

ELISA Validation

The right strategy saves much costs!

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ELISA are used as bioanalytical methods in pharmaceutical research and many other fields of life sciences. After successful development and optimisation, an adequate validation is necessary to make the assay suitable for routine use. The validation is important to evaluate the reliability of results of the assay.

■ Only a good validated assay can give trustfulness in its results. No one can believe in any result without the right validation. But sometimes user don't know how to do their validation correctly. In this article main aspects of validations are discussed. Choosing a good validation strategy becomes easier.

For a meaningful validation, you have to know the intended use of the assay. You have also keep track of the costs. Smaller pre-validation with specimens in the context of development can save costs and increase the safety.

An assay is always developed and validated for a special problem. This problem is called the intended use of the assay. The intended use is the lynchpin of all decisions concerning the complexity of the validation. The validation gives answers to questions like: In which range can the ELISA quantify the substance? What is the minimum concentration to measure the substance correctly?

Is the measured value conform to the real value and how large are the variations? Is there any interfering substance which makes all results not reliable if it is in the specimen?

The validation gives you a feeling when you can trust in the results or when you have to act with caution. Validation does not mean, that you will get always

correct results with a validated assay. Anyway, we recommend orientating according to official guidelines for validation, independent of the “intended use”. This makes your own work reproducible and therefore reliable for others. Also a short and simple validation can be in compliance with Q2A^[1] and Q2B^[2]. Q2A defines essential terms and definitions. Q2B gives methodical advice. For definitions we want to refer to further technical literature^[3,4].

FDA report

If the results of the ELISA should be used in a report for the FDA, one has to follow the “Guidance for Industry – Bioanalytical Method Validation”^[5]. The advantage of using this guidance is a good estimation and high reliability of the results for third persons. This advantage is independent of using the ELISA for research or for routine purposes. The guidance does not regulate a precise mandatory procedure. It gives strong recommendations which can be adapted to practical needs. Any adaptation has to be explained by a technical statement.

Level of validation and parameters of validation

The guidance describes full validation,

partial validation and cross validation. It distinguishes between a new assay e.g. for a new drug candidate, a modified assay or a comparison between two methods describing the same analyte. The parameters to validate are: selectivity, accuracy, precision, recovery, calibration curve and stability of analyte. Each of these parameters is elucidated up to the number of specimen and acceptable percentage of variation.

Remarkable are the specific requirements for immunoassays concerning the performance of the assays in specimens. For the stability testing of the analyte is for instance an interference free biological matrix required. This matrix should correspond with the analysed matrix (e.g. blood serum). Already this can be a great challenge for some assays.

A part validation is always required if the analytical procedure of the assay was modified. Also for changes of critical materials like ELISA plates, tracer or buffers. In addition different cross reactivities have to be tested. Matrix effects have to be tested in comparison with biological matrix and pure buffer matrix as well as in the context of dilution experiments. Unspecific binding should also be analysed. The postulation that all used experiments in the validation have to be reported is also valid. This includes also those experiments which have shown unwanted effects. This shows clearly the complexity of the validation if the Intended Use requires an exact knowledge of the reliability of the assay. Therefore the validation can become a great expense factor.

Are cost savings possible?

How can I save costs in validation? The experience shows that negative results in validation, particularly in selectivities and matrix effects, force regularly a modification of ELISA procedures. The required modifications need a re-validation or respectively a part validation with additional labour, costs and documentation inputs. The avoidance of revalidation saves the most costs and time in development. The easiest ways of savings are a good selection of materials and dealing with the problem of selectivity and matrix effects at an early stage.

Selection of material

In assay development it's important to guarantee constant standards for all used materials and their purchasing. All parameters which can have influence on manufacturing should be described in the beginning of all self made materials. Any own manufacturing should be well documented. The production procedures have to be in compliance with guidelines. This is possible by explicit operating procedures as well as standard operating procedures. Every substance e.g. used for tracer or buffer production should be documented by order or batch number. The purchase of manufactured goods is more economic for most labs. You get more safety if you purchase goods from certified manufacturers. In addition they will inform you if changes in manufacturing have been done, if requested by you. For almost any material beginning from plate to buffer certified suppliers are available (**fig. 1**). There are very good reasons, why in regulated areas purchasing from certified suppliers is obligatory.

Negative effects due to selectivity or matrix effects

These problems are often described and generally known and appear in almost every assay. The success of strategies to improve the selectivity and to avoid matrix effects has to be examined within the validation. Generally known



▲ **Fig. 1:** Ready-to-use goods for ELISA are available in great variety - even from certified manufacturers.

and easy to use are the validation methods to analyse matrix effects. The "Guidance for Industry"^[5] refers to the comparison of standard curves in biological matrices and pure buffers as well as to experiments with comparative dilution linearity. Interference caused by cross reacting substances has also to be analysed.

Concerning selectivity there are HAMA (human anti mouse antibodies) blockers, which act only very specifically on HAMAs as well as other solutions which are generally useable for problems. One example is the new sample and antibody dilution buffer LowCross-Buffer which helps to reduce cross reactivities and matrix effects. The use of adequate sample dilution buffer increases the costs per sample only marginal while reliability and expressiveness of ELISA increase strongly. The avoidance of matrix effects leads in many cases to a lower detection limit. Normally cost savings for validation and for loops in development after detection of matrix effects rise above multiple the costs of innovative buffer systems with practicable blocking and assay buffers.

