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Development and Application of a Time-Resolved Fluorescence Immunoassay for Quantification of Rabies Virus Glycoprotein Antigen

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ABSTRACT

Objective: To establish and validate a time-resolved fluorescence immunoassay (TRFIA) for quantifying rabies virus glycoprotein antigen, thereby enhancing the quality control capabilities of rabies vaccines. **Methods:** A novel detection method was developed using a TRFIA-based kit. The kit's performance was comprehensively evaluated through method validation and comparative analysis against results obtained from an enzyme-linked immunosorbent assay (ELISA) kit. **Results:** The TRFIA method met all predetermined validation criteria. Assay results demonstrated a strong correlation with ELISA outcomes, while providing a broader quantitative range, greater resistance to interference, and superior methodological robustness. **Conclusion:** The TRFIA-based method for rabies virus glycoprotein quantification delivers reliable quality control results when applied to in-process samples and final rabies vaccine products..

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Key Words: Time-Resolved Fluorescence Immunoassay; Rabies Virus; Glycoprotein; Vaccine; Quality Control

Abbreviations: TRFIA, Time-Resolved Fluorescence Immunoassay; RV, rabies virus; GP, glycoprotein; ELISA, enzyme-linked immunosorbent assay; MAb, monoclonal antibody; RSD, Relative standard deviation.

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Introduction

Rabies virus antigen is a key quality indicator of rabies vaccines, with the glycoprotein being the only viral protein capable of inducing neutralizing antibody production and binding to such antibodies in the host. To a certain extent, the concentration of RV GP (referred to as "antigen content") determines the protective potency of the vaccine[1]. The NIH test, which measures vaccine potency, reflects the in vivo biological activity of the vaccine but has limitations including a 28-day test cycle, requirement for large numbers of animals, and poor result reproducibility, making it unsuitable for in-process quality control during vaccine production[2]. A sandwich ELISA using monoclonal antibodies (MAbs) has been developed for the rapid quantification of RV GP in rabies vaccines, with results showing a positive correlation with NIH potency values. Compared with the NIH test, ELISA offers advantages such as good reproducibility, low cost, and rapid turnaround time[3]. Based on the premise that MAbs can recognize correctly folded glycoproteins, RV GP content can serve as a surrogate indicator for vaccine potency evaluation[4]. The Chinese Pharmacopoeia specifies that ELISA may be used for antigen content determination of rabies vaccine bulk[5], and commercial ELISA kits are widely adopted by domestic manufacturers for this quality control purpose.

Long-term application of ELISA has revealed several limitations: the activity of enzyme-labeled antibodies is susceptible to labeling conditions, leading to batch-to-batch variability; numerous factors influence enzyme-catalyzed reactions, including enzyme/substrate properties, temperature, pH, and product inhibition[6]; as a result, the signal intensity is linearly correlated with enzyme concentration only within a narrow low-concentration range, and the Hook effect is prominent[7], which post challenges for accurate quantification. TRFIA shares the same immunological reaction basis as ELISA but incorporates lanthanide element-labeled antibodies and a complementary time-resolved fluorescence detection system, representing a next-generation immunoassay technology with distinct advantages: bifunctional europium chelates are used as small-molecule labels, minimizing interference with the spatial conformation of antibodies and thus preserving their biological activity[8]; the signal detection system distinguishes specific signals from background noise through temporal resolution, fundamentally addressing background interference issues[9]; detection signals are generated via photon counting, eliminating product inhibition effects and significantly extending the upper limit of signal detection.

In this study, a TRFIA kit developed for RV GP quantification was evaluated for

various performance parameters and compared with a domestic ELISA kit.

Materials and methods

1. Vaccine Samples and Reference Standards

Rabies vaccine samples (bulk, in-process products, final products, etc.) and antigen content reference standards were prepared by Guangzhou Baiyunshan Biologics Co., Ltd. in accordance with the commercial production process for freeze-dried human rabies vaccine (Vero cell-derived).

