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In both scientific research and clinical diagnostics, the sensitivity, specificity, and repeatability of ELISA kits are critical to ensuring the reliability and accuracy of experimental results. To support thorough evaluation and optimization of ELISA kit performance, we offer the "Comprehensive Guide to Key Performance Metrics of ELISA Kits" This guide covers the following essential topics：

- **Sensitivity & Limit of Detection** – How to ensure accurate detection of low-concentration analyte
- **Inter-assay and Intra-assay precision** – Analysis of data variability and common troubleshooting
- **Recovery rate & linear range** – Practical tips for optimizing sample preparation
- **Specificity evaluation** – Cross-reactivity validation method and case study

The key performance metrics for ELISA analysis include sensitivity, detection limit, precision, specificity, recovery rate, and accuracy. This article focuses primarily on methods for sensitivity analysis.

Sensitivity & Limit of Detection (LOD)

Sensitivity refers to the lowest concentration of an analyte that can be reliably detected—commonly known as the limit of detection (LOD)—or the ability to distinguish between samples with low analyte levels. Sensitivity is typically classified into two types: analytical sensitivity and clinical sensitivity.

- **Analytical sensitivity** generally corresponds to the LOD, indicating the lowest concentration of an analyte that the assay can consistently and accurately detect.
- **Clinical sensitivity** describes the assay's ability to correctly identify true positive cases in real-world samples. It is closely linked to the false negative rate and must be validated using clinical specimens.

This section focuses on the experimental design and calculation methods used to assess analytical sensitivity.

Experimental Protocol

Experimental Steps	Key Operations	Technical Requirements
Blank Sample Testing	Repeat testing using matrix identical to test samples	≥20 replicates
Low Concentration Sample Preparation	Prepare concentrations near expected LOD	Recommend 3-5 gradients
Testing & Analysis	Test each concentration with 10 replicates	Record signal values
Data Processing	Calculate mean, standard deviation	Statistical signification

Key Parameter Calculations

Limit of Blank (LOB) = $Mean_{blank}$ + 1.645 × SD_{blank}

Limit of Detection (LOD) = LOB + 1.645 × SD low concentration sample

Limit of Quantitation (LOQ) = LOB + 10× SD_{blank} (optional)

Example Data and Results Analysis

Blank sample: This refers to the sample in ELISA that does not contain the antigen. A total of 20 wells' OD_{450} values were measured.

0.045	**0.046**	**0.052**	**0.06**	**0.05**	**0.054**	**0.051**	**0.048**	**0.047**	**0.052**
0.052	**0.053**	**0.045**	**0.052**	**0.055**	**0.049**	**0.055**	**0.052**	**0.051**	**0.049**

Mean value of blank samples：0.0509

SD value of blank samples：0.004

CV value of blank samples = SD value / Mean value×100% = 0.004/0.0509 × 100% = 7.3%

LOB = Mean value of blank samples + 1.645 × SD value of blank samples = 0.0509 + 1.645 × 0.004 = 0.057

Low concentration sample (e.g. 0.5 pg/mL): Dilute the antigen sample to a theoretical concentration of 0.5 pg/mL. Prepare 5 separate sample aliquots, and test each in quadruplicate, resulting in a total of 20 OD_{450} readings.

0.075	**0.080**	**0.075**	**0.078**	**0.082**	**0.075**	**0.080**	**0.079**	**0.078**	**0.078**
0.082	**0.081**	**0.076**	**0.083**	**0.083**	**0.077**	**0.077**	**0.082**	**0.080**	**0.081**

Mean value of low -concentration samples: 0.079

SD value of low-concentration samples: 0.003

CV value of low-concentration samples = SD value of low-concentration samples/Mean value of low-concentration samples × 100% = 0.003/0.079 × 100% = 3.4%

LOD = LOB + 1.645 × SD value of low-concentration samples = 0.057 + 1.645 × 0.003 = 0.062

LOQ = LOB + 10 × SD value of blank samples = 0.057 + 10 × 0.004 = 0.097.

LOD verification:

Preparation of low-concentration samples (0.5 pg/mL): Use antigen samples or samples with known concentrations and dilute to a theoretical concentration of 0.5 pg/mL. Prepare 5 individual samples and test each in duplicate, resulting in a total of 10 OD_{450} measurements.

0.075	**0.077**	**0.082**	**0.079**	**0.083**	**0.079**	**0.075**	**0.079**	**0.078**	**0.083**

The OD_{450} values of the low-concentration samples > LOD of 0.062, meeting the criteria for LOD verification.

Precision & Reproducibility

Precision refers to the degree of consistency among the measurement results obtained from multiple repeated measurements of the same sample under the same conditions. It reflects the repeatability and stability of the detection method, and is usually expressed by the coefficient of variation (CV%). Precision can be divided into two types:

Intra-assay Precision: The degree of variation when the same sample is repeatedly measured by the same operator within a short period of time in the same batch.

