THESIS PROGRESSION

Thesis title: Development Of Quality-Controlled Anti-Interferon-Gamma Autoantibodies Quantification By Inhibitory Elisa

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1. Background and rationale of research

Adult-onset immunodeficiency (AOID) is an uncommon condition characterized by the presence of autoantibodies against interferon-γ (IFN-γ), known as anti-interferon-γ autoantibodies or AIGAs. These autoantibodies, produced by the host's immune cells, neutralize interferon-γ and inhibit its essential immunoregulatory functions. AOID was initially identified as a sporadic late-onset immunodeficiency affecting patients with recurrent opportunistic infections, particularly nontuberculous mycobacteria (NTM) and other intracellular pathogens (Chetchotisakd et al., 2007). The first documented case was reported in Thailand in 2003, with international recognition following around 2004 (Höflich et al., 2004). Current epidemiological data suggest an estimated incidence rate of 0.5-1.0 per million people (Li et al., 2024). Worldwide incidence indicates that more than 600 individuals have been diagnosed with AIGAs to date. (Y. C. Chen et al., 2022), with a significant demographic pattern: most are adults of Asian descent, and more than two-thirds of all reported cases originate from Thailand and Taiwan (Browne et al., 2012).

It is complicated to diagnose AOID syndrome due to inconsistencies in the analytical assays. Although there have been some methods developed, such as enzyme-linked immunosorbent assay (ELISA) and flow cytometry (available at select laboratories). Unfortunately, there is no standardized approach, as the variability between these methods also complicates the result comparison. (Khositnithikul et al., 2022).

A previous study demonstrated that the inhibitory ELISA for detecting AIGAs is highly sensitive and specific to lymphadenopathies frequently observed in patients with NTM infection. This method is a semi-quantitative assay determination of AIGA titers, offering diagnostic utility for autoantibody-associated NTM infections and aiding in both the prediction and detection of active disease. Additionally, AIGAs titers in non-immunomodulated patients remained stable throughout the infection (Nithichanon et al., 2020).

Although numerous studies have explored the use of anti-IFN-γ autoantibodies as a laboratory biomarker for diagnosing adult-onset immunodeficiency syndrome, research on their plasma stability and the impact of temperature variations on AGAIs' integrity remains limited. Understanding how specific storage temperatures (−80°C, −20°C, 4°C, and room temperature) and prolonged storage affect the concentration and functional stability of these antibodies will help address this knowledge gap. Such insights are critical for establishing optimal sample-handling protocols in laboratories and improving the diagnosis of AOID-related immune deficiency.

In addition, measuring AIGAs is crucial for clinical decision-making and monitoring treatment in AOID patients. However, the lack of an accurate quantitative method for AIGA levels, combined with the absence of quality control samples, poses a significant challenge to the reliability and accuracy of test results. This study aims to move beyond semi-quantitative titers by enabling accurate quantification of AIGAs. Our study ensures scientific rigor and relevance in developing a robust method for AIGA quantification, its validation, and practical considerations for sample storage.

2. Research questions

2.1. Can the AIGA level be accurately quantified using ELISA, rather than semi-quantitatively measuring the AIGA titer?

2.2. How can a quantitative inhibitory ELISA be standardized through defined steps and key parameters to ensure consistent and reproducible measurement of AIGA levels?

2.3. How does sample quality, including storage time and temperature affect the accuracy and reliability of quantitative inhibitory ELISA for AIGA quantification?

3. Objectives

3.1. To establish a quantitative ELISA that can provide an accurate measurement of AIGA levels from plasma samples.

3.2. To standardize a quantitative inhibitory ELISA for AIGAs detection and ensure its consistent, reproducible quantification.

3.3. To investigate the impact of sample quality, specifically storage time and temperature, on the accuracy and reliability of AIGAs quantification using the quantitative inhibitory ELISA.

4. Hypothesis

4.1. The quantitative inhibitory ELISA can provide accurate measurement of AIGA levels from plasma samples, showing improved precision and reduced variability compared to semi-quantitative or titer-based methods.

4.2. Systematic standardization of the quantitative inhibitory ELISA for AIGA detection, through defined key steps and parameters, is expected to yield a robust, reproducible, and consistent assay with high agreement across repeated trials and different operators, enabling reliable and accurate quantification of AIGA levels.

4.3. Sample quality, including storage time and temperature, significantly affects the accuracy and reliability of AIGA quantification by quantitative inhibitory ELISA, with suboptimal conditions reducing assay performance and increasing result variability.

