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Research paper

Rapid fluorescent focus inhibition test optimization and validation: Improved detection of neutralizing antibodies to rabies virus

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ABSTRACT

The rabies rapid fluorescent focus inhibition test (RFFIT) is the most widely used cell-based assay for detecting and quantitating rabies virus neutralizing antibodies (RVNA) in human serum. However, it is a complex, labor intensive, and somewhat subjective manual assay, the performance of which may be affected by a number of factors including the quality of cells and virus, variability of assay reagents and the skill and expertise of analysts. This study sought to identify and evaluate conditions that may impact RFFIT performance and RVNA detection by evaluating assay parameters including: different serial dilution scheme of serum samples in a 96-well microplate using semi-automated pipetting systems, the range of dose of challenge virus standard (CVS-11) strain of rabies virus, the effect of complement (C′), the effect of cell seeding density and passage number, the effect of diethylaminoethyl (DEAE) dextran concentration on virus infectivity, and the assay incubation period prior to immunostaining. In addition the evaluation of counting fluorescent foci using a microscope versus using scanned images from a cell imaging reader was performed in an effort to ease the reading of slides and have permanent records of the raw data. The results from optimization of each parameter are presented along with

Abbreviations: ATCC, American Type Culture Collection; BRC', Baby Rabbit Complement, manufactured by Pel Freez Biologicals (catalog #31061–3) and stored at −80 °C to −40 °C; BHK-21, Baby Hamster Kidney Cells, originated from ATCC (catalog #CCL-10): master and working bank produced in-house at SP; BioTek Cytation™ Cell Imager and Stacker, Manufactured by BioTek (catalog # CYT3V and BIOSTACK4); BioTek Precision Microplate Pipetting System, Manufactured by Biotek (catalog # PRC384U, model: Universal Precision); C′, Complement; CBER, Center for Biologics Evaluation and Research; $CCID_{50}$, 50% Cell Culture Infectious Dose; CDC, Centers for Disease Control and Prevention; CI, Confidence Interval; %CV, % Coefficient of Variation; CVS-11, Challenge Virus Standard-11 strain of rabies virus. The virus was aliquoted into single-use aliquots and stored at −80 °C to −40 °C. Produced by SP, MLE, originated from virus stock from CDC, Atlanta; ddPCR, Droplet Digital Polymerase Chain Reaction; DEAE, Diethylaminoethyl dextran, manufactured by Sigma (catalog # D9885-10G): a 0.1% DEAE stock solution was prepared using Hanks Balanced Salt Solution; ED_{50}, 50% Neutralization Endpoint Titer; ELISA, Enzyme-Linked Immunosorbent Assay; EMEM, Eagle's Minimum Essential Medium, manufactured by ATCC (catalog # 30–2003); Evan's Blue, Evans Blue Powder, manufactured by Santa Cruz Biotechnology (catalog # SC-203736); FBS, Fetal Bovine Serum, manufactured by HyClone (catalog # SH3007001); FDA, Food and Drug Administration; FITC, Fluorescein Isothiocyanate; FITC-conjugated mAb, FITC-conjugated mouse anti-rabies IgG2a monoclonal antibody, manufactured by LIGHT DIAGNOSTICS™ Rabies DFA II, FITC Conjugate clone 1037 & 5022 (Millipore, catalog #5500) with a working dilution of FITC conjugate 1:100, diluted in PBS, filtered through a 0.22 μm filter, protected from light and stored at 2 °C to 8 °C; GCV, Geometric Coefficient of Variation; GCLP, Good Clinical Laboratory Practice; Geq, Genome equivalents; GPC', Guinea Pig Complement, manufactured by Rockland, Inc. (catalog # C200–0005) and stored at −80 °C to −40 °C; GMC, Geometric Mean Concentration; GMT, Geometric Mean Titer; HEPES, N-(2-Hydroxyethyl) piperazine-N′-(2-ethanesulfonic acid), manufactured by Sigma (catalog # H0887); HIPAA, Health Insurance Portability and Accountability; IU, International Units; KSVDL, Kansas State Veterinary Diagnostic Laboratory at Kansas State University College of Veterinary Medicine (Manhattan, KS, USA); KSU, Kansas State University; LLOQ, Lower Limit of Quantitation; MEM, Minimum Essential Medium, manufactured by Gibco Life Technologies (catalog # 11090–081); MLE, Marcy L'Etoile; NIBSC, National Institute of Biological Standards and Controls; PBS, Phosphate Buffered Saline; PEP, Post-Exposure Prophylaxis; PI, Post Infection; PM-IN rabies virus, Inactivated Pittman Moore rabies virus, received from SP (Marcy L'Etoile) at a concentration of $10^{8.8}$ $CCID_{50}$/mL before inactivation; RFFIT, Rapid Fluorescent Focus Inhibition Test; RVNA, Rabies Virus Neutralizing Antibody; SAGE, Strategic Advisory Group of Experts; SP, Sanofi Pasteur; SRIG, Standard Rabies Immune Globulin; T75, 75 cm^2 Tissue culture flask; SVN, Serum-Virus Neutralization; $TCID_{50}$, 50% Tissue Culture Infectious Dose; VSV-IN, Inactivated vesicular stomatitis virus, obtained from KSVDL at a concentration of 10^7 $TCID_{50}$/mL before inactivation; WHO, World Health Organization; WHO-1 SRIG, 1st WHO International SRIG Reference Serum, prepared by FDA/CBER (Lot R-3 59 IU/ampoule); WHO-2 SRIG, 2nd WHO International SRIG Reference Serum, prepared by NIBSC (code RAI 30 IU/ampoule)