Inter-assay Precision: The degree of variation when the same sample is measured by different batches, different operators, or at different times.

Experimental Protocol

Step	Key Operations	Technical Requirements
Sample selection	Select samples of high, medium and low concentrations (covering the detection range)	Perform at least 3 repetitions for each concentration (or use Standard sample)
Intra-assay Precision	Repeat the same sample on the same plate multiple times (e.g. 10 times)	Calculate the average, SD, and CV%
Inter-assay Precision	Test the same sample using different batches, different operators, or different dates (e.g. 3 batches, 3 repetitions per batch)	Calculate the average, SD, and CV%
Data analysis	Use statistical software (such as Excel, GraphPad Prism) to calculate the coefficient of variation	CV% is usually required to be <10%

Calculation of key parameters

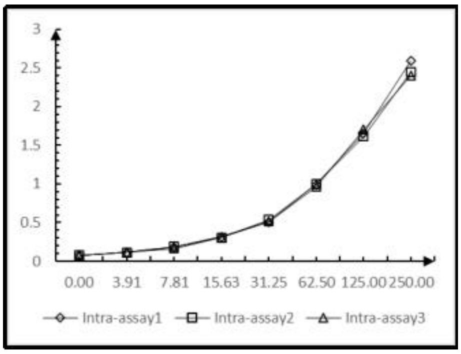
Coefficient of variation (CV) = Sample standard deviation(SD) / Sample mean x 100%

Example Data and Results Analysis

The precision was conducted by selecting standard samples and clinical samples with known concentrations. Each concentration point on the standard curve was tested, with 9 samples prepared for each point, including 3 intra-assay samples and 6 inter-assay samples (with duplicates for inter-assay samples). Clinical samples with known concentrations (covering high, medium and low concentrations within the detection range) were also used, with 3 samples prepared for each concentration. Each sample was tested 8 times intra-assay and 4 times inter-assay(with duplicates for inter-assay samples).

Intra-assay Precision of the standard curve

	Concentration (pg/mL)	OD_{450} value of the standard curve for Intra-assay precision			Average value	SD value	CV value
		1	2	3			
A	250.00	2.592	2.444	2.403	2.480	0.099	4.01%
B	125.00	1.654	1.617	1.703	1.658	0.043	2.60%
C	62.50	0.991	0.994	0.961	0.982	0.018	1.86%
D	31.25	0.507	0.534	0.514	0.518	0.014	2.70%
E	15.63	0.318	0.303	0.307	0.309	0.008	2.51%
F	7.81	0.182	0.187	0.164	0.178	0.012	6.81%
G	3.91	0.115	0.116	0.113	0.115	0.002	1.33%
H	0.00	0.075	0.073	0.071	0.073	0.002	2.74%



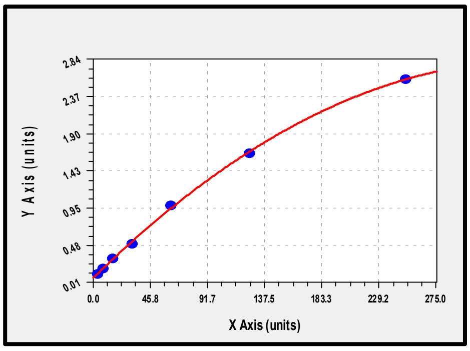
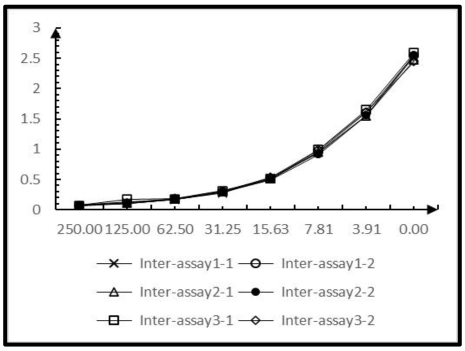


Figure 1 shows the standard curves within each Intra-assay. Figure 2 shows the standard curve after calculating the average values for each concentration.