5. Conceptual framework

The conceptual framework of the thesis is described in the table below:

Table 1. Conceptual framework

Problems	1. Lack of an accurate quantification method for AIGAs 2. The lack of standardized quantitative inhibitory ELISA in AIGA measurement

	3. Impact of pre-analytical factors on sample quality remains unclear
Research plan	1. IQC for inhibitory ELISA: Create IQC sample and set up the Levey-Jenning chart 2. Evaluation of inhibitory ELISA for quantifying AIGAs: Linearity, LOQ, Precision,… 3. Correlation with reference method 4. Storage effect against AIGAs: keep internal quality control samples at temperature variations (-80°C, -20°C, 4°C, 25°C), and measure the concentrations of the AIGAsat each time point (3 months, 6 months)
Inputs	1. Sample: Blood heparinized from AOID patients 2. Equipment: inhibitory ELISA kits, freezers, refrigerators, room temperature setups, data collection tools, and temperature monitoring devices 3. Laboratory personnel: Skilled technicians trained in ELISA methods 4. Protocols: Standardized procedures for the Inhibitory ELISA assay
Outputs	1. Having the accurte quantitative inhibitory ELISA assay for quantifying the AIGAs 2. A standardized quantitative inhibitory ELISA established for consistent and reproducible quantification of AIGAs

	3. Quantitative evidence established showing that sample storage temperature and time reduce the accuracy and reproducibility of AIGA quantification by inhibitory ELISA
Outcomes	1. Laboratory staffs are able to develop internal quality control sample preparation procedures for quantitative AIGA testing, thereby providing evidence of the reliability of test results and helping doctors and patients have confidence in the outcomes 2. Clear guidelines on how to store plasma samples at various temperatures to preserve anti-IFN-γ autoantibody stability
Impacts	1. Medical doctors can confidently use quantitative AIGAs test results from the laboratory to diagnose, monitor, and predict the prognosis of AOID, thereby improving treatment effectiveness and reducing the burden on patients and society. 2. Fill critical gaps in understanding the effects of storage conditions on the stability of AIGAs

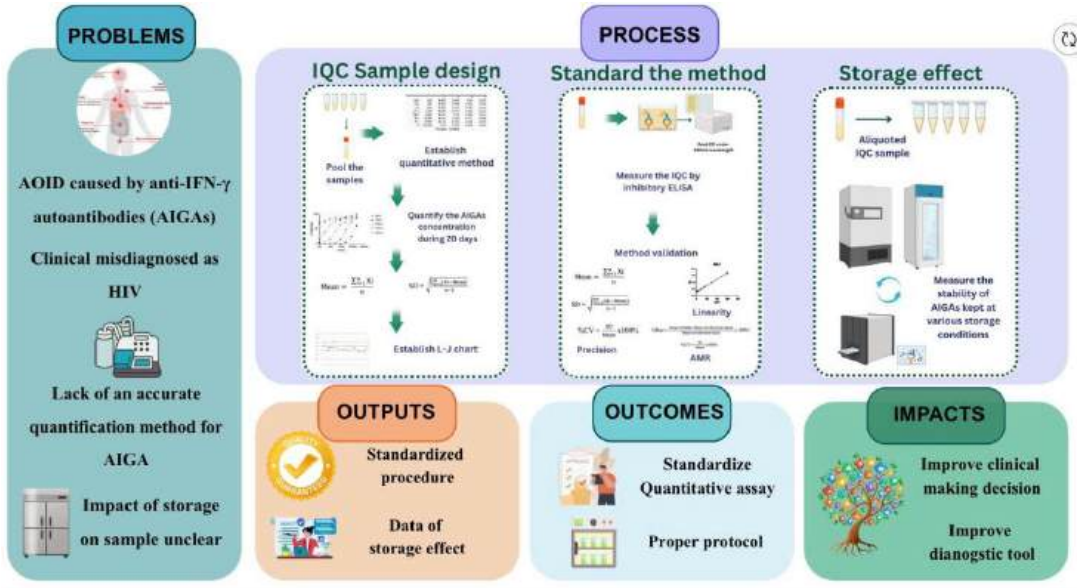


Figure 1. Conceptual framework

6. Scope and limitations.

6.1. Scopes

6.1.1. Target: Investigate the stability of AGAIby storage conditions (temperatures and duration), and create an accurate quantitative ELISA assay.