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subsequent assay validation in accordance with the International Conference on Harmonization (ICH) guidelines. The improved and optimized RFFIT accuracy, linearity and sensitivity was demonstrated by testing World Health Organization (WHO)-1 and WHO-2 Standard Rabies Immune Globulins (SRIGs) and complete assay development and validation was performed in compliance with Good Clinical Laboratory Practice (GCLP) guidelines.

1. Introduction

Rabies continues to be a major public health concern in most developing countries in Asia, Africa, Latin America and the Caribbean (World Health Organization, 2018). It is estimated that 59,000 human deaths occur annually, with the majority of cases occurring in Africa and Asia. The major burden of the disease falls on those living in poor rural communities, and children in particular (World Health Organization, 2018).

Although rabies infection almost always leads to fatal encephalitis in humans once clinical symptoms manifest (World Health Organization, 2018), rabies virus neutralizing antibodies (RVNA), both exogenous and endogenous, play an essential role in protection from the disease. Pre-exposure prophylaxis with rabies vaccination for those at continual, frequent or increased risk of exposure to rabies is strongly recommended (World Health Organization, 2018). Booster vaccinations are recommended in those at increased risk of infection when RVNA concentration falls below 0.5 IU/mL (World Health Organization, 2018). Post-exposure, the disease can almost always be prevented if the World Health Organization (WHO) recommended post-exposure prophylaxis (PEP) protocol is immediately implemented, including proper wound management (washing/cleaning) and prompt administration of rabies-immunoglobulins in conjunction with active immunization with rabies vaccines (World Health Organization, 2018).

The evaluation of RVNA requires reliable serological methods to help ascertain immune status and determine efficacy of pre- or post-exposure vaccination. There are several different assays that are used to measure immune responses to rabies vaccination, including calculating RVNA titers via cell-based methods, such as the Rapid Fluorescent Focus Inhibition Test (RFFIT), the fluorescence readout based antibody virus neutralization test, the simplified fluorescence inhibition microtest and pseudotype virus micro-neutralization assays, or enzyme-linked immunosorbent assay (ELISA) based methods, such as the indirect fluorescence antibody test, the immunoperoxidase inhibition assay, and the counter immunoelectrophoresis test (World Health Organisation/Department of Control of Neglected Tropical Diseases, 2018). Although cell-based and ELISA-based methods produce comparable results, differences in vaccine equivalency have been demonstrated between the two methods, especially during the primary vaccination phase (Moore et al., 2016).

The cell-based RFFIT is an in vitro functional assay and is considered the “gold standard” assay for the measurement of RVNA in serum samples (Smith et al., 1973; Moore and Hanlon, 2010; World Health Organisation/Department of Control of Neglected Tropical Diseases, 2018). RVNA binds to rabies virus (challenge virus standard [CVS-11] strain adapted to cell culture) and blocks infection to baby hamster kidney (BHK-21) cells susceptible to rabies virus. Fluorescein isothiocyanate (FITC) conjugated mAb to the rabies virus is added to determine if any infected BHK-21 cells are present.

