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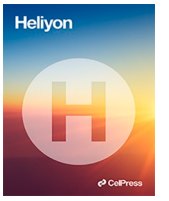

Research article

Novel envelope protein time-resolved fluoroimmunoassay as an alternative in vitro potency assay for quality control of inactivated Japanese encephalitis virus vaccine

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ABSTRACT

Japanese encephalitis (JE) vaccination is the most effective way to prevent JE. Plaque reduction neutralization test (PRNT) as the standard method for potency testing for inactivated JE vaccine could not provide the exact potency value. Envelope (E) protein of JE virus induces the body to create neutralizing antibodies. There is a potential for using the determination of E protein to assess the immunogenicity and efficacy of JE vaccine. In this study, an automatic time-resolved fluoroimmunoassay for detection of E protein in JE vaccine was established as a simple and rapid in vitro potency assay to complement PRNT, including the expression and paired screening of monoclonal antibodies, the establishment of assay method and performance verification. A pair of anti-E protein neutralizing antibodies (L022 and L034) were screened to construct the sandwich detection pattern. After pre-treating the vaccine sample, the entire analysis was performed using a fully automated machine, which had a little detection time and eliminated manual error. The results of the validation experiment met the requirements for quality control. The linear range was from 0.78125 U/mL to 25 U/mL, the sensitivity was 0.01 U/mL, the intra-assay coefficient of variation was less than 5 %, and the inter-assay coefficient of variation was less than 10 %. The recovery from the dilution was between 90 % and 110 %. This present TRFIA shown good stability and effectiveness in quality control for samples related to JE vaccine production. The outcomes demonstrated that the present TRFIA could be an alternative in vitro potency assay in quality control for inactivated JE vaccine.

1. Introduction

Japanese encephalitis (JE) is a zoonotic disease caused by the JE virus, which is the most prevalent form of epidemic encephalitis globally and is extensively distributed throughout the Asia-Pacific region [1]. The World Health Organization has estimated that JE

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accounts for a substantial number of cases worldwide, with an approximate range of 68,000 cases annually, leading to a significant number of deaths, ranging from 13,000 to 20,000, primarily in Asia [2]. Despite the absence of a pandemic in the past decade, China still experiences a considerable burden of JE, with an estimated annual incidence of 20,000 to 30,000 cases [3]. Patients diagnosed with clinical Japanese encephalitis (JE) exhibit a mortality rate ranging from 20 % to 30 %, while those who survive face a 30 %–50 % probability of experiencing enduring and severe neurological consequences, including but not limited to paralysis, speech impairment, and various chronic and severe neurological abnormalities [4].

There are no specific drugs for JE virus infection [5,6]. In the absence of antiviral drugs, the incidence of JE can only be reduced by palliative care and preventive measures [7,8]. Depending on the manner of transmission, prevention methods for JE virus include controlling mosquitoes, avoiding human contact, and vaccine. Unfortunately, it is impractical to completely eradicate mosquito breeding, particularly in the Asia-Pacific region where rice paddies are widely spread [9]. Moreover, preventing human contact with infected mosquitoes is only a temporary fix. In order to effectively prevent and manage JE transmission over the long term and sustainably, vaccination with high-quality JE vaccine is recommended [10,11].

Controlling the quality of vaccinations is a very important issue given the advantages and significance of vaccines in the prevention of JE. The most important quality control indicators are antigen content and vaccination potency. National Institutes of Health (NIH) test and plaque reduction neutralization test (PRNT) are designated for the assessment immunogenicity and potency testing of inactivated JE vaccine [12]. These methods not only require secure biosafety experimental environment, expensive experimental animals, lengthy testing procedures, and high operational skills, but also increased exposure in human beings to live and virulent JE virus and cannot guarantee the stability of test results [13]. The most troubling aspect of quality control of JE vaccine is that the PRNT method doesn't give an exact potency value, but only a conclusion as to whether the JE vaccine sample is effective. Development of a simple and rapid in vitro potency assay to complement existing in vivo potency assay is needed to enhance the efficiency of quality control for JE vaccine [14]. E protein on the envelope surface of JE virus, also known as viral hemagglutinin, is the most significant structural element of the virus surface and has a molecular weight of around 53 kDa [15,16]. Three functional structural areas of the E protein control the virus's capacity to enter cells, its pathogenicity, and its immunogenicity [17]. The primary target of JE virus neutralization in vitro is the E protein, which is also where JE virus-specific antibodies work [18]. There is a potential for using the E protein determination to assess the immunogenicity and efficacy of JE vaccines. It has been reported that an in vitro ELISA was used to assess the immunogenicity of the JE vaccine's antigen E protein [19]. Nevertheless, there are too many variables affecting the enzyme activity, which leads to ELISA's drawbacks like low sensitivity, unstable, and limited detection range [20].