6.1.2. Methodology: Inhibitory ELISA for measuring the AIGAs concentrations.

6.1.3. Sample: Blood plasma from AOID patients.

6.2. Limitations

6.2.1. The stability of AIGAs could not be measured after one month due to the loss of IFN-γ standard samples.

6.2.2. Using the freezing samples from the previous study, not using the fresh ones.

6.2.3. Measure the stability of AIGAs only in plasma samples, do not extend to other blood samples.

6.2.4. The study assesses AIGAs stability only within a 6-month storage period.

6.2.5. The clinical validation could not be performed because of the limited sample.

6.2.6. Due to limitations in reagents, we are unable to measure repeatability according to CLSI standards (10–20 times per sample). We only measure each sample in duplicate. In addition, it is not possible to measure the individual 5 samples for each concentration level before pooling, limiting the analysis to the pooled sample.

7. Materials and reagents

7.1. ELISA reader: SUNRISE-BASIC TECAN

7.2. Incubator BD OptEIA™ Recombinant human IFN-γ lyophilized

7.3. BD OptEIA™ Anti-human IFN-γ monoclonal antibody

7.4. BD OptEIA™ Biotinylated Anti-human IFN-γ monoclonal antibody

7.5. BD OptEIA™ Streptavidin-horseadish peroxidase conjugate

8. Methods

8.1. Biological sample in this study

Our retrospective study protocol has been reviewed and approved by the Institutional Review Board of Khon Kaen University with the approval number HE671128. This study will obtain leftover heparinized samples from a previous research project, which collected samples at Srinagarind Hospital, Khon Kaen University (Thailand) from May 2022 to February 2023.

The participants were patients with a history of dNTM, defined as a positive blood culture for NTM or detection of NTM in more than one organ accompanied by reactive skin disorders (Sweet syndrome, pustular psoriasis, erythema nodosum), and concurrent or subsequent opportunistic infections; exclusion criteria included NTM infection limited to the lungs, HIV-positive status, and nosocomial infections. Additionally, healthy controls were enrolled for comparison; healthy control eligibility required no history of NTM or other systemic infections, HIV-negative status, no known immunodeficiency or use of immunosuppressive medications, and no acute infection at the time of sampling.

Clinically, samples were classified as “Inactive” (no signs of active infection and off antimycobacterial therapy for ≥30 days before sampling) or “Active” (requiring oral or parenteral antimycobacterial therapy within 30 days prior to sampling); the Active group was further divided into “Non-progressive” (stable symptoms, ongoing treatment, and no hospital admission in the prior 12 months) and “Progressive” (worsening despite treatment and hospital admission within the prior 12 months).

8.2. Creating internal quality control (IQC) samples

We will prepare pooled control samples as follows:

- Negative control will be generated by pooling plasma from four healthy control subjects. All the samples were quantified for AIGAs titer by inhibitory ELISA with a result of "undetectable".
- Low positive control sample will be generated by pooling plasma from five dNTM patients. All the samples were quantified for AIGA titer by inhibitory ELISA, with a result of the titre from <10 to 100.
- Medium positive control sample will be generated by pooling plasma from five dNTM patients. All the samples were quantified for AIGA titer by inhibitory ELISA, with a result of the titre from 100 to 1000.
- High positive control sample will be generated by pooling plasma from five dNTM patients. All the samples were quantified for AIGA titer by inhibitory ELISA, with a result of the titre from 5000 to 10000.
- Very high positive control sample will be generated by pooling plasma from five dNTM patients. All the samples were quantified for AIGA titer by inhibitory ELISA, with a result of the titre from 10000 to 100000.

AIGA titers will be measured at least 20 times across the 20 days, with the mean and standard deviation calculated. Measurement variability will be tracked using a Levey–Jennings control chart, and the acceptable internal quality control range will be set at ±2 SD.

8.3. Inhibitory ELISA for AIGA detection

Ninety-six well immunoassay plates will be coated with 50 µl of 1:250 diluted Capture Antibody Purified Anti-human IFN-γ monoclonal antibody (BD OptEIA™, BD Biosciences Pharmigen, US) in bicarbonate coating buffer (pH 9.6) overnight at 4 °C. Next day, the plates will be washed three times with washing buffer, 0.05% Tween-20 in phosphate buffer saline (PBS), before being blocked with 100 µL of 1% Bovine Serum Albumin in PBS at room temperature for 2 hours.

During the incubation time, plasma samples will be incubated with 300 pg/ml of Recombinant human IFN-γ lyophilized standard (BD OptEIA™, BD Biosciences

Pharmigen, US) in U-bottom plates. The first well of each sample will be diluted to 1:100, then undergo 3-fold serial dilutions. The Recombinant human IFN-γ lyophilized standard (BD OptEIA™, BD Biosciences Pharmigen, US) will also be prepared at 300, 150, 75, 37.5, 18.75, 9.375, and 4.6875 pg/ml. Only the sample diluent will be tested and referred to as "blank control". All the diluted mixture will be incubated at 37 °C for 1 h.