The serum neutralization endpoint titer (ED_{50}) is defined as the reciprocal dilution of the highest serum dilution at which 50% reduction in the number of fluorescent positive fields is observed as compared to the virus control. This RVNA titer (50% neutralization, ED_{50}) is converted into IU/ml by calibrating against the reference standard.

The RFFIT method has evolved over the years such that alternative variations to the test procedure are used at different laboratories (Smith et al., 1996; World Health Organisation/Department of Control of Neglected Tropical Diseases, 2018). Due to the complexity of the assay, its performance is affected by many factors (SAGE Working Group on Rabies Vaccines and Immunoglobulins/World Health Organization, 2017) and the assay needs to be optimized, standardized and validated. In addition, there is inherent subjectivity in the counting of fluorescent fields of virus infection which can vary between microscopes and analysts. This method of counting is also time consuming and cumbersome.

Here we detail the optimization and validation of the in-house RFFIT used at Sanofi Pasteur (SP). Several experiments were undertaken to evaluate conditions that may impact RFFIT sensitivity, accuracy, precision, and robustness. Specifically, we assessed the impact on RFFIT performance using 2-fold semi-automated microplate pipetting system serial dilution versus conventional 5-fold manual serial dilution to determine if assay precision would be impacted. We assessed the effect of exogenous complement, cell seeding density, cell passage, diethylaminoethyl (DEAE) dextran concentrations, and incubation time post-infection before immunostaining, on RFFIT performance. In addition, the use of a cell imaging reader to scan slides eased the reading of slides and allowed permanent records of the raw data. The intention was to improve the robustness and sensitivity of the RFFIT performance to support rabies vaccine development.

2. Materials and methods

2.1. Serum samples

RVNA human serum samples were obtained from Imovax® Rabies vaccinated SP employees in compliance with Health Insurance Portability and Accountability (HIPAA) regulations and Sanofi Policies and Procedures. All samples were prepared, coded, and randomized (blinded) by a SP biostatistician in compliance with informed consent, and aliquoted by an independent sample management team to create 12 × 150 µL single use aliquots for each sample. The serum samples were heat inactivated at 56 °C for 30 min prior to use.

2.2. Cells

The working and master banks of BHK-21 cells were produced in-house from American Type Culture Collection (ATCC) cells (catalog #CCL-10). Cell banks were qualified before use and confirmed to be free of any microbial, mycoplasma and viral contamination. The acceptable cell passage range was established based on the performance in the RFFIT and susceptibility to rabies virus infection. BHK-21 cells were sub-cultivated at 37 °C in an atmosphere of 5% CO_2 in T75 flasks using cell culture medium (EMEM supplemented with 10% fetal bovine serum [FBS], without antibiotics) for up to 21 passages and periodically tested for contamination.

2.3. Rabies virus

Challenge virus standard 11 (CVS-11) strain of rabies virus was produced by SP at Marcy L'Etoile (MLE) using virus stock received from Centers for Disease Control and Prevention (CDC), Atlanta. For use in the RFFIT, CVS-11 strain of rabies virus was diluted in test medium (MEM supplemented with 10% FBS, 2 mM [1%] L-glutamine, and 10 mM [1%] HEPES) to target a challenge virus dose of 50 (tissue culture infectious dose [TCID]50)/100 µL. The actual input of rabies virus in $TCID_{50}$/100 µL was determined by a virus back-titration in each

assay run and was calculated using the Reed and Muench method (Reed and Muench, 1938).

2.4. Standard rabies immune globulin (SRIG)

WHO-1 SRIG (lot R-3, 59 IU) and WHO-2 SRIG (code RAI, 30 IU), both diluted to 2 IU/mL according to manufacturer's instructions, were used in this study. WHO SRIGs were heat inactivated at 56 °C for 30 min prior to use. WHO-1 SRIG was used as a calibrator to calculate the RVNA titers in IU/mL in test serum samples. WHO-1 and WHO-2 SRIGs were used to evaluate the accuracy and linearity of the improved/optimized SP RFFIT.