High-level quality control system for JE vaccine relies on accurate, stable, and fully automated analytical method. Since its first application to rabies vaccine quality control, the time-resolved fluoroimmunoassay has gradually proven to be an excellent analytical platform for quality control in vaccine production due to its good specificity, high accuracy, wide linear range and simple operation [21]. TRFIA is significantly better than ELISA, which is commonly used for vaccine quality control at this stage, in terms of sensitivity, accuracy and detection range [22]. In this study, a paired neutralizing antibodies against E protein was prepared to establish an automated time-resolved fluoroimmunoassay for determination of E protein in JE vaccine. The performance indices and application value were preliminary verified and evaluated, including the verification of repeatability, specificity, sensitivity, accuracy, and feasibility. The outcomes demonstrated that the present TRFIA could be an alternative in vitro potency assay in quality control for inactivated JE vaccine.

2. Materials and methods

2.1. Virus, animals, and samples

P3 strain JE virus, inactivated JE virus, inactivated JE vaccine and national references of inactivated JE vaccine were supplied by Liaoning Cheng Da Biotechnology Co., Ltd (Shenyang, China). Female BALB/c mice aged 6–8 weeks were bred in Southern Medical University's Laboratory Animal Center (Guangzhou, China). BHK21 cells were provided by the Institute of Antibody Engineering, Southern Medical University (Guangzhou, China). Animal experiments were approved by the Laboratory Animal Welfare and Ethics Committee of Southern Medical University.

2.2. Reagents and instruments

Bovine serum albumin (BSA), Freund's complete adjuvant, Freund's incomplete adjuvant, TEMED, Tween-20, and glutaraldehyde were purchased from Sigma-Aldrich (St. Louis, MO, USA); Europium labeling reagent, enhancement solution were purchased from Guangzhou You Bo Biotechnology Co. Ltd. (Guangzhou, China); Ultrafiltration centrifuge tubes were purchased from Millipore Corp. (Billerica, MA, USA); Protein purification column filler Sephadex G-50 was purchased from Amersham Pharmacia Biotech (Piscataway, NJ, USA). Ultra-pure water preparation system and ultramontane electronic balance were purchased from Sartoruis (Gottingen, Germany); Microplate thermostatic oscillator was purchased from Thermo Fisher Scientific (Waltham, MA, USA); DR6660 was purchased from Guangzhou DARUI Biotechnology Co. Ltd. (Guangzhou, China).

2.3. Solutions

Coating solution: 50 mmol/L Tris-HCl buffer, pH 7.2; Blocking solution: 1 % BSA was added to 50 mmol/L Na_2CO_3–$NaHCO_3$ buffer system, and the pH was adjusted to 9.6. Antibody labeling buffer: 155 mM NaCl, 50 mM Na_2CO_3–$NaHCO_3$, adjusted pH to 8.5; Washing

buffer: 0.84 % NaCl, 0.01 % NaN_3, 0.06 % Tween 20 were added to 50 mmol/L Tris-HCl buffer system, and the pH was adjusted to 7.8. The column regeneration solution was added with 0.01 % DTPA in 10 mmol/L hydro phthalate, and the pH was adjusted to 4.0. Assay buffer: 0.02 % BSA, 0.05 % Tween-20 and 0.05 % NaN_3 were added to 50 mmol/L Tris-HCl buffer system, and the pH was adjusted to 7.8. Standard diluents: 0.1 % NaN_3 and 0.2 % BSA were added to 50 mmol/L Tris-HCl buffer system, and the pH was adjusted to 7.8. Enhancement solution: The pH was adjusted to 7.8 by adding 50 μM trin-octyl phosphine oxide, 15 μM β-naphthyl trifluoroacetate, and 0.1 % Triton X-100 to 100 mM acetate-phthalate buffer.

2.4. Establishment of hybridoma cell lines

The immunization was performed on 7 healthy female BALB/c mice. The serum titer was found using an indirect ELISA. At a concentration of 3 μg/mL, the purified inactivated JE virus was coated in 96-well plates, and mouse serum was utilized as a gradient dilution sample. The fusion could be performed if the titer reached 1:64000. After fusing with SP2/0 myeloma cells, splenocytes were cultivated for 7 days. Indirect ELISA was used to identify antibody secretion and identify positive clones.