After incubation, the supernatant in the blocked plates will be discarded prior to transferring 50 µL of each diluted and incubated sample into the plates. After incubation for 1 h at room temperature, the plates will be washed three times with washing buffer, 50 µL of detector mixture (1:250 Antibody Biotin Anti-Human IFN-γ monoclonal (BD OptEIA™, BD Biosciences Pharmigen, US) with 1:250 Enzyme reagent SAv-HRP (BD OptEIA™, BD Biosciences Pharmigen, US)) will be introduced and incubated 1 h in the dark at room temperature. After that, the plate will be washed five times with washing buffer, 50 µL TMB substrate (Substrate Reagent A + Substrate Reagent B (BD OptEIA™, BD Biosciences Pharmigen, US)) will be added for 20 min in the dark at room temperature. The reaction will be stopped by adding 30 µL of 2N H_2SO_4. Absorbance will be measured at 450 nm using Microplate reader SUNRISE-BASIC TECAN (Tecan Austria GmbH, Austria).

8.4. Analysis for AIGAs concentration

Standard curve of detectable recombinant human IFN-γ by inhibitory ELISA will be created by GraphPad Prism 10 (GraphPad Software, US). The results will be accepted if they have an R-squared of the linear curve > 0.97.

The concentration of AGAIs is expressed as IC50 (half-maximal inhibitory concentration) by GraphPad Prism 10 (GraphPad Software, US). To calculate IC50, open GraphPad Prism and create a new data table, selecting "Enter 2 replicate values in side-by-side subcolumns". And normalize the OD of the samples by adding the highest OD of the standard on the first row and the lowest OD of the standard on the tenth row, put the OD of the sample in the second row to the ninth row, click "Analyze" → "Normalize". Copy the normalized sample data into a new table, enter the sample concentrations (100, 300, 900, 2700, 8100, 24000, 72000, 216000) in the X column, then run Nonlinear

Regression using “Sigmoidal, 4PL, X is concentration”. The IC50 results will appear in “Nonlin fit of Data”, and the corresponding graph in “Graphs-Data”.The results will be accepted if the R-squared of the non-linear curve ≥ 0.95.

8.5. Analysis for AIGA concentration quantification precision

The precision will be evaluated by measuring each concentration in duplicate once per day for 20 consecutive days (n = 2 × 20 = 40 measurements per concentration). The results will be accepted if the R-squared of the non-linear curve ≥ 0.95. For each concentration, we will calculate the daily mean, overall means, and the pooled within-run variance (repeatability) from the duplicate SDs; intermediate precision will be estimated by one-way ANOVA (day as factor) to derive the between-day variance component and its SD, and total precision will be computed. For each precision level, we will report mean, SD, and %CV, with typical acceptance criteria of %CV < 15% for repeatability.

8.6. Determine AMR and limits of quantification

Based on the precision of each concentration analyzed above, I will determine the AMR from the LLOQ and the ULOQ. The LLOQ is defined as the lowest concentration with a CV% ≤ 15%, and the ULOQ is defined as the highest concentration with a CV% ≤ 15%. The AMR or reportable range will be the interval from LLOQ to ULOQ.

8.7. Impact of sample quality, specifically storage time and temperature, on the accuracy and reliability of AIGAs quantification using the quantitative inhibitory ELISA

The stability of AIGAs in plasma samples will be evaluated over time. Quality control aliquots will be frozen and stored under the following conditions: −80 °C (deep freezing; reference standard), −20 °C (conventional freezing), 4 °C (refrigerated), and room temperature (~25 °C; unstable storage). Samples will be analyzed in duplicate at 3 and 6 months after being stored using an inhibitory ELISA to quantify AIGAs concentrations at each time point. The results will be accepted if the R-squared of the non-linear curve ≥ 0.95.

For each antibody concentration, measurements will be performed in duplicate at each time point and storage condition, and the mean of the duplicates will be used to reduce technical variability. Because only a single sample is available for each concentration level, statistical tests could not be applied. Therefore, antibody stability was assessed descriptively by calculating the percent change from baseline (day 0) for each sample under the different storage conditions:

$$\% \text{ Remaining} = \frac{\text{Mean } (Time\ point)}{\text{Mean } (Day\ 0)}$$

Results will be presented as the mean of duplicate measurements and visualized using the Levey-Jenning chart to illustrate changes over time and between storage conditions. This approach allows a clear assessment of the stability trends for each antibody concentration. Additionally, we will use Ordinary one-way ANOVA to highlight conditions where antibodies remained stable or showed significant decreases over the 3- and 6-month storage periods by comparing the p-value.