2.5. RFFIT method

The RFFIT described by Smith et al. (1973) was conducted in 8-well LabTek chamber slides. Serial dilutions of heat inactivated test samples were incubated with a pre-determined amount of CVS-11 strain of rabies virus. DEAE-treated BHK-21 cells were then added to the serum-virus mixture. After a further incubation post-infection, the presence of un-neutralized rabies virus was detected in the infected BHK-21 cells by immunostaining with FITC-conjugated mouse anti-rabies nucleocapsid protein monoclonal antibody (mAb). The number of positive fields with rabies virus infected cells was then counted and recorded. The RVNA titer (50% neutralization, ED_{50}) of a test sample was then mathematically interpolated using the Reed and Muench method (Reed and Muench, 1938) and was further calibrated and converted into IU/mL against the WHO-1 SRIG.

Modifications to these test parameters (Smith et al., 1973) were made as described in the following section to assess optimal assay conditions.

2.6. RFFIT optimization

2-fold serial dilutions of the serum samples were assessed using the semi-automated microplate pipetting system versus conventional manual 5-fold serial dilutions, the optimal challenge dose of the rabies CVS-11, the use of exogenous baby rabbit complement (BRC') and guinea pig complement (GPC'), BHK-21 cell density and cell passage, DEAE concentrations, the incubation time post-infection before immunostaining and the use of a cell imaging reader to scan slides.

2.6.1. 2-fold versus 5-fold serial dilutions of serum samples

Serial dilutions of serum samples in test medium with a starting dilution of 1:2.5 were prepared in a microtiter plate using a semi-automated BioTek Precision Microplate pipetting system (2-fold dilutions) or manual pipetting (5-fold dilutions). One sample per 8-well LabTek chamber slide was tested in the RFFIT.

2.6.2. Selection of CVS-11 optimal virus dilution

We aimed to use in the RFFIT assay a CVS-11 rabies virus that was well characterized with a high concentration of infectious virus particles. Rabies virus titrations (performed by at least 4 analysts and read at 22 h post-infection), genomic analysis and droplet digital polymerase chain reaction (ddPCR) (to determine the ratio of infectious viral particles to total viral particles) were conducted.

The optimal working dilution of the CVS-11 virus to target a challenge dose of 50 $TCID_{50}$/100 µL (with an acceptable range of 30–100 TCID50/100 µL) and its impact on RVNA titers were evaluated.

2.6.3. Effect of exogenous complement on RVNA titers

Exogenous BRC' or GPC' was added to the serum-virus mixture at 0%, 0.25% or 0.5% concentrations to evaluate the possible impact on RVNA titers.

2.6.4. Impact of BHK-21 cells on RFFIT performance

The impact of the BHK-21 cells on the performance of RFFIT and impact on RVNA titers was evaluated by assessing cells from different phases of cell growth and cells from different passages (P61, P68, P77, and P88). Two seeding densities, 5×10^6 and 3×10^6 cells per T75 flask at Day 2 and Day 3 post-seeding, respectively, were used for RFFIT.

2.6.5. Effect of DEAE on rabies virus infectivity of BHK-21 cells

BHK-21 cells were treated with 0, 10, 15, 25, or 50 µg/mL of DEAE and incubated for 15 min at ambient temperature before being added to the serum-virus neutralization mixture for use in the RFFIT. A panel of human serum samples were used which included: up to 10 RVNA positive samples, 1 RVNA negative sample, and WHO-1 or WHO-2 SRIG which was used as the calibrator.

2.6.6. Effect of incubation time post-infection before immunostaining

We evaluated the impact of 1 day (22h) vs 2 day (46 h) incubation on RVNA titers. The serum-virus mixture was mixed with DEAE-treated BHK-21 cells (1×10^5 cells/chamber) and incubated at 37 °C in 5% CO_2 atmosphere for 22 h or 46 h post-infection. Subsequently, the culture medium was removed, and the slides were washed once with cold (2–8 °C) 85% acetone before fixing with cold (2–8 °C) 85% acetone at ambient temperature for 15 min. After fixing, the slides were air dried. The slides were then stained with fluorescein isothiocyanate (FITC)-conjugated mAb and with Evan's blue counterstain at 0.0002% and incubated for an additional 40 min at 37 °C in 5% CO_2 atmosphere. After incubation the slides were rinsed, first in PBS + 0.05% Tween 20, then in water, and air dried.

2.6.7. Comparison between counting methods of fluorescent fields

After immunostaining, the fluorescent rabies virus positive fields on the 8-well chamber slides were counted both manually under the microscope by qualified analysts at Kansas State Veterinary Diagnostic Laboratory (KSVDL) and manually read off the scanned images obtained with the automated BioTek Cytation3 Imaging System by qualified analysts at SP.