2.5. Preparation of monoclonal antibodies(Mabs)

The hybridoma cell line that stably secretes monoclonal antibody was expanded and cultured, and then inoculated into the peritoneal cavity of BALB/c mice at a cell number of 1–2^10^6 per mL. Thus, a large number of ascites samples containing monoclonal antibodies were obtained, and then ascites was purified by Protein G affinity chromatography to obtain Mabs.

2.6. Neutralization tests for Mabs

500 plaque forming units (PFU) per milliliter P3 strain JE virus sample was utilized, and 300 μL of test antibody was combined with the same volume of diluted virus suspension. Viral combinations and neutralizing antibodies were applied to BHK21 cell culture wells. The wells of cell culture plates were stained with crystal violet after 5 days of culture, then cleaned and given time to dry. The neutralizing inhibition rate was then determined after counting the plaques in each well.

2.7. Selection of paired antibodies

2.7.1. Labeling of the detected antibodies

According to the Europium labeling kit instructions, pretreatment of the labeled antibody needed 0.2 mg of labeling reagent to label 1 mg of antibody, or a mass ratio of 5:1. Thereafter, the antibody and the europium-labeled reagent were fully combined in the centrifuge tube. To prevent evaporation, the opening of the centrifuge tube was sealed with a sealing membrane. The centrifuge tube was then incubated on a shaker for 18 h or overnight at room temperature. The europium-labeled antibody was purified using a Sephadex^{TM} G-50 gel chromatography column. Finally, 10 % BSA was added to the collected, purified antibody, bringing the labeled antibody's BSA concentration down to 0.1 %. The EP tube's opening was sealed with a sealing membrane and kept at −20 °C for storage.

2.7.2. Coating of the capture antibody

Each antibody strain was diluted to a concentration of 3 μg/mL using coating buffer, and then the diluted antibody solution was applied at a dose of 100 μL per well in a 96-well plate. The 96-well plates were shaken for 1 h at room temperature, then washed four times, dried, 300 μL of blocking solution were added to each well, and the plates were finally put in the refrigerator for 2–8 °C overnight. The blocking solution was discarded from the 96-well plates on the second day and kept at 2–8 °C until needed.

2.7.3. Selection of paired antibodies by TRFIA

The most effective capture antibody to combine with the detection antibody was found using cross-square titration. The Inactivated JE vaccine was added to the wells at 100 μL per well and incubated for 1h at room temperature with vibration on an oscillator. Each europium standard detection antibody was diluted with assay solution by a factor of 1:500, and 100 μL was added to each well. The mixture was then discarded, washed four times, dried, and incubated for 1 h at room temperature with oscillator shaking. The liquid was thrown away, cleaned six times, and dried afterward. 100 μL enhancement solution was added to each well, shaken for 5min, and then the fluorescence value was detected on the detector.

2.7.4. Specificity identification of Mabs

To identify the specificity of antibodies for JE virus E protein, Western blot was performed with purified JE virus sample for L022 and L034 Mabs.

2.8. TRFIA protocol

The full analysis procedure was carried out by an automated time-resolved fluoroimmunoassay system DR6660, as depicted in Fig. 1. The specific steps are as follows: The labeled detection antibody was taken out of −20 °C, diluted with assay buffer to the proper working concentration, added to the appropriate location of the instrument for subsequent use. The automatic analyzer was loaded

with the standard or sample. During detection, the apparatus sucked 100 μL of the tested sample or standard into the reaction well and incubated it there at room temperature with shaking for 1 h. In the reaction well, the test sample's antigen and the capture antibody were combined to create a complex, which was then washed four times. The reaction well was added with 100 μL of the detection antibody, which was then incubated at room temperature for 1 h while being shaken. The detection antibody formed complex with the above substances. The 96-well plate was washed 6 times and dried. The response wells received 100 μL of the enhancement solution and were shaken for 5 min 100 μL of the enhancement solution was sucked into the reaction wells and shaken for 5 min. The fluorescence signal of each well was collected and analyzed by the instrument at the excitation wavelength.