9. Results

9.1. Establish a quantitative inhibitory ELISA assay

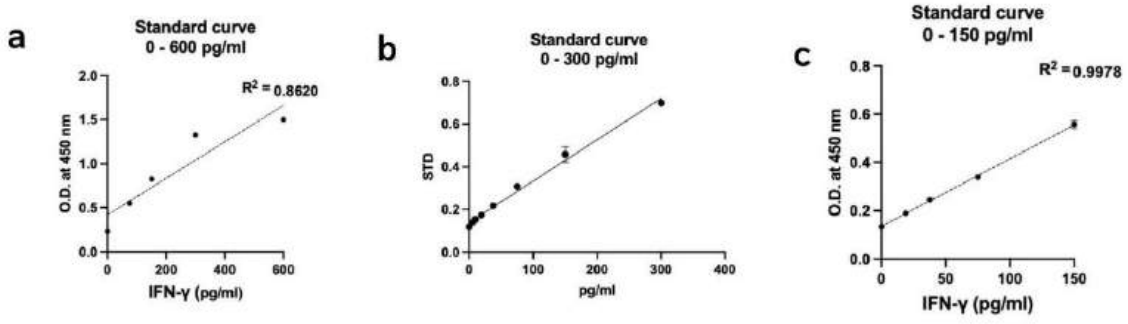
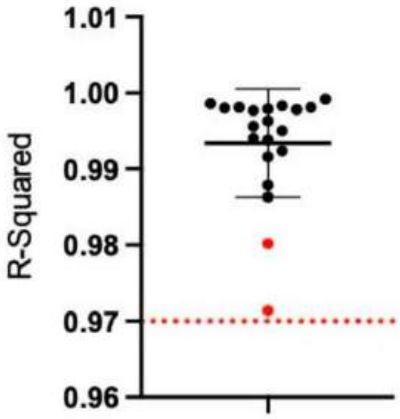


Figure 2. IFN-gamma standard curves

The 0–600 pg/mL range was removed because it showed poor linearity, which reduces the accuracy of IFN-γ quantification. The 0–150 pg/mL range was too narrow and could not cover higher sample concentrations. Therefore, the 0–300 pg/mL range was selected as it offers good linearity while covering a wider and more practical concentration range (Figure 2).



	20 independent experiments
Minimum	0.9714
Maximum	0.9992
Range	0.02780
Mean	0.9934
Std. Deviation	0.007136
Std. Error of Mean	0.001596

Figure 3.R-squared by 20 independent experiments

Results from 20 independent experiments show a high mean R-squared (0.9934) with low variability, the lowest value is 0.9714 (Figure 3). Setting the threshold at 0.97 helps exclude poorly fitted curves and ensures accurate and reliable quantification of IFN-γ.