In the traditional RFFIT method, RVNA titers are determined using 5-fold titrations of test samples and by manually scoring the number of fluorescent positive fields within each chamber under a microscope with a 160 × magnification. An experienced qualified analyst typically has to read 80 non-overlapping fields in four chambers for each sample. If a 2-fold titration RFFIT is performed then 160 non-overlapping fields in eight chambers per sample have to be manually read. This manual reading process is labor intensive, subjective, and without audit trail capability.

The BioTek Cytation3 cell imaging system is an automated image scanner composed of a high resolution microscope lens (made by Olympus) and a high resolution camera (made by SONY). The BioTek Cytation3 is capable of scanning fluorescent images from an 8-chamber LabTek slide and can be programmed to evenly divide each chamber into 20 fields covering ~95–99% of the chamber. This imaging system allows analysts to view the scanned images on a computer screen and score the number of fluorescent positive fields. The non-overlapping fluorescent fields from the whole chamber can be manually counted more easily than using a microscope. These factors allow for a more robust and accurate determination of RVNA titers and generate consistent results on the same test, when read by multiple analysts. In addition, the BioTek Cytation3 creates a digital visual record of the scanned results, which is in compliance with audit trail requirements and counting can be automated.

A total of 60 LabTek slides, 50 human RVNA-positive serum samples and 10 WHO-1 SRIG at various concentrations, were used to compare the manual reading process to reading using the automated cell imaging system. The number of positive fluorescent viral fields (total of 20 fields covering the entire chamber) was recorded and the RVNA ED_{50} titer

interpolated using the Reed and Muench method (Reed and Muench, 1938). The ED_{50} titer reported for each test sample was the average value of the ED_{50} titer from two independent assay runs, converted into IU/mL against the WHO-1 SRIG only included in the same assay run with an assigned value of 2.0 IU/mL.

2.6.8. RFFIT robustness

RFFIT robustness was evaluated by varying four assay conditions: (i) DEAE treatment time of 15–30 min (while the serum-virus neutralization [SVN] time, post-infection time, and FITC mAb incubation time were kept at a constant, 90 min, 22 h, and 40 min, respectively) (ii) SVN time of 90 ± 15 min, (iii) incubation time post-infection of 22 ± 2 h, and (iv) incubation time with FITC-conjugated mAb of 40 ± 10 min. A total of 8 RVNA positive human serum samples, including the WHO-1 SRIG and WHO-2 SRIG, were tested in singleton in two independent assay runs by two qualified analysts. Percent differences in RVNA titers (IU/mL) between standard and varied assay conditions were computed, with titer differences within ±30% considered equivalent.

2.6.9. Stability of rabies virus and RVNA in serum samples

The short-term stability of rabies virus and RVNA in serum samples were assessed under a number of experimental manipulations. RVNA in serum samples were assessed following five freeze-thaw cycles, and after 2 and 4 weeks storage at 4 °C, and after 4 h at ambient temperature. The short-term stability of rabies virus was evaluated after keeping for 15 min at ambient temperature before use. The percent differences between the observed RFFIT results (IU/mL) of 5 test samples for each experimental manipulation versus no manipulation were calculated. RVNA titer differences within ±30% were regarded as equivalent.

2.7. RFFIT validation

The factorial experiment was designed by biostatisticians to evaluate precision (intra-assay precision [repeatability] and intermediate precision), dilutability, lower limit of quantitation (LLOQ), accuracy/linearity and specificity of the RFFIT. We observed a < 30% variability during assay optimization and robustness assessment; this variability is consistent with Kostense and group (Kostense et al., 2012). Therefore, 30% was used in the assay validation.

2.7.1. RFFIT precision, dilutability, and LLOQ

Precision expresses the closeness of measurements obtained from multiple testing of the same homogeneous sample and was considered at two levels: intra-assay precision (repeatability) and intermediate precision. Dilutability is the ability to obtain test results which are directly proportional to the amount of analyte in a sample, and LLOQ is the lowest level of analyte in a sample that can be quantitatively determined with a defined level of precision and accuracy.