2.9. Preparation of standard and quality control

National reference of inactivated JE vaccine (Assign a value of 100 U/mL) was diluted to the following concentrations with 1.5 % dilution of standard substance to obtain standard substance: 0 U/mL, 0.78125 U/mL, 1.5625 U/mL, 3.125 U/mL, 6.25 U/mL, 12.5 U/mL, 25 U/mL, respectively, were divided into 1 mL per bottle, lyophilized and stored at 4 °C, and re-dissolved with 1 mL pure water before use. The quality control materials were diluted to 0.8 U/mL, 4 U/mL and 20 U/mL with 1.5 % dilution of standard. The quality control materials were divided into 1 mL/bottle, lyophilized and stored at 4 °C, and re-dissolved with 1 mL pure water before use.

2.10. Optimization and evaluation of the Reaction System

2.10.1. Selection of the optimal capture antibody concentration

The capture antibody was diluted to 0.5, 1, 2, 3, 5, 8 μg/mL using the coating buffer. Different concentrations of the diluted capture antibody were then used to coat 96-well plates. The labeled antibody dilution was chosen to be 1:200 and 1:400, with a reaction time of 60 min. A 20 U/mL standard was tested.

2.10.2. Selection of the Optimal Labeled Antibody Dilution

The labeled antibody was diluted to 1:50, 1:100, 1:200, 1:400, 1:800, and 1:1600 using the assay buffer. A capture antibody concentration of 3 μg/mL was chosen, with a reaction time of 60 min 20 U/mL and 0 U/mL standards were tested.

2.10.3. Selection of the optimal reaction time

The reaction times were set to 20 min, 30 min, 40 min, 50 min, 60 min, and 80 min, with a capture antibody concentration of 3 μg/mL and a labeled antibody dilution of 1:200. A 20 U/mL standard was tested.

2.11. Performance evaluation of novel TRFIA

2.11.1. Standard curve

10 replicate wells were set up for standards of different concentrations (0 U/mL, 0.78125 U/mL, 1.5625 U/mL, 3.125 U/mL, 6.25 U/mL, 12.5 U/mL, 25 U/mL) and the measured values are obtained using the TRFIA method established above. Statistical Product and

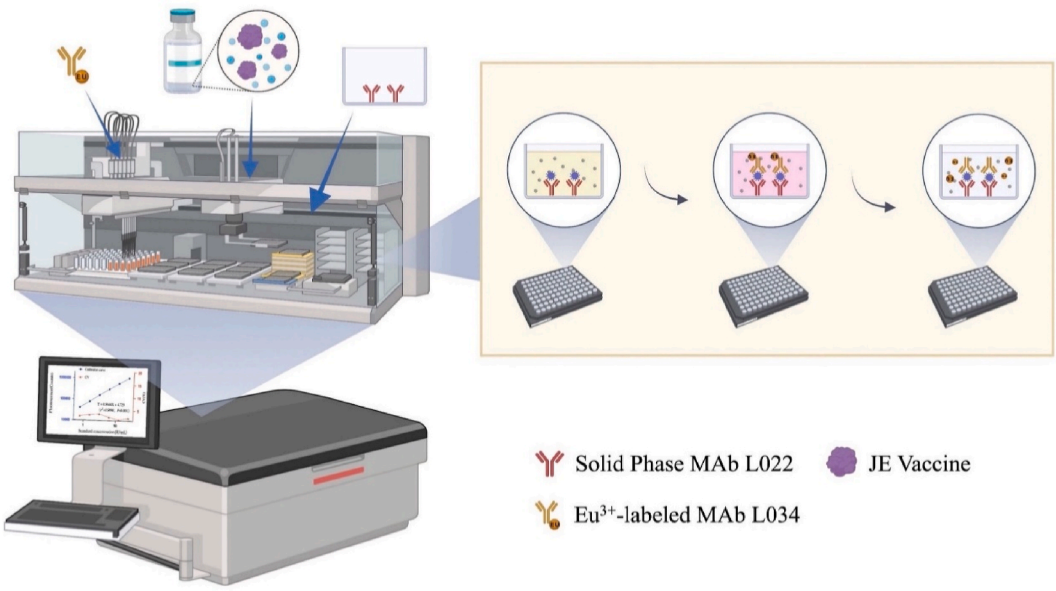

Fig. 1. Schematic diagram of the novel TRFIA.

Service Solutions (SPSS) software was used to analyze the data (version 20.0; SPSS Inc., Chicago, IL, USA). Each test had two tails, with $\alpha = 0.05$ significance level. Statistics were considered significant if $P < 0.05$. The data were fitted using the following formula using the logarithm of the fluorescence signal values (Y) plotted against the logarithm of JE vaccine potency (X) as a standard curve in the Prism GraphPad software: Plotting a standard curve, LogY = a *LogX + b.

