
- Your standards should be run at the same pH as your samples. If needed run them at the irregular and standard (e.g. 7.4) pH to correlate them to physiological conditions, if needed.
- If you are making your standards outside the plate, you should use a new pipet tip to add the samples to the plate. If you are making your samples in the plate, you can use the same pipet tips for moving the volume and mixing in the well.
- Mix your solutions well.
 - If you are making large volumes and pipetting into a well then mix it well with a pipettor or a vortex.
 - Measure your amounts accurately.
 - Try to minimize your samples dilutions if you have to create a highly concentrated samples and dilute it down to get into the range of your curve. For example, if you have to make a 1mg/mL solution and your highest point is 1 ug/mL, try a 1:1000 (1uL in 999uL) dilution, rather than several 1:10 dilutions.

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- Use a 96-well schematic to keep track of your samples and standards. [Link](#)
- Be careful of splashing to the lid/film or from the lid/film.

Question: Should I use the intercept and slope or just the slope of the best fit line?

Generally the intercept represents the background or zero concentration value. This value takes into account the plastic (or glass) of the measuring entity (e.g. plate) as well as the solution the standard is ran. If the intercept and slope is used, then the value at the zero point should not be subtracted. If the background is subtracted, then the intercept should not be used. The simplest way to use your standard is to use the entire line equation and negate any background subtraction.

Question: What should my n be for my standard curve?

Generally each concentration is ran in at least two wells, perhaps three. Then the average across the wells can be used to determine the value at that concentration as well as if any of the values are outliers. For example, if you have three values and one is >> or << then the others you can perhaps omit that value if it is < or > 2 standard deviations from the average. The more points you have to perform this analysis, the easier and more confident you can be in your data. Of course excessive n's for a standard curve (or samples for that matter) is just excessive and wasteful.

Question: Is there an easy way to make standard curves?

One of the simplest ways to make a standard curve in a 96-well plate is to use a multi-channel pipettor. If you are doing half-dilutions down the length of the plate at a volume of 100uL per a well, place your highest dilution at the top of the plate at a volume of 150uL. Add 50uL to the remaining wells of your standard, and 100 uL of solution at the zero standard. Set the multi-channel to 50 uL and pipette up 50 uL from the highest well, and deposit it in the second well. Mix by pipetting up and down ~5 times and repeat all the way down to the well just before the zero point. If you are doing the same with 10-fold dilutions, simply start with 110uL and pipette down 10uL. Mix well and minimize bubbles.

Question: Should I truncate my curve to better fit my data?

If you have an excess of standard curve points and your sample points might be affected by extraneous points then yes, as long as you have two points above and two points below. This might be most impactful in curves that have 10-fold diluted samples.