Inter-assay precision of the standard curve

Concentration (pg/mL)	Inter-assay 1 OD450 value		Inter-assay 2 OD450 value		Inter-assay 3 OD450 value		Average value	SD value	CV value
250.00	2.53	2.543	2.472	2.562	2.592	2.444	2.524	0.056	2.21%

125.00	1.55	1.608	1.553	1.559	1.654	1.617	1.590	0.043	2.68%
62.50	0.943	0.967	0.95	0.911	0.991	0.994	0.959	0.031	3.28%
31.25	0.53	0.513	0.508	0.498	0.507	0.534	0.515	0.014	2.74%
15.63	0.274	0.294	0.302	0.297	0.318	0.303	0.298	0.014	4.83%
7.81	0.164	0.177	0.177	0.169	0.182	0.187	0.176	0.008	4.77%
3.91	0.128	0.108	0.108	0.11	0.172	0.116	0.124	0.025	20.11%
0.00	0.071	0.071	0.073	0.069	0.075	0.073	0.072	0.002	2.91%



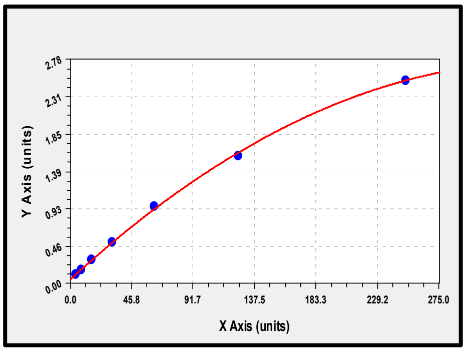


Figure 3 shows the standard curves within each Intra-assay. Figure 4 shows the standard curve after calculating the average values of each concentration.

Intra-assay precision of clinical samples

	OD450 value of clinical samples for Intra-assay precision									Average value	SD value	CV value
High concentrations	A1	2.344	2.401	2.421	2.379	2.290	2.365	2.199	2.418	2.352	0.075	3.20%
	A2	2.581	2.682	2.577	2.577	2.431	2.440	2.621	2.508	2.552	0.087	3.41%
	A3	2.015	1.975	2.135	2.089	1.896	2.103	2.098	2.038	2.044	0.080	3.89%
Medium concentrations	B1	0.982	0.887	0.996	0.936	0.987	0.987	0.946	0.986	0.963	0.038	3.91%
	B2	1.325	1.355	1.255	1.305	1.325	1.369	1.201	1.398	1.317	0.064	4.83%
	B3	1.231	1.225	1.300	1.298	1.256	1.199	1.285	1.265	1.257	0.037	2.92%
low concentrations	C1	0.075	0.078	0.076	0.088	0.087	0.079	0.065	0.077	0.078	0.007	9.24%
	C2	0.121	0.112	0.129	0.125	0.099	0.118	0.102	0.115	0.115	0.011	9.14%
	C3	0.085	0.088	0.079	0.085	0.089	0.090	0.075	0.088	0.085	0.005	6.22%

Inter-assay precision of clinical samples

		OD450 value of clinical samples for Inter-assay precision								Average value	SD value	CV value
		Inter-assay 1		Inter-assay 2		Inter-assay 3		Inter-assay 4				
High Concentrations	A1	2.405	2.399	2.333	2.401	2.198	2.235	2.251	2.295	2.315	0.082	3.56%
	A2	2.615	2.566	2.572	2.592	2.444	2.403	2.592	2.444	2.529	0.084	3.30%
	A3	2.09	2.106	1.926	2.025	2.006	1.964	2.016	2.031	2.021	0.059	2.94%
Medium Concentrations	B1	0.991	0.994	0.961	0.991	0.994	0.961	0.943	0.967	0.975	0.020	2.02%
	B2	1.355	1.391	1.301	1.288	1.222	1.181	1.241	1.257	1.280	0.069	5.42%
	B3	1.241	1.196	1.222	1.254	1.178	1.201	1.221	1.231	1.218	0.025	2.06%
Low Concentrations	C1	0.074	0.082	0.076	0.072	0.063	0.074	0.061	0.075	0.072	0.007	9.59%
	C2	0.124	0.132	0.114	0.128	0.138	0.116	0.113	0.109	0.122	0.010	8.48%
	C3	0.094	0.088	0.092	0.078	0.088	0.084	0.092	0.107	0.090	0.008	9.35%

Recovery rate

Recovery Rate , also known as spiked recovery rate, is determined by adding a known amount of standard substance to a subsample of the sample during the measurement process. The recovery rate of the added standard substance is then calculated by subtracting the measured value of the original sample from the measured result of the spiked sample. It is further divided into blank spiked recovery rate and sample spiked recovery rate.

The blank spiked recovery rate involves adding a quantitative standard substance to a blank sample matrix without the analyte. Analyze according to the sample processing steps to obtain the result. The ratio of this result to the theoretical value is the blank spiked recovery rate, which is mainly used to evaluate the influence of experimental conditions, instrument performance, reagent quality, and operational procedures on the measurement results. By determining the blank spiked recovery rate, one can check for systematic errors or contamination, thereby ensuring the accuracy of the analysis results.

For the sample spiked recovery rate, two identical samples are taken. Add a quantitative amount of the analyte standard substance to one of them, and both samples are analyzed simultaneously using the same analytical steps. The result of the spiked sample is subtracted from that of the non-spiked sample, and the difference is divided by the theoretical value of adding the standard substance to obtain the sample spiked recovery rate. By determining the sample spiked recovery rate, one can verify the accuracy and reliability of the analytical method.