2.11.2. Sensitivity assay

The blank standard (0 U/mL) was repeatedly detected 20 times in accordance with the TRFIA operating method, and the mean and standard deviation (SD) of the fluorescence signal values obtained were computed. Then the fluorescence value generated by mean + 2*SD was substituted into the standard curve equation, and the calculated concentration was the sensitivity of the time-resolved fluoroimmunoassay reagent for the E protein of the JE vaccine.

2.11.3. Hook effect

The national reference of inactivated JE vaccine (100 U/mL) was diluted with 1.5 % dilution of standard to make different concentrations: 0 U/mL, 0.78125 U/mL, 1.5625 U/mL, 3.125 U/mL, 6.25 U/mL, 12.5 U/mL, 25 U/mL, 30 U/mL, 35 U/mL, each standard was repeated three times, and the curve was fitted and drawn.

2.11.4. Accuracy assay

A crucial parameter for assessing accuracy is the sample recovery. Two batches of JE vaccine samples with different concentrations were used for accuracy detection with 3 different dilutions (8 times, 16 times, 32 times). Each sample was tested in triplicate. The accuracy of the detection method was analyzed by comparing the measured value of the sample with the theoretical value to calculate the sample recovery rate (recovery rate = measured value/theoretical value × 100 %).

2.11.5. Precision assay

Precision of this assay was evaluated based on intra-assay and inter-assay. 3 quality control samples (0.8 U/mL, 4 U/mL, and 20 U/mL) were measured 10 times/day for 3 sequential days. The fluorescence signal values were collected and analyzed. The inter-assay coefficient of variation (CV) and intra-assay CV of fluorescence signal values were calculated by standard deviation divided by mean.

2.11.6. Specificity assay

Sucrose, bovine serum albumin and human serum albumin, as conventional components of JE vaccine, were used to assess the specificity of our TRFIA method. According to the TRFIA procedure, JE vaccine samples added with high concentration sucrose, Bovine serum albumin and human serum albumin respectively were detected. The detection concentration was compared with the theoretical concentrations to evaluate specificity (recovery rate = detection value/theoretical value × 100 %).

2.11.7. Application in quality control of JE vaccine

Three test operators tested three lots of purified JE virus samples and JE vaccine semi-finished products at different days using this present TRFIA of the moment to analyze TRFIA stability and validity.

3. Results

3.1. Antibody screening and identification

Results of serum potency assay after immunization of mice with purified inactivated JE virus were showed in Table S1. Results of indirect ELISA for identifying positive clones were shown in Table S2. For the following antibody pairing experiment, 9 monoclonal antibodies against E protein of JE virus with inhibition rates greater than 50 % were obtained. Table 1 displayed the outcomes of neutralization tests performed on monoclonal antibodies with inhibition rates greater than 50 %. The next phase can involve the

Table 1
Result of neutralization test for MAbs.

Sample	Rate of inhibition
L015	50.00 %
L021	58.33 %
L022	58.33 %
L023	66.67 %
L024	54.17 %
L025	58.33 %
L034	54.17 %
L041	50.00 %
L049	50.00 %
Positive control 10 ×	100.00 %
Positive control 100 ×	100.00 %
Positive control 500 ×	58.33 %

creation of time-resolved fluorescent immunoreagents using monoclonal antibodies with inhibition rates greater than 50 % (L015, L021, L022, L023, L024, L025, L034, L041 and L049). These antibodies have good neutralizing effects. Results of PRNT for antibodies L022 and L034 were displayed in Fig. 2.

3.2. Screening pairing of capture and detection antibodies

The coating and labeling of nine monoclonal antibodies were done in pairs. Purified JE virus sample (100 μg/mL) was used as the detection standard. As shown in Fig. 3, the fluorescence signal value was the highest when the capture antibody was L022 and the detection antibody was L034, therefore this antibody combination was chosen for the following creation of TRFIA. As shown in Fig. S1, specific reaction bands appeared at the 53 kDa size of the E protein, indicating the specificity of the reaction of L022 and L034 with the E protein.