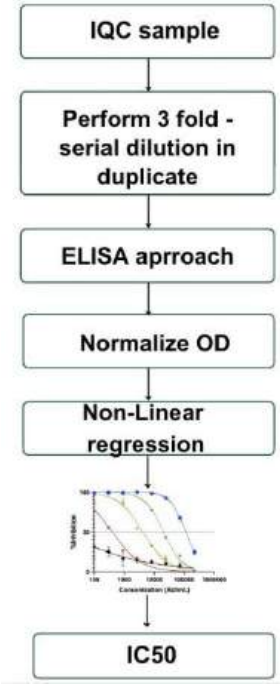


Figure 4. Flowchart of quantifying AIGA concentration from IQC sample

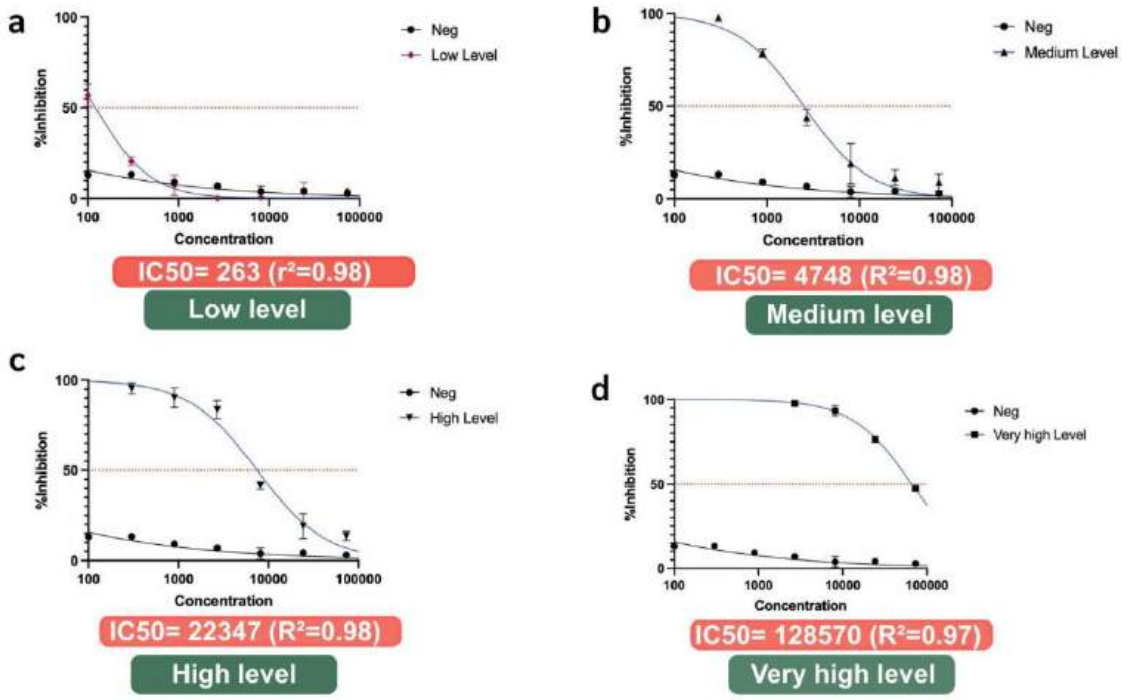
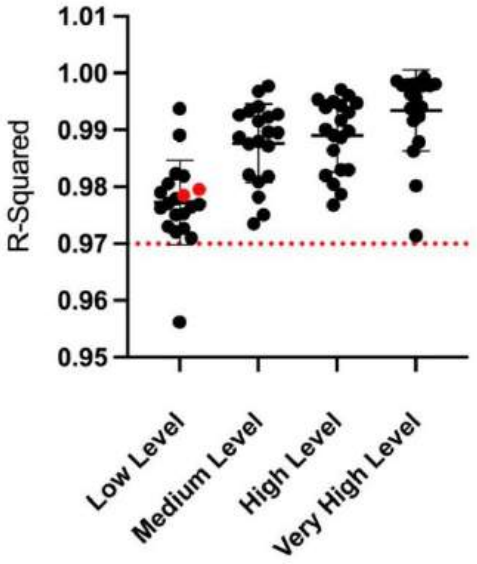


Figure 5.R-squared of the IQC samples in an independent experiment



R-squared	Mean -3SD	Mean +3SD
Low level	0.955	0.999
Medium Level	0.967	1.008
High Level	0.97	1.008
Very high level	0.972	1.015

Figure 6. R-squared of the IQC samples during 20 independent experiments

An R^2 cut-off value of 0.95 was selected for AIGA quantification based on the performance observed across 20 independent experiments. The results demonstrate that, at

all AIGA concentration levels (low, medium, high, and very high), the majority of standard curves achieved R^2 values well above 0.95, indicating a strong and consistent relationship between analyte concentration and assay response (Figure 5-6).