2. Main Reagents and Instruments

TRFIA kit: "Rabies Virus Glycoprotein Assay Kit (Time-Resolved Fluorescence Immunoassay)", purchased from Guangzhou Darui Biotechnology Co., Ltd.

ELISA kit: "Reagent for Relative Antigen Content Detection in Rabies Vaccines (Enzyme-Linked Immunosorbent Assay)", an in-house quality control reagent used for internal quality control of the company (Guangzhou Baiyunshan Biologics Co., Ltd.), purchased from Wuhan Biological Products Research Institute Co., Ltd.

Instruments: DR6606 Time-Resolved Fluorescence Immunoanalyzer (Guangzhou Darui Antibody Engineering Technology Co., Ltd.) and H1F Microplate Reader (BioTek Instruments, Inc., USA).

3. Detection Method

3.1 Assay Procedure

a) Dilute the antigen content reference standard to prepare serial standard solutions. Dilute test samples to ensure their fluorescence intensity falls within the linear range of the standard curve.

b) Add aliquots (100μl/well) of diluted reference standards, standard solutions, and test samples to microplate wells. Include negative controls. Seal the plate with a plate sealer and incubate for 1 hour.

c) Wash the plate 5 times with washing buffer. Dilute europium-labeled antibodies with antibody buffer, add 100 μl/well to the plate. Seal the plate and incubate for 1 hour.

d) Wash the plate 5 times with washing buffer. Add enhancement solution (100 μl/well), with gentle shaking.

e) Measure fluorescence intensity.

3.2 Calculation of Results

A standard curve was constructed with the common logarithm of the serial standard concentrations as the x-axis and the common logarithm of the corresponding signal (S) values as the y-axis, using the regression equation $Y = kX + b$ to determine k and b values. The antigen content of test samples was calculated based on their signal (S) values.

4. Validation of the TRFIA Method

4.1 Specificity

Key solutions used in the vaccine production process (e.g., cell maintenance medium, human albumin solution, sucrose solution, PBS), as well as blank formulations, were tested to assess potential interference with the assay.

4.2 Linear Range

The in-house reference standard was diluted to 0.2835EU/ml with sample dilution buffer and serially diluted 3-fold to 0.0035 EU/ml. A standard curve was plotted with the common logarithm of reference standard concentrations as the x-axis and the common logarithm of signal values as the y-axis, and the correlation coefficient (R^2) was calculated.

4.3 Accuracy

Known amounts of antigen (final concentration: 0.04725 EU/ml) were spiked into different dilutions of vaccine samples. The final dilution factor of spiked samples was consistent with that of test samples. Spike recovery rates were calculated based on simultaneous measurements of spiked and unspiked samples.

4.4 Precision

Repeatability: One operator performed 3 independent assays on the same vaccine samples.

Intermediate precision: Two additional operators performed independent assays to verify consistency across different operators.

4.5 Robustness

Fluorescence intensity was measured immediately after assay completion (0 minutes) and 10 minutes later. The results were compared to assess potential differences.

5 Comparison of the Two Methods

Vaccine samples (bulk, in-process products, final products) were tested using both the ELISA and TRFIA kits. The correlation between the two sets of results was analyzed. Comprehensive performance comparison of the two kits was conducted based on TRFIA validation data and practical application outcomes.

Results

1. Validation of the TRFIA Method

1.1 Specificity

Signals from key production process solutions and blank formulations were comparable to those of negative controls, indicating no significant interference with the assay.

1.2 Linear Range

Within the concentration range of 0.0035–0.2835 EU/ml, the linear correlation coefficient (R^2) of the standard curve was more than 0.98 in all 5 independent experiments, meeting linearity requirements (Table 1; Figure 1).

1.3 Accuracy

Add a known amount of antigen (final concentration: 0.04725 EU/ml) to different dilutions of vaccine samples. The spike recovery rates ranged from 90.9% to 118.8%, meeting the acceptance criterion of 80%-120% (Figure 2).