3.3. Optimization of the Reaction System

From Fig. 4A, it can be observed that when the capture antibody concentration increased to 3 μg/mL, there was no significant increase in fluorescence value, indicating that the coating concentration of the capture antibody was nearing saturation. Therefore, 3 μg/mL was selected as the optimal capture antibody concentration. As shown in Fig. 4B,as the dilution concentration of the detection antibody increased from 1:1600 to 1:200, the fluorescence value gradually increased. When the dilution degree of the detection antibody exceeded 1:200, the fluorescence value no longer increased, suggesting that the binding of the detection antibody approached saturation. Therefore, 1:200 was chosen as the optimal dilution for the labeled antibody. From Fig. 4C, the fluorescence value significantly increased when the reaction time was between 10 and 60 min. However, when the reaction time was between 60 and 80 min, the increase in fluorescence counts became less pronounced, indicating that the specific binding reaction between the antigen and antibody was nearing equilibrium. Therefore, 60 min was selected as the optimal reaction time for subsequent experiments.

3.4. TRFIA standard curves and limit of detection

Logarithmic and linear regression were used to determine the standard curve. As shown in Fig. 5, the following equation represents the best match calibration: LogY = 0.9444 LogX +4.729(r^2 = 0.9990), $P < 0.001$. The linear range for the TRFIA technique was up to 25 U/mL with the sensitivity of 0.01 U/mL. When the standard concentration exceeded 25 U/mL, the signal was saturated (Fig. 6).

3.5. Accuracy and precision results

The sample recovery rate was calculated by comparing the measured value of the sample with the theoretical value (recovery = measured value/theoretical value*100 %), and the results are shown in Table 2. Each recovery rate ranged from 90 % to 110 %, which is indicative of good accuracy. According to the results in Table 3, the inter-assay coefficient of variation was less than 10 %, and the intra-assay coefficient of variation was less than 5 %, which showed good precision and met the relevant requirements of detection reagents.

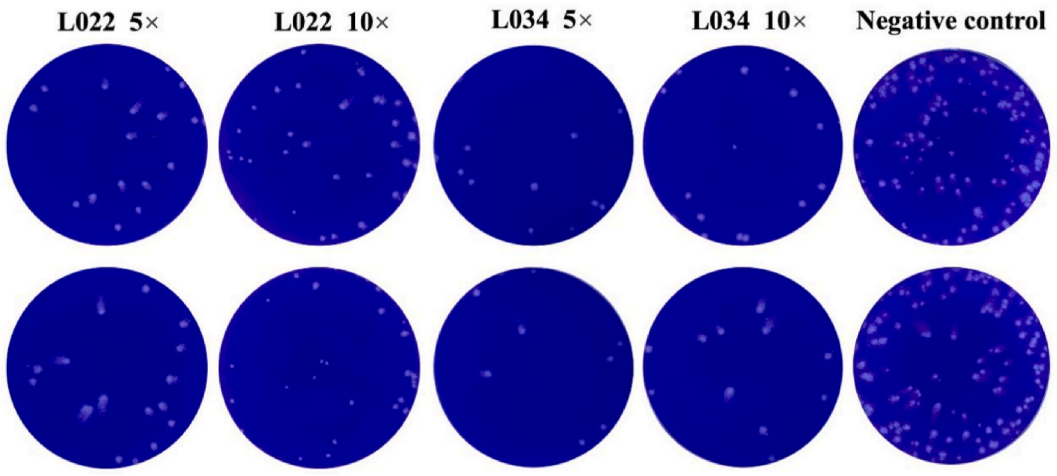

Fig. 2. PRNT results of L022 and L034.

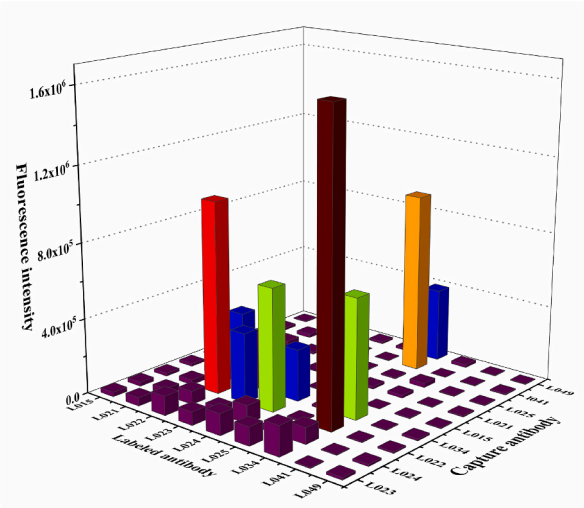

Fig. 3. Screening of optimal antibody pairings by the checkerboard method.

