BRIEF

Eight limitations of ELISA and how to overcome them using alternative technologies

Introduction

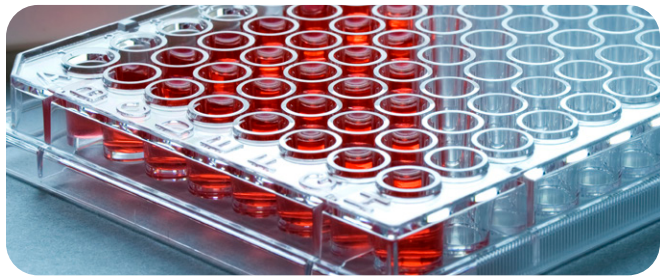
The introduction of the ELISA (enzyme-linked immunosorbent assay) in 1971 expanded the tools available to scientists by offering a non-radiometric alternative to radioimmunoassays without compromising sensitivity. Since its introduction nearly 50 years ago, ELISAs have been used as a diagnostic tool in medicine and biotechnology research, as well as quality control in various industries, and continue to remain an important tool. However, as the demand for greater throughput and sensitivity has evolved over the decades, more robust immunoassays have been developed to address some of the limitations of the standard colorimetric ELISA.

Key limitations of an ELISA

1. **Labor intensive wash-based assay.** Despite the advent of automated plate washers, ELISA is still a labor-intensive assay with numerous tedious wash steps. Inadequate washing can introduce large variation across the wells and result in poor agreement among replicates. This can make it difficult to fit the linear standard curve or could introduce large error bars to your samples confounding the results.

2. **Time to results.** Due to multiple wash steps and incubation times, the assay time is generally on the scale of 4-6 hours. This does not include assay plate preparation, which includes overnight coating of the assay plate with capture antibody.

3. **Large sample volume required.** Typically, an ELISA is performed in 96-well format and requires 100-200 µL of sample for testing. Such a large demand for sample volume will hinder how many targets can be quantified from the test sample and severely limit the ability to add replicates for more accurate, reliable results.



4. **Lack of scalability.** Routinely performed in a traditional 96-well plate format, ELISA lacks the ability to scale through miniaturization to increase throughput.

5. **Narrow dynamic range.** ELISA is an absorbance-based readout which has a narrow window for linear dynamic range as dictated by the optical density (OD), typically ~2 logs. This requires testing samples at different dilutions to fall within the linear portion. Again, this speaks to the amount of sample required for testing and has the potential for samples to be out of range which results in a costly repeat run of the entire assay.

6. **High background.** ELISAs have the potential for high background which hurts the sensitivity of the assay. This could come from TMB substrate contamination, poor washing steps or cross reactivity. High background can lead to data loss or false negative/positive data.

7. **Signal Stability.** ELISA suffers from short signal stability requiring reading within minutes of adding the stop solution. Often the reaction is not fully stopped, and the data will drift over time as seen with additional reads of the plate.

8. **Detection of weak interactions.** Due to the numerous wash steps in ELISA assays, weak protein-protein (or antibody-protein) interactions may not be detected.

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ELISA alternative technologies

In a standard colorimetric ELISA, an antibody specific for the target of interest is coated to the wells of the assay plate and used to capture the ligand in the sample. After incubation with the sample and multiple wash steps, a second antibody toward the target is added for detection. The detection antibody is typically conjugated to horseradish peroxidase (HRP) which reacts with a tetramethylbenzidine (TMB) substrate to produce a color change. Upon stopping the reaction with the addition of acid, the amount of target in the sample can be quantified by absorbance (optical density, OD) as determined from the standard curve (see Figure 1 Assay Workflow).

Today, there are several alternative technologies available that serve the same purpose of traditional ELISAs. Here are examples of three different immunoassay formats that can be used as ELISA alternatives and how they can overcome some of the limitations of ELISAs mentioned above.

Time-Resolved Fluorescence (TRF). The assay workflow is similar to the sandwich ELISA with reagent addition steps (sample capture and detection) and washing (see Figure 1 Assay Workflow). Detection relies on dissociation and enhancement of the lanthanide signal from the detection antibody and measuring fluorescence intensity at the appropriate wavelength. Lanthanides include Europium (615 nM), Terbium (545 nM) and Samarium (645 nM). The time-resolved component comes by way of a delay from excitation to detection which is possible due to the long-lasting emission signal from each lanthanide. As a wash-based technology, TRF is amenable to several complex sample types which include tissue, serum, plasma, or whole blood.