Table 1 Concentration Range and Linear Correlation Coefficients of Standard Curves

Concentration	Antigen Content （EU/ml）	Test Serial No.	Linear Correlation Coefficient (R^2)
1	0.2835	1	0.9984
2	0.0945	2	0.9986
3	0.0315	3	0.9984
4	0.0105	4	0.9993
5	0.0035	5	0.9992

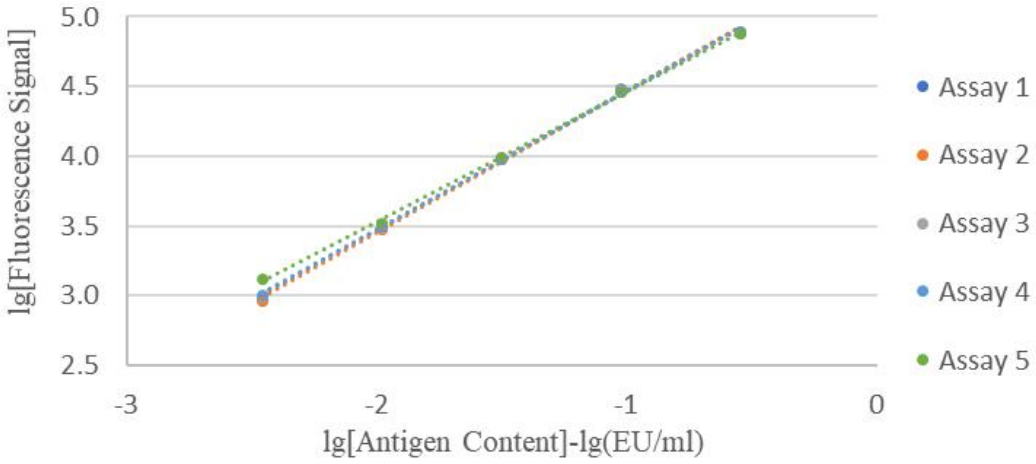

Figure 1: Standard curves (antigen concentration range: 0.0035–0.2835 EU/ml)

1.4 Precision

1.4.1 Repeatability: Relative standard deviation (RSD) of results from 3 independent assays by Operator 1 ranged from 0.7% to 7.2%, meeting the requirement of RSD ≤ 20%.

1.4.2 Intermediate precision: RSDs of results from 5 independent assays by 3 operators ranged from 0.9% to 7.1%, also meeting the requirement of RSD ≤ 20% (Figure 3).