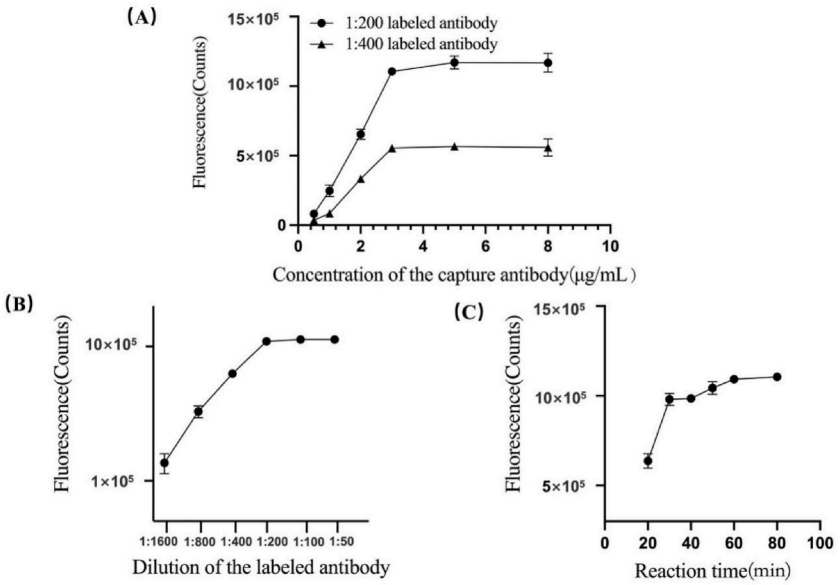

Fig. 4. Optimization of the reaction system. (A) Selection of the optimal capture antibody concentration. (B) Selection of the optimal labeled antibody dilution. (C) Selection of the optimal reaction time.

3.6. Specificity results

As shown in Table 4, the recovery ranged from 90 % to 110 % at the interference of high concentration sucrose, bovine serum albumin and human serum albumin, which proved that the specificity of present TRFIA was good.

3.7. Application in quality control of JE vaccine

As shown in Table 5, this present demonstrated good stability and effectiveness in quality control for samples related to JE vaccine production.

4. Discussion

It is frequently necessary to monitor the potency in time during the production process of JE vaccine. However, PRNT as the "gold standard" for quality control of inactivated JE vaccine potency cannot give an exact potency. In addition, its disadvantages, such as

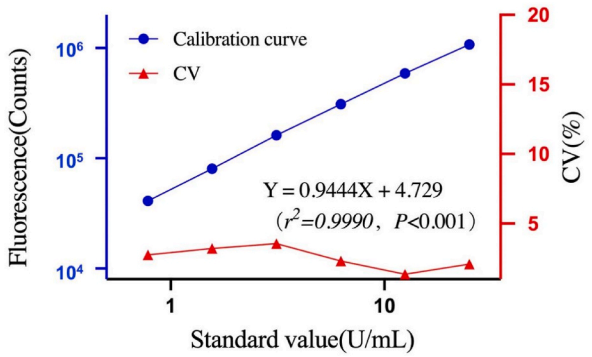

Fig. 5. Calibration curve of the novel TRFIA (each point was based on 10 replicates). The Log function value of the standard concentration was set to the X axis, and the Log function value of the fluorescence value was set to the Y axis.

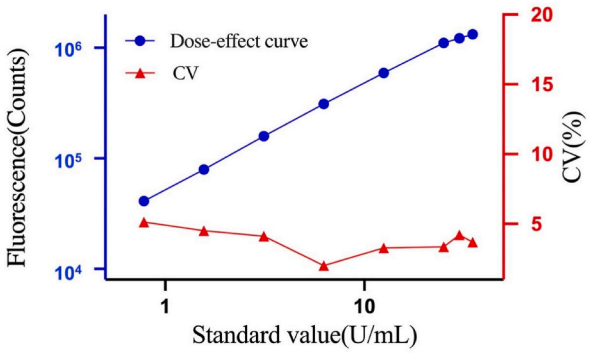

Fig. 6. High-dose signal saturation (hook-effect) for the present TRFIA.

Table 2
Accuracy assay of the present TRFIA (n = 3).

Sample	Original concentration (U/mL)	Multiple of dilution	Expected value (U/mL)	Mean (U/mL)	Recovery (%)
A	128	8	16	16.88	105.5
		16	8	7.93	99.13
		32	4	3.95	98.75
B	80	8	10	10.46	104.60
		16	5	5.10	102.00
		32	2.5	2.47	98.80

Table 3
Precision assay of the present TRFIA.