Precision, dilutability and LLOQ were evaluated using results generated within a single design of experiment (DOE) using the same sample panel. This sample panel consisted of 52 RVNA-positive (26 from Imovax® Rabies vaccinated SP employees and a 1:10 dilution of each sample spiked into RVNA-negative human serum) and 4 RVNA-negative samples (obtained from either SP-employee donors who did not receive the rabies vaccine or as a human pooled serum sample purchased from Sigma, catalog # H4522). See Table 1 for acceptance criteria.

2.7.2. RFFIT accuracy/linearity

Accuracy/linearity is the closeness of agreement between the true or reference value and the reported value.

Table 1
Acceptance criteria and testing method for each validation parameter.

Validation parameter	Testing method	Acceptance criteria
Intra-assay precision (repeatability)	52 RVNA positive and 4 RVNA negative human serum samples	% GMC ≤ 30%
Intermediate precision		% GMC ≤ 30% All negative serum samples must remain negative.
Dilutability	25[a] paired RVNA positive human serum samples (undiluted and 1:10 diluted)	≥80% of the positive samples must have an absolute value of percent difference ≤ 30% between the GMC for each diluted and undiluted (baseline control) serum sample. Linear regression slope (GMC of pre-diluted vs. undiluted) must be 0.80–1.25, and R^2 must be ≥0.95.
LLOQ	12 RVNA positive human serum samples with a GMC of < 0.5 IU/mL and ≤ 4 RVNA negative human serum	Acceptance criteria same as for the intra-assay and intermediate precision. Accuracy criteria should be met for the samples near the LLOQ level.
Accuracy/linearity	WHO-1 and WHO-2 SRIGs (8.0, 4.0, 2.0, 1.0, 0.5, 0.2, and 0.1 IU/mL)	80% of the spiked SRIGs with results ≥ LLOQ, must have percent recovery of 70–130% for both WHO-1 and WHO-2 SRIGs. Linear regression slope (observed GMC vs expected) must be 0.80–1.25 and R^2 must be ≥0.95 for both WHO-1 and WHO-2 SRIGs.
Specificity – competition studies	7 positive human serum samples (0.5–5.0 IU/mL) including the WHO-1 and WHO-2 SRIGs competed with homologous ($10^{7.1}$, $10^{6.1}$, $10^{5.8}$, $10^{5.1}$, and $10^{4.8}$ $CCID_{50}$/100 μL) and heterologous antigens ($10^{3.0}$ $TCID_{50}$/100 μL) to the rabies virus and assay medium (baseline control)	Competition with homologous antigen A dose-dependent inhibition must be observed for high titer samples when a concentration series of homologous competitor is employed. The highest concentration of the homologous competitor must decrease the RVNA titer values of high titer serum samples by ≥80%. Serum samples with low titers must have a value less than the LLOQ (< 0.2 IU/mL) following competition with the highest concentration of the homologous competitor. Competition with heterologous antigen Heterologous (unrelated) competitors must not cause the RVNA titer to reduce by >30% as compared to the RVNA titer obtained without any competition (baseline control).
Specificity – matrix effect studies	7 RVNA positive human serum samples, including the WHO-1 and WHO-2 SRIGs spiked with different serum matrices (hemolytic lipemic, and icteric) and normal human serum (RVNA negative sample, baseline control)	For each matrix, ≥ 80% of the samples spiked into lipemic, hemolytic, and icteric serum matrices must have an absolute value of percent difference ≤ 30% when compared to the sample spiked into normal human serum (baseline control).

[a] One pair was eliminated from statistical analysis as per a validation protocol deviation. $CCID_{50}$, cell culture infectious dose; GMC, geometric mean concentration; LLOQ, lower limit of quantitation; RVNA, rabies virus neutralizing antibodies; R^2, coefficient of determination; SRIG, standard rabies immune globulins; $TCID_{50}$,50% tissue culture infectious dose; WHO, World Health Organization.

Accuracy/linearity was evaluated by testing WHO-1 and WHO-2 SRIGs spiked into RVNA-negative serum samples at seven concentrations (8.0, 4.0, 2.0, 1.0, 0.5, 0.2, and 0.1 IU/mL), each. The SRIGs at each spiked concentration were tested in singleton and repeated in 6 independent assay runs by at least two qualified analysts. See Table 1 for acceptance criteria.

2.7.3. RFFIT specificity

Specificity assesses only the analyte in the presence of other components in the sample.