- **Low sample volume.** TRF typically uses about half the sample volume of a standard ELISA (50-100 µL) in the same 96-well format.
- **Scalable.** TRF can be performed in 96-well, 96-well ½ Area and even 384-well assay plates making it easily scalable for higher throughput demands.
- **Wide dynamic range.** TRF provides large dynamic ranges ~4-5 logs (see Figure 2). TRF assays typically have a higher concentration limit compared to the ELISA which is constrained by the optical density at the top of the curve. Combined with the typical low background this expands the dynamic range 2-3 logs farther than a standard ELISA.
- **Low background.** TRF offers low background stemming from the time-resolved functionality. The delay between excitation and signal capture allows a reduction of autofluorescence and other interfering signals. This yields excellent signal-to-background values and improves overall sensitivity of the assay.
- **Signal Stability.** After initial equilibrium in the plate is reached, TRF assays provide long, stable signal which can persist for hours and up to days (barring evaporation issues).

Time-Resolved Fluorescence Resonance Energy Transfer (TR-FRET). TR-FRET is a homogeneous, no wash technology that relies on fluorophore labelled antibodies to detect analytes in a sample matrix (see Figure 1 Assay Workflow). When both acceptor and donor fluorophore labelled antibodies are bound to the target, excitation of the donor label emits energy that is transferred to the nearby acceptor label via FRET (~10 nM spacing limit). The acceptor fluorophore then emits signal for detection that is proportional to the concentration of the target analyte in the sample.

- **No-wash.** TR-FRET is a homogeneous no-wash assay which in turn results in a simplified protocol. Typical TR-FRET assays offer the convenience of being a 1-step assay where sample and antibodies are added together with one incubation step.
- **Faster time to results.** TR-FRET has short incubation steps in the workflow and therefore rapid time to results. Results can be obtained in two hours.
- **Low sample volume.** TR-FRET-based assays require small volumes of sample (only 15 µL of sample for a 384-well assay). This allows added replicates for more accurate, reliable determination of the target concentration in the sample.
- **Scalable.** TR-FRET-based assays are scalable and amenable to high throughput and have been shown to give accurate results in 1536-well plates.
- **Wide dynamic range.** TR-FRET-based assays a show large dynamic range ~4 logs (see Figure 2).

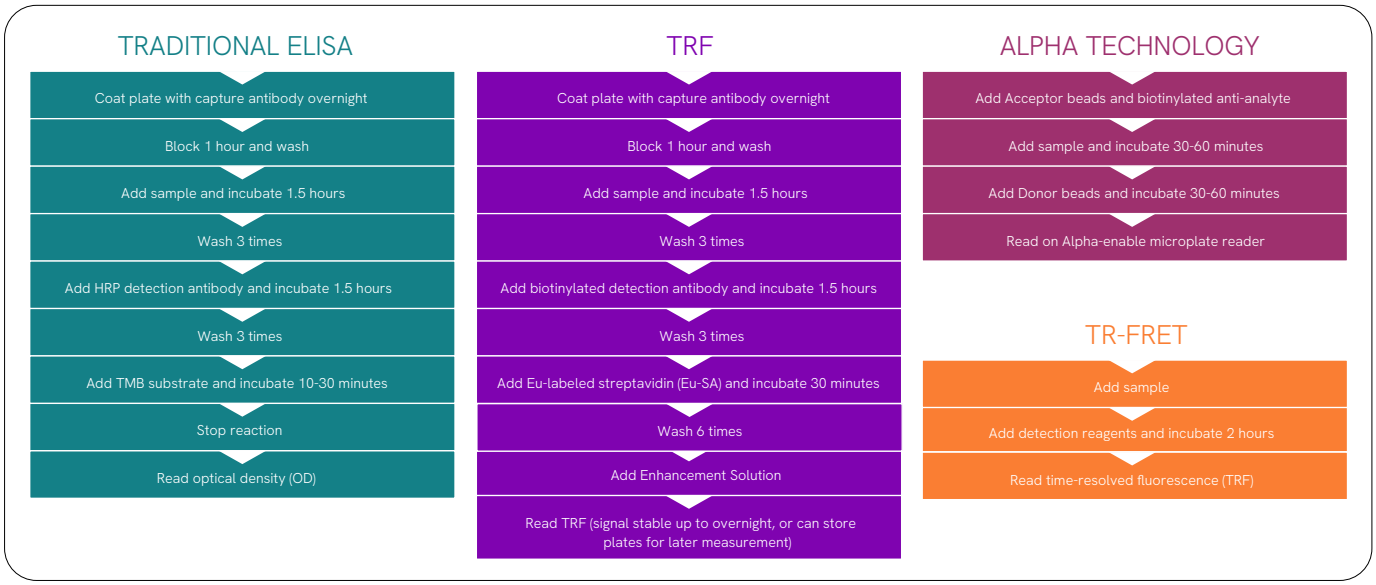

Figure 1: Assay workflow of ELISA compared to alternative technologies.