Principles of Recovery Rate Experiment Design

Sample selection Select standard samples with known concentrations or clinical samples. Add volumes accounting for ≤10% to reduce dilution interference.

Concentration design Design 3-4 different concentration standard samples to be added to different types of samples to be tested, and prepare them as spiked samples. It should be noted that the minimum spiked concentration should not be lower than twice the LOQ.

Repeatability Repeat the determination at each concentration point 2-3 times, and calculate the recovery rate range and the average recovery rate.

Experimental Protocol

Step	Key Operations	Technical Requirements
Sample selection	Select 5 standard samples with high, medium and low concentrations (covering the detection range)	Prepare 3-5 samples for each concentration, with 3 samples for testing and 2 samples for backup.
Matrix Selection	Blank sample matrix or regular sample matrix (serum, cell culture supernatant, etc.)	Prepare 4-6 samples each for blank sample matrix and regular sample matrix, with 1 sample as the control sample and the rest as the dilution solution.
Sample Preparation	Combine the selected high, medium and low concentration standard samples with the blank sample matrix or regular sample matrix.	The volume of the standard sample added should be ≤ 10% and the minimum spiked concentration should not be lower than twice the LOQ.
Test Sample	Perform ELISA analysis on the spiked samples prepared and the blank sample matrix or regular sample matrix.	Calculate the spiked sample recovery range and average recovery rate for each concentration sample.
Data Analysis	Use statistical software (such as Excel, GraphPad Prism) to calculate the recovery rate(R). R = (measured value of spiked sample - measured value of blank sample matrix or regular sample) / spiked amount ×100%.	Recovery rate range of 80%-120% is qualified. Average recovery rate between 95% and 105% is optimal. CV value of repeated detection of spiked samples ≤ 10%.

Example Data and Results Analysis

Sample type	Spiked concentrations	CV value	Average recovery rate (%)	Recovery range (%)
Blank sample matrix (n=5)	250 pg/mL	3.3%	89.5-108.9	96.6
	100 pg/mL	6.5%	88.6-106.5	95.5
	150 pg/mL	4.5%	85.9-110.6	98.8
	50 pg/mL	6.6%	90.2-109.9	101.2
	15 pg/mL	2.5%	87.5-105.5	94.7
Serum(n=5)	250 pg/mL	3.5%	89.6-107	98.5
	100 pg/mL	6.6%	84.5-103.6	95.6
	150 pg/mL	6.2%	79.9-99.8	94.9
	50 pg/mL	4.3%	80.6-103.5	95.1
	15 pg/mL	5.5%	81.2-104.9	95.9

Specificity

The specificity of ELISA detection reagents primarily encompasses two distinct aspects. The first is assay specificity, which refers to the ability of the antibodies in the ELISA kit to bind exclusively to the target protein without cross-reacting with non-target proteins, particularly those homologous to the target. The second is diagnostic specificity, a statistical measure in medical diagnostics where any diagnostic test is characterized by two fundamental parameters: sensitivity and specificity. Sensitivity reflects the probability of correctly identifying individuals with the disease (i.e., avoiding false negatives), while specificity indicates the probability of correctly identifying individuals without the disease (i.e., avoiding false positives).

Experimental Protocol

Step	Key Operations	Technical Requirements
Screening cross-reactive proteins	**Select proteins that are:** ● Highly homologous to the target protein. ● Known or suspected to cross-react with the target protein. ● The same target protein derived from different species.	● Search for proteins highly homologous to the target protein sequence through NCBI as cross-reactive proteins. ● Proteins belonging to the same family. ● Proteins of the target protein from different species.

Detection time of cross-reaction	During antibody screening, the selected cross-reactive proteins serve as negative controls in the initial specificity assessment. Upon completion of ELISA development, the specificity should be further validated against the same panel of proteins.	Select antibodies that bind specifically to the target protein while demonstrating no detectable binding to the negative screening agents. Identify which proteins within the cross-reactivity panel show no significant reactivity with the antibodies selected for the ELISA kit.
Cross-reaction design	Use the target protein as the positive screening agent or positive control, and use cross-reactive protein as the negative screening agent or test group. Blank buffer solution must be included as the procedural negative control to monitor background signal and assay validity.	During the screening of antibodies, the target protein, negative screening agent and negative control were coated. The immunizing serum was used as the primary antibody, and the HRP-labeled Fc or Fab antibody was used as the secondary antibody for antibody screening. For specific detection, the screening antibodies were coated, and direct ELISA or sandwich ELISA was performed to detect cross-reactive proteins.
Data analysis	The positive screening agent or positive control binds to the antibody, while negative screening agent or negative control or test group does not bind to the antibody.	