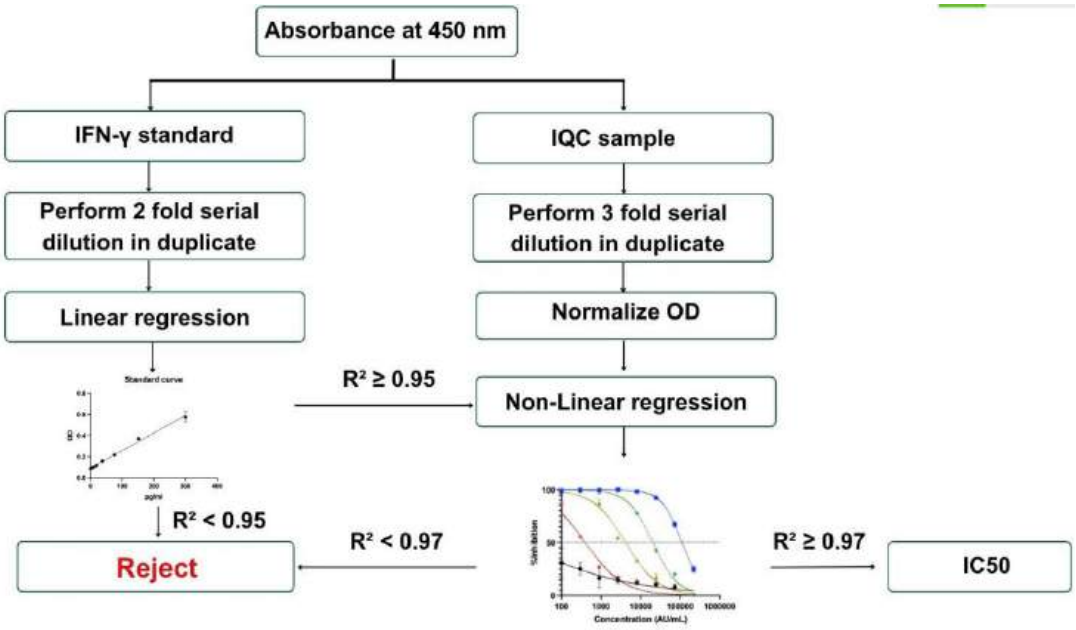


Figure 7. Criteria for IQC analysis

IQC samples are serially diluted, normalized, and analyzed using non-linear regression only when the IFN-γ standard curve shows linear regression with $R^2 \geq 0.97$. Subsequently, only non-linear regression curves with $R^2 \geq 0.95$ are accepted for IC50 calculation, ensuring accurate and reliable AIGA quantification (Figure 7).

9.2. Standardize a quantitative inhibitory ELISA assay

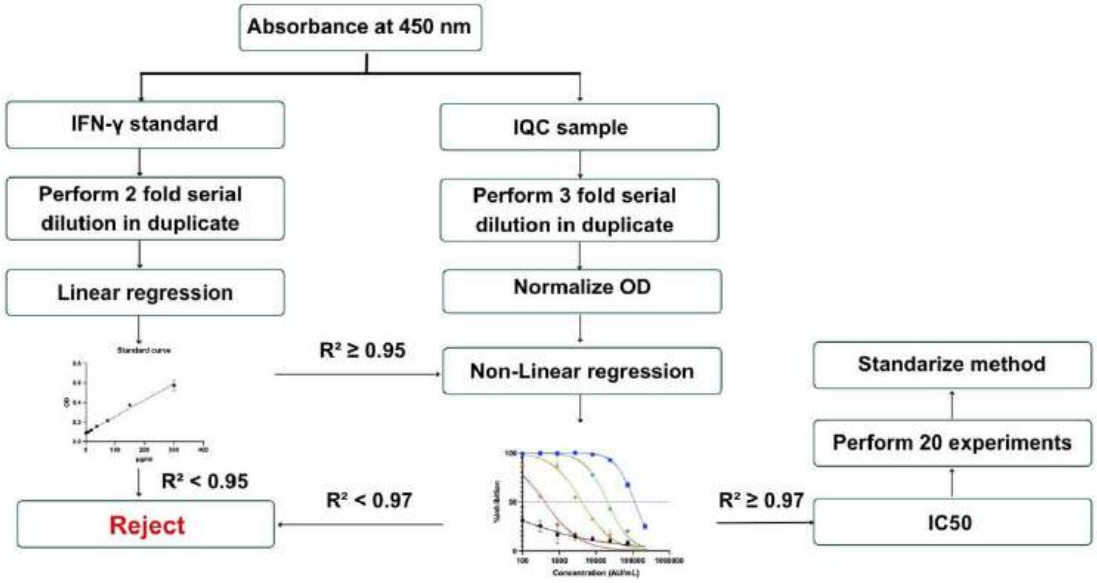


Figure 8. Standardize the inhibitory ELISA assay flowchart

After establishing the IQC analysis criteria, 20 independent experiments were conducted to collect data and standardize the method (Figure 8).

