ELISA troubleshooting tips – Poor standard curve

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This is the first post in a new series looking at ELISA troubleshooting tips – Poor standard curve

ELISAs, generally, are pretty straightforward assays to carry out. And they can be pretty robust as too.

However, anyone who has spent anytime developing them from scratch will know that there are numerous ways in which they can go wrong as you hunt the right balance of reagents, buffers and analytes to get an ELISA which does what you want.

This often means that when a single thing goes wrong there can be any number of possible reasons for it.

So, if you are having trouble with an ELISA we hope that this new series of blog posts will be helping-hand in troubleshooting them and getting everything back on track.

This first post will look at the possible causes and potential solutions behind a getting a poor standard curve on your ELISA.

Quality Controls (QCs)

The standard curve is perhaps the most important part of your ELISA. It’s what tells you the concentration of whatever you’re interested in in your sample. It might not always be apparent that your standard curve is incorrect. So having confidence in it, is the first step.

We highly recommend using or preparing quality control (QC) samples for each ELISA you're doing.

Ideally, these should be at low, medium and high concentrations, spanning the length of the standard curve, and placed at the start and the end of the ELISA plate.

They should also be at different concentrations to those of the points on your standard curve; we're treating them as actual samples, but with a known concentration against which we can check their accuracy.

Again, in an ideal world QC samples would match your real sample as closely as possible. So try to prepare them in the same matrix as your sample, e.g. serum or plasma.

It's also best to prepare a job-lot of QC samples ahead of time and store them as you would your samples (usually -80°C). Doing this also allows you to check how your assay is performing over time, or between manufacturing lots, assuming your analyte is stable in your storage conditions.

If you find you'll need to make new QC samples in the future, remember to do it before you run out of the older batch, so that you can run both in a test assay and compare them to make sure they're equivalent.

Problems

There are a lot of possible problems you could see with your standard curve, lower than expected absorbance values (low ODs) leading to a flattening of the bottom of the curve, or higher than expected absorbance (high OD's) leading to a flattening of the upper portion of the calibrations curve.

Below we'll list some common causes of poor standard curves, how they might manifest in your results and possible solutions.

Cause: Incorrect standard solution.

Using the wrong concentration of your starting stock for a standard could lead to standards that are either under or over diluted, raising or lowering ODs and shifting your standard curve out of range.

Solution:

Firstly, double check standard stock concentration, dilutions calculations and your dilutions.

Also, if you are reconstituting a standard from a lyophilized vial, ensure that you have followed the manufacturer's instructions. It's generally good practice to vortex vials to ensure all of the material has been reconstituted.

Cause: Degraded standard

If your standard is old or hasn't been stored correctly, it may be possible that it has degraded. This could manifest in a number of ways depending on how the protein degrades and the type of ELISA you're running. But, generally, you'd expect to be seeing lower ODs than with a non-degraded standard.

Solution:

Double check that the standard has been reconstituted in the correct buffer and stored correctly.

Cause: Curve doesn't fit scale.

This can be a tricky one, especially if you're otherwise confident that the curve was prepared correct (i.e. no dilution errors etc). It depends on your familiarity with the ELISA you're performing.

Sometimes, you might just get higher or lower ODs than normal due to using, for example, a different batch of detection reagent (e.g. TMB). So, while your absorbance/OD values may fluctuate assay to assay, the assay itself should still be reliable if everything else is the same.

Again, having run QC samples would help hugely with confidence in the assay here.

Solution:

If this is the first time you've run this ELISA from a specific manufacturer, make sure you're using the suggested curve fitting model. Usually, this is a 4 or 5 parameter logistic curve fitting model (4-PL or 5-PL). However, different software may give slightly different fits, so you could try others (e.g. log-log) to see if that gives you a better fit.

The key is consistency, however.

So, if you've always used a 4-PL curve fit before on this assay *(not every assay from a manufacturer uses the same curve fit – it's just a model of the assay) and suddenly that doesn't work, we'd recommend running the assay again. Suddenly switching to a different curve fit may mean your results are no longer comparable between assays. Especially if you require absolute values.*

Cause: pipetting error

Pipetting errors, such as too much or too little sample, or high CVS, can easily occur when adding samples to a plate.

Solution:

It may be obvious that you've transposed one standard for another on the curve (i.e. the 10 ng/mL sample has a higher OD than the 30 ng/mL standard, but otherwise it all seems normal). If that's the case this can be easily fixed in your curve analysis software.

Poor CV's in standard duplicates can be a sign of pipetting error and reduces the reliability of your standard curve. This can't really be fixed after the fact, meaning the assay may have to be repeated.

Also, be sure to double check that your pipettes are calibrated, especially when using a multichannel pipette.

General tip: Keep your tubes until you have analysed your results!

Try to keep any tubes used for preparing standards, QC samples and samples to one side until you have finished your assay and analysed the results.

This can be an invaluable way of double checking you haven't made a dilution mistake or accidentally mixed up tubes when you subsequently spot an error. For example, sample and tube transpositions can be noticed, or a higher or lower residual volume in a tube can indicate a preparation error.