- **Low background.** TR-FRET offers low background in part due to the time-resolved readout which greatly reduces any fluorescent background from autofluorescence or other interfering signals. This yields excellent signal-to-background values and improves overall sensitivity of the assay.
- **Signal Stability.** TR-FRET-based assays can be re-read multiple times over many hours without loss of signal.

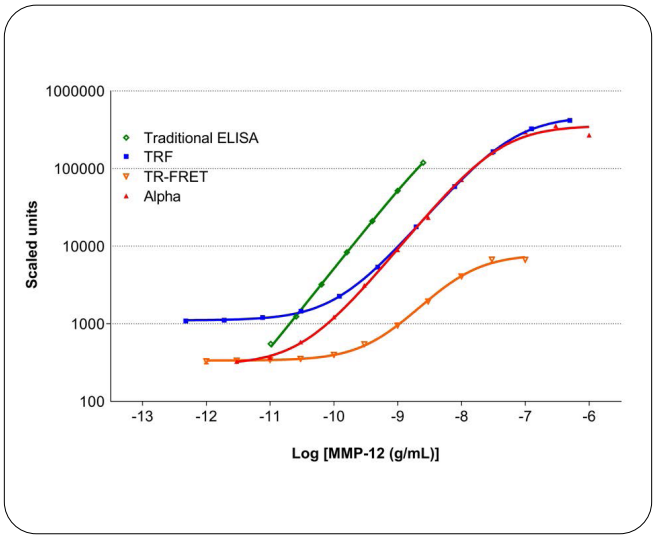

Figure 2: Overlay of Standard Curves. The same antibody was used to develop each of the four assays such that assay metrics could be directly compared including dynamic range, sensitivity and signal to background.

Amplified Luminescence Proximity Homogeneous Assay (Alpha). Alpha is a bead-based assay technology used to study biomolecular interactions in a microplate format (see Figure 1 Assay Workflow). When in proximity to each other due to binding to a target analyte, excitation of the donor beads releases singlet oxygen which activates the acceptor beads and results in a chemiluminescent signal. The Alpha signal is proportional to the concentration of the target analyte in the sample.

- **No-wash.** Alpha is a homogeneous no-wash assay which leads to a simplified protocol. Typical Alpha assays offer the convenience of having only two incubation steps.
- **Faster time to results.** Alpha has short incubation steps in the workflow and therefore rapid time to results. Results are easily obtained in two hours.
- **Low sample volume.** Alpha assays require small volumes of sample (only 5 µL of sample for a 384-well assay). This allows great flexibility in the number of targets that can be measured in parallel from the same sample as well as including replicates for precise quantification of the analyte tested.
- **Scalable.** Alpha assays are scalable and amenable to high throughput and have been shown to give accurate results in 1536-well plates.

- **Wide dynamic range.** Alpha assays show a large dynamic range ~4-5 logs (see Figure 2).
- **Low background.** Alpha offers a low background, excellent signal-to-background values and overall sensitivity.
- **Signal Stability.** Alpha assays offer stable signal over many hours.
- **Detection of weak interactions.** Since it is a homogeneous assay, Alpha can measure weak protein-protein interactions that might otherwise be lost in a washed-based format.

About Revvity

Whatever approach you choose, having a supplier that has the resources and expertise to partner with you on your assay development can help you avoid potential pitfalls, and get the quality results you need, faster. Revvity offers a range of assay technologies to address the shortcomings and limitations of a standard colorimetric ELISA (see Table 1. Assay Logistics).

Table 1: Assay Logistics

	ELISA	TRF	TR-FRET	ALPHA
		DELFIA	"HTRF® LANCE®*Ultra*™	AlphaLISA®
Wash Steps	Yes	Yes	No	No
Time of Assay	4-6 Hours	4-6 Hours	1-2 Hours	2 Hours
Sample Volume	100-200 µL	25-00 µL	5-16 µL	5 µL
Scalable/Miniaturization	No	Yes	Yes	Yes
Dynamic Range	~2 Log	~4-5 Log	~4 Log	~4-5 Log
Sensitivity	10 pg/mL	10 pg/mL	10 pg/mL	1 pg/mL
Signal Stability	< 15 min	Hours to Days	Hours to Days	Up to 24 Hours

Technology selection will depend on the need of the end user. For instance, many complex samples require wash steps restricting your choice to ELISA and TRF assays. In cases like this, to improve overall assay performance and cut down on sample material used, a simple conversion to TRF will suffice. In other cases, if higher throughput is required or sample is limiting, homogeneous no-wash assays such as Alpha and TR-FRET can be used. Depending on the ELISA limitation that is holding back your work, Revvity can offer the right solution to propel your research to the next level.

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