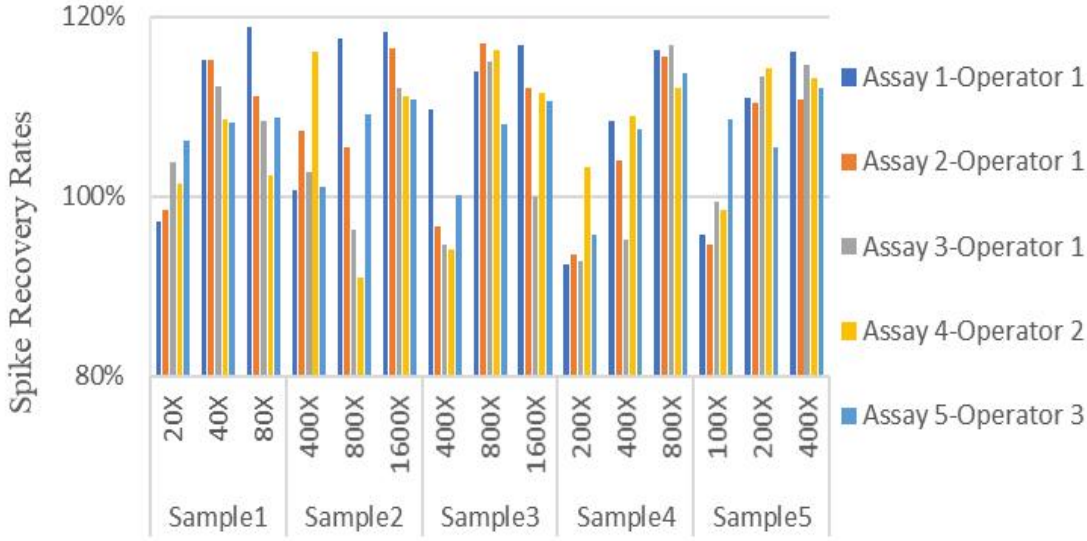

Figure 2： Accuracy, spike recovery rates

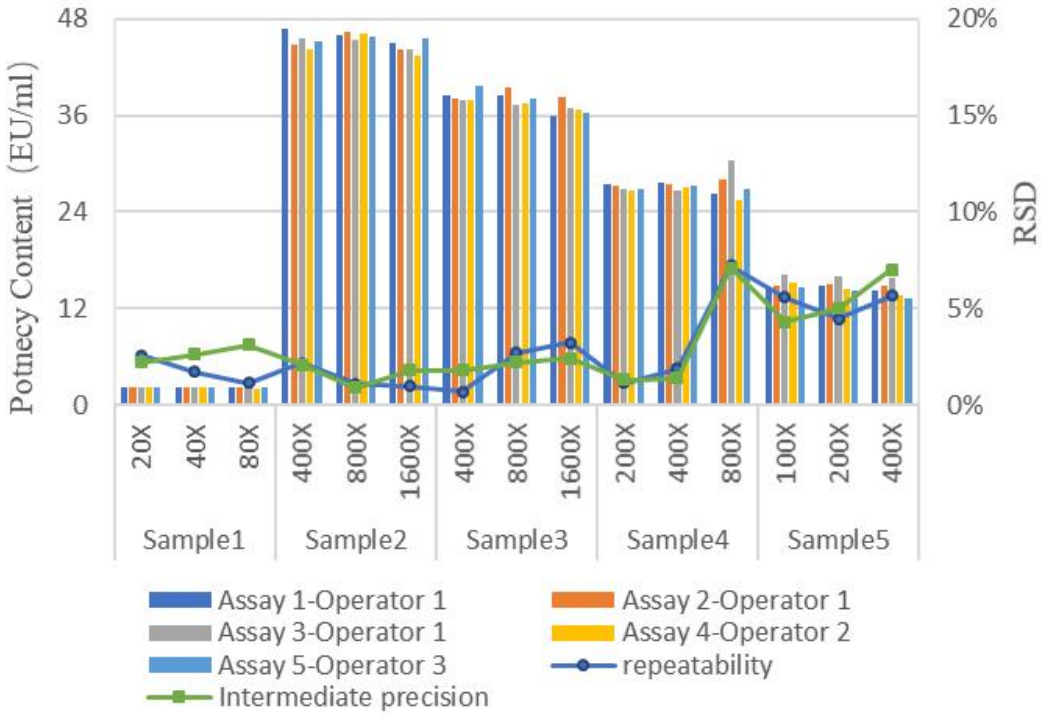

Figure 3: Precision, repeatability and intermediate precision

1.5 Robustness

The ratio of results obtained at 10 minutes to those obtained at 0 minutes ranged from 99.4% to 101.4% across 3 independent comparisons, indicating no significant differences (Figure 4).

2 Comparison with ELISA

2.1 the Result of correlation

A total of 50 vaccine samples (bulk, in-process products, final products) were tested by both methods. The correlation coefficient (r) between ELISA and TRFIA results was 0.936, indicating a high degree of correlation (Figure 5).