	Sample	Theoretical value (U/mL)	Mean ± SD (U/mL)	CV (%)
Intra-assay (n = 10)	A	0.8	0.76 ± 0.04	5.26
	B	4	4.13 ± 0.20	4.84
	C	20	19.64 ± 0.75	3.82
Inter-assay (n = 30)	A	0.8	0.79 ± 0.05	6.33
	B	4	4.22 ± 0.27	6.39
	C	20	19.79 ± 0.82	4.14

lack of accuracy, time-consuming and tedious, and high cost, constrain the quality control process. For example, the physiological state of the cells will be highly influenced by the experimenter's competence and accuracy, which will cause the experimental results to deviate. It is difficult to consistently ensure the dependability of varied laboratory workers [23]. Unfortunately, there is still a lack of precise and trustworthy quantitative detection method for JE vaccine effective antigen that can be used for quality control.

E protein is the major structural protein of JE virus and plays an important role. As the surface membrane protein of JE virus, it plays an important role in mediating virus adsorption, determining virus hemagglutination activity, cellular tropism, virus virulence

Table 4
Specificity assay of the present TRFIA (n = 3).

Sample	Added concentration (ng/mL)	Theoretical value (U/mL)	Mean of measured value (U/mL)	Recovery (%)
Sucrose	2000	4	3.91	97.8
BSA	2000	4	3.92	98.0
HSA	2000	4	4.17	104.3

Table 5
Quality control testing of the present TRFIA for multiple lots of JE vaccine related samples.

Sample	Measured value (U/mL)			CV (%)
Purified JE virus sample	559	502	507	6.1 %
	566	507	520	5.8 %
	572	508	533	6.0 %
JE vaccine semi-finished product	139	134	138	1.9 %
	140	125	133	5.7 %
	142	125	135	6.3 %

and inducing host protective immune response [24]. As the key for JE treatment and prevention, evaluation of vaccines and the diagnosis of infections depend greatly on the detection of JE virus E protein [25,26]. The glycoprotein of rabies virus has a similar function to that of the E protein, and rabies vaccines have achieved quality control by quantitative analysis of the glycoprotein to assess vaccine efficacy [27]. TRFIA is regarded as a sensitive, quick, convenient, high-throughput, and reliable detection technique. We have successfully applied the time-resolved fluoroimmunoassay to the quantitative application of rabies virus glycoprotein, nucleoprotein, host cell protein of Vero from rabies vaccine and rabies antibody titer [21,22,28,29]. Hence, we propose the idea of achieving in vitro potency assay by developing a novel TRFIA for determination of E protein, which is expected to be an additional supplement to PRNT for JE vaccine quality control.

The detection range of the novel TRFIA was 0.01 U/mL-25 U/mL with the sensitivity was 0.01 U/mL, The intra- and inter-assay coefficients of variation were less than 10 %, and the dilution recovery was between 90 % and 110 %. Compared with previously reported ELISA methods for JE virus E antigen detection, this novel TRFIA provides wider detection range, higher accuracy, excellent precision and application of automation [19,30]. However, the present TRFIA still relied on the traditional 96 well plate for detection. There are limitations in terms of simplicity and operating time. In the future, we considered the optimization of magnetic beads as the coating carrier, there might be a higher improvement in time and detection performance [31]. Quality control application of the present TRFIA for purified JE virus samples and JE vaccine semi-finished products demonstrated good stability and effectiveness. Although additional vaccine samples and longer validation are needed, the available findings provide preliminary evidence that this novel TRFIA might be applied to monitor the quality of JE vaccine production and determine the stability and efficacy of the vaccine.

In conclusion, we established an alternative in vitro potency assay for JE vaccine based on an automated JE virus E protein time-resolved fluoroimmunoassay. Based on the positive results of the validations in this study, it is expected that the new TRFIA will provide vaccine makers and national control laboratories with automated quality control procedures that are more useful, efficient, and accurate as a supplement to in vivo testing techniques.

Data availability statement

The datasets generated and/or analyzed during this study are available from the corresponding authors.

CRediT authorship contribution statement

Zhaoyue Li: Writing – original draft, Methodology. **Hui Zhao:** Methodology. **Xuzhe Gao:** Resources, Methodology. **Feifei Sun:** Resources, Methodology. **Shiyuan Liu:** Methodology. **Zhigao Zhang:** Software, Methodology, Data curation. **Xiangming Zhai:** Validation, Software. **Yue Cao:** Validation. **Yingsong Wu:** Writing – review & editing, Project administration, Conceptualization. **Guanfeng Lin:** Writing – review & editing, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e33015.

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