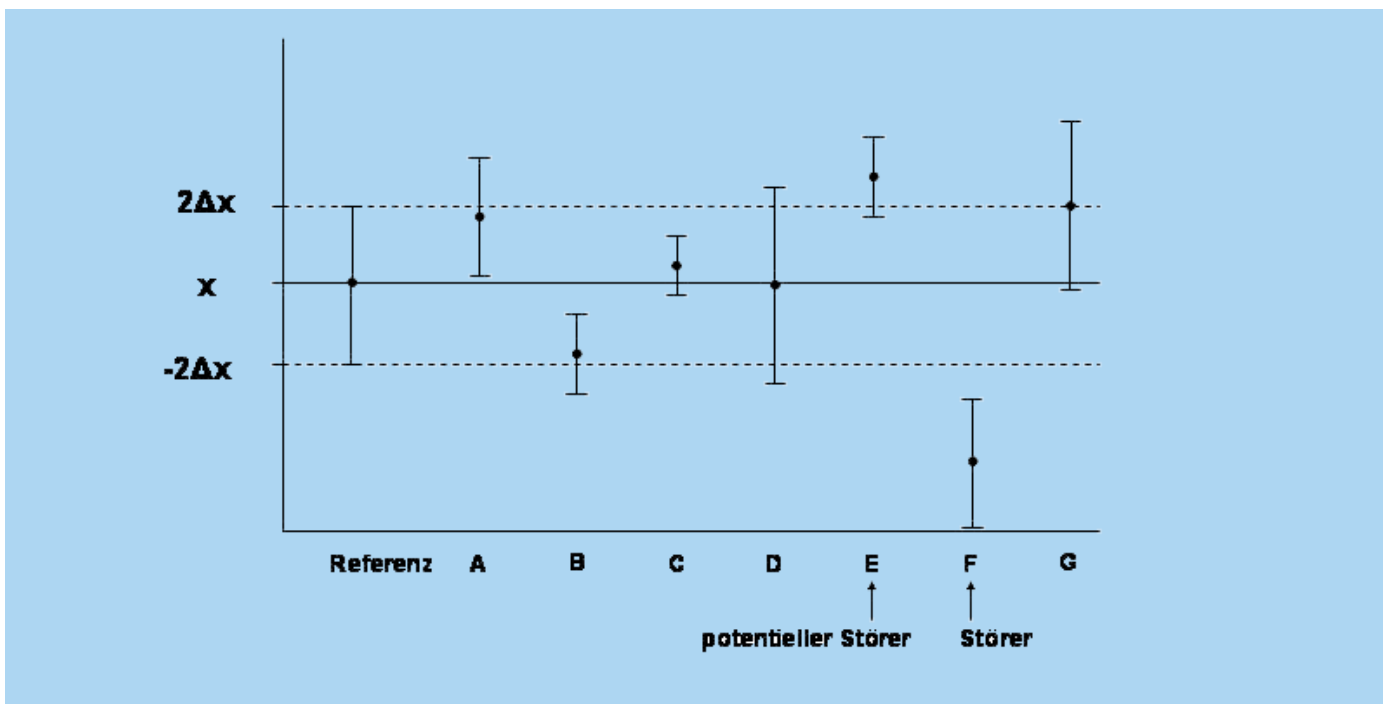
Interference testing

For validation specimen without analyte are measured to test the specificity

and interference susceptibility of the assay. But this test searches only for false positives. That's not sufficient because false negatives and false low values are also very critical. Normally many potential interfering substances are a priori known. These have to be observed alone or sometimes in combination of different substances. You can work with spiking experiments in specimens without interfering substances and with the addition of defined test substances. Therefore you add the analyte in different concentrations (low, middle and high) to the sample with the potential interfering substance. Then you carry out multiple measurements. For every sample you calculate the median and the confidence interval. Now you plot the received medians with confidence intervals against interfering substances or combinations thereof. The result is a diagram with the substance specific confidence intervals (**shown in fig. 2**). The confidence interval of the reference sample has to be compared with the confidence intervals of the interfering samples. The question is: Do the confidence intervals overlap?

- Every potential interfering substance which median lies within this confidence belt is inconspicuous and therefore not an interfering substance.
- Every potential interfering substance which median lies outside this confidence belt but which confidence interval lies partly within the confidence interval of the reference is maybe not an interfering substance. But this decision is unclear. In this case you have to consider measuring again with a higher falling number for making a clear decision.
- Every potential interfering substance which median and confidence interval lies outside this confidence belt is definitely an interfering substance of the assay.

In many cases you have clear decisions concerning interfering substances. But in some cases you can only detect "potential" interfering substances.



▲ **Fig. 2:** Interference testing with A to G as testing substances. Substance E is detected as a potential interfering substance and substance F is identified definitely as an interfering substance.

Based on intended use and economical aspects you have to decide if these potential interfering substances have to be analysed with more test samples or not. In either case are these results an integral part of the validation report.

Conclusion

A validation is neither right nor wrong. At best it's plausible. After successful validation you can't be sure that the assay works correctly in any specimen. But for the user it's possible to estimate the risk of false results. Validations can cost a lot and due to new detected unreliability of the assays you obtain new optimisation steps and loops. Avoiding of interference and predictable faults can save costs if you look after these facts in the very beginning of assay development. It makes sense to realise development and validation as possible in one certified laboratory. Important for the planning of validation is the clearness of the intended use of the ELISA. The selection of guidelines also has to be defined exactly.

Literature

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