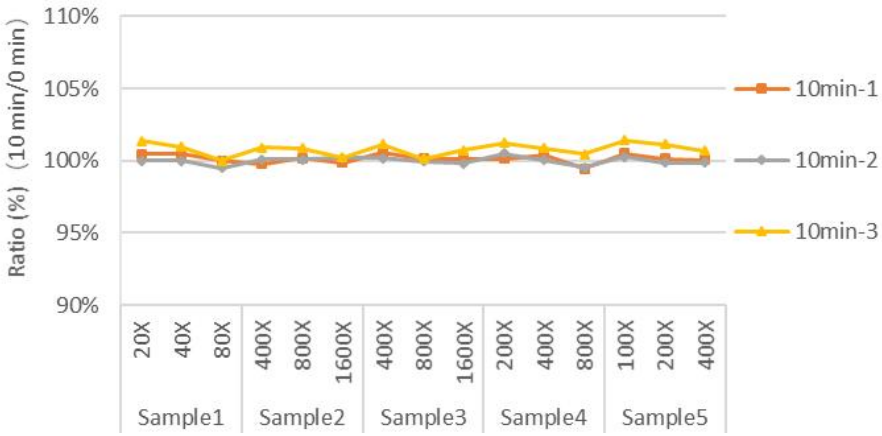

Figure 4: Robustness

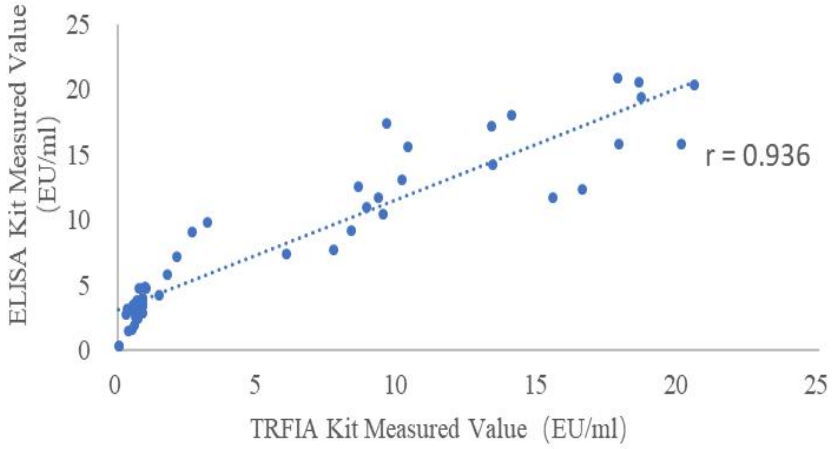

Figure 5: Comparison of measured values between the ELISA kit (y-axis) and TRFIA kit (x-axis)

2.2 Kit Performance Comparison

The TRFIA kit exhibited excellent linearity over a wide concentration range, strong anti-interference ability, and stable signals that persisted for an extended period without significant attenuation, resulting in superior method robustness.

In practical applications, this effectively reduces the need for retesting due to sample composition/content variations or signal values outside the standard curve range, improving work efficiency. Additionally, the TRFIA kit components showed greater stability, with a shelf life of 12 months (Table 2).

Discussion

The TRFIA-based method for RV GP quantification has demonstrated satisfactory quality control results in testing both in-process and finished rabies vaccine products. TRFIA and ELISA share the same immunological reaction principles, with improvements primarily in antibody labeling and signal detection, leading to significant enhancements in quality control performance. Therefore, replacing the conventional ELISA kit with the TRFIA kit for RV GP quantification can effectively enhance the quality control capabilities of rabies vaccine products[10].

Table 2: Performance Comparison of ELISA and TRFIA Kits

Performance Parameter	ELISA Kit	TRFIA Kit
Quantitative Range	16-fold range: 0.007–0.110 EU/ml Limit of quantification: 0.007 EU/ml	81-fold range: 0.0035–0.2835 EU/ml Limit of quantification: 0.0035 EU/ml
Anti-Interference Ability	Weak; variations in sample buffer salt concentration may cause result deviations	Strong; generally unaffected by sample buffer conditions
Method Robustness	Strict temperature control required; color development and reading after termination must be timed precisely to avoid signal distortion	Room temperature operation; signals remain stable over a wide time window before and after reading
Shelf Life	6 months	12 months

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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