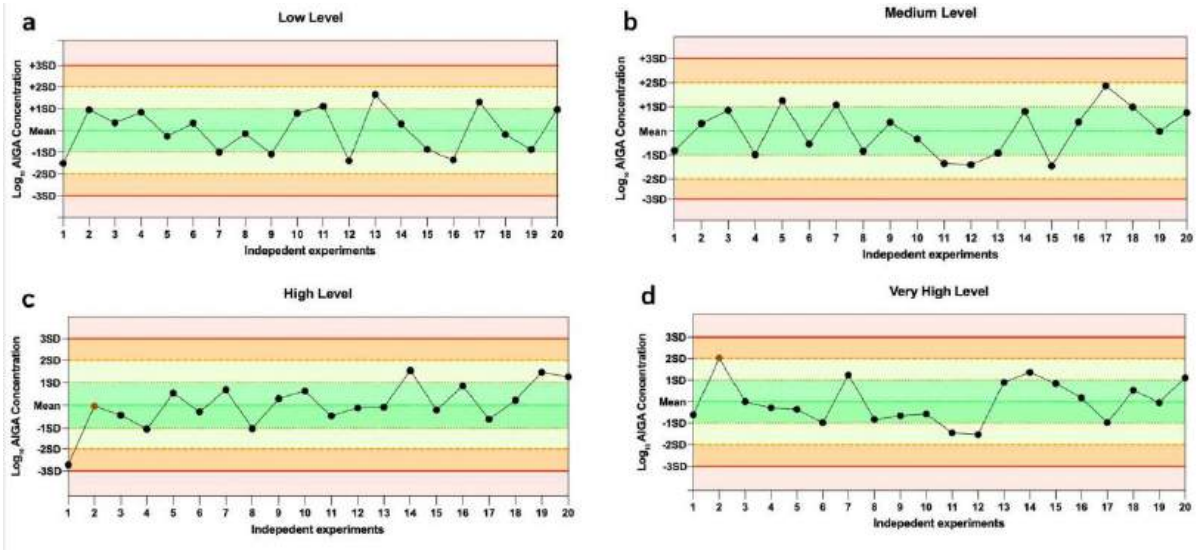


Figure 9. Levey-Jennings charts of IQC samples after 20 independent experiments

Precision evaluation through Levey–Jennings analysis demonstrated that the assay consistently meets acceptable performance criteria. All four IQC levels showed results

within ±2SD boundaries, and most measurements fell within ±1SD, indicating stable assay behavior across concentration ranges (Figure 9a-d). Occasional 1_{2s} Westgard warnings were observed at higher concentrations; however, these did not progress into more serious rule violations and therefore did not indicate analytical instability (Figure 9c,d). These findings confirm that the prepared IQC materials are suitable for routine internal quality control and that the assay performs reliably on a day-to-day basis. Reproducibility testing revealed a typical analytical pattern in which relative variability decreases as analyte concentration rises.

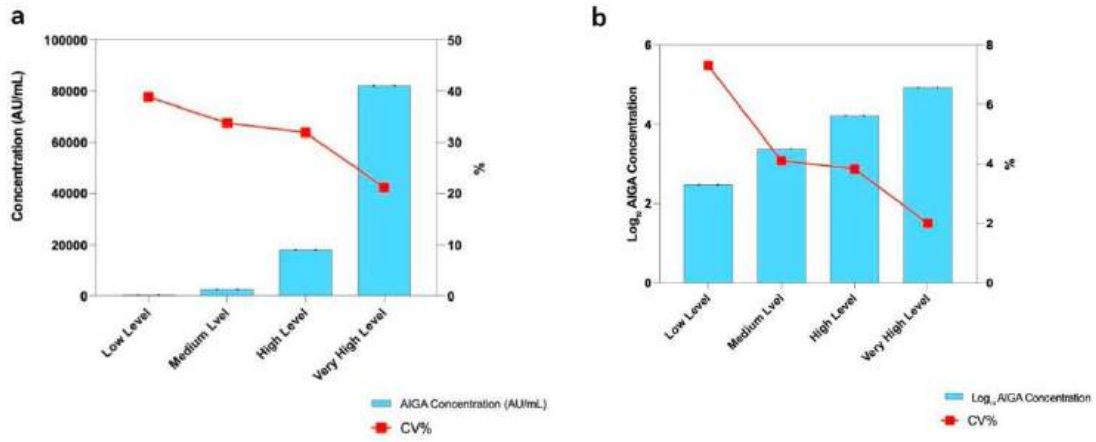


Figure 10. Units for AIGAs concentration reporting

AIGA concentrations expressed in AU/mL exhibited very high variability, with CV% ranging from approximately 20% to nearly 40% (Figure 10a), indicating that this unit is not suitable for reliable quantification using this method, unlike the result values expressed as $\log_{10}$-transformed units. Although the CV% was highest at the Low IQC level and lowest at the Very High level, all CV values remained below 10% (Figure 10b), which satisfies internationally accepted criteria for immunoassays. This suggests that even at low AIGA concentrations, where variability is most expected, the assay maintains acceptable precision).

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