Specificity was demonstrated through competition studies with multiple antigens (homologous and heterologous competitors to the rabies virus) and through matrix effect studies (spike and recovery experiment). See Table 1 for acceptance criteria.

For competition studies, 7 RVNA-positive human serum samples which included the WHO-1 and WHO-2 SRIGs (i.e., 0.5–5.0 IU/mL) were pre-incubated with $10^{7.1}$, $10^{6.1}$, $10^{5.8}$, $10^{5.1}$, and $10^{4.8}$ $CCID_{50}$/100 µL of homologous inactivated Pittman-Moore rabies (rabies PM-IN) antigen, or $10^{3.0}$ $TCID_{50}$/100 µL heterologous inactivated vesicular stomatitis virus (VSV-IN) antigen, or assay medium (baseline control). VSV was used as the heterologous competitor as it belongs to the same family as the rabies virus. The samples were tested in one assay run. Specificity was evaluated as the % reduction (or difference) of the observed ED_{50} titers in the presence of competitors as compared to the expected ED_{50} titers in the absence of competitors (baseline control).

For matrix effect studies, 7 RVNA positive human serum samples, including the WHO-1 and WHO-2 SRIGs, were spiked at a 1:1 ratio into different matrix samples (lipemic, hemolytic, and icteric) or spiked with a RVNA negative human serum sample (baseline control). The observed recovery results were examined to determine if spiking with different matrices would result in a significant change in RVNA titers as compared to the expected titer (baseline control). The samples were tested in one assay run. Assay specificity using matrix effect studies was analyzed by determining the number of samples having a percent difference ≤ 30% in RVNA titer between spiking into the lipemic, hemolytic, and icteric matrices versus the normal human serum matrix (baseline control).

3. Results

3.1. RFFIT optimization

3.1.1. 2-fold versus 5-fold serial dilutions of serum samples

We compared RFFIT precision and accuracy using 2-fold versus 5-fold serial dilutions of WHO-1 SRIG and other RVNA positive serum samples. RFFIT precision using 2-fold serial dilutions was slightly better (lower % geometric coefficient of variation [GCV]) than using 5-fold serial dilutions (Table 2). On this basis, we selected 2-fold serial dilutions scheme for testing of serum samples in RFFIT.

Table 2
Two versus five-fold serial dilutions of serum samples.

	2-Fold Serial Dilutions	5-Fold Serial Dilutions
RFFIT Precision		
Intra-Assay (Repeatability)		
Number of Samples [a]	36	16
Estimated % GCV (95% CI)	20.3% (17.2, 24.7)	23.1% (19.3, 28.7)
Intermediate Precision		
Number of Samples [b]	12	20
Estimated % GCV (95% CI)	23.1% (19.4, 28.6)	24.0% (17.4, 38.7)

GCV, Geometric Coefficient of Variance (95% Confidence Interval); RFFIT, Rapid Fluorescent Focus Inhibition Test.

[a] 12 RVNA positive samples tested in 3 independent assay runs were considered as different samples resulting in a total of 36 samples.

[b] Intra-assay and intermediate precision in two separate experiments using 16 and 20 samples, respectively.

3.1.2. Selection of CVS-11 optimal virus dilution

The CVS-11 rabies virus lot with the highest infectious virus titer ($10^{6.0}$ $TCID_{50}$/mL) and % infectious particle content (10.17 log_{10} genome equivalents [Geq]/mL) was selected for use in the RFFIT.

Dilutions were used to target 50 $TCID_{50}$/100 µL virus input in the RFFIT, to determine which dilution yielded the most consistent RVNA measurements. Of 3.7 log_{10} (1:5000), 4.1 log_{10} (1:12,000), 4.2 log_{10} (1:15,000), 4.3 log_{10} (1:20,000) and 4.7 log_{10} (1:50,000) virus dilutions tested, the 4.2 log_{10} virus dilution achieved the most consistent results in terms of minimal percent difference in RVNA ED_{50} and IU/mL titers and resulted in $TCID_{50}$/100 µL values within the acceptable range of 30–100 and was therefore chosen for use in RFFIT.

3.1.3. Effect of exogenous complement on RVNA titers

Complement-mediated non-specific killing of the rabies virus was observed when ≥0.3% complement was added to the serum-virus neutralization mixture (Table 3A). The addition of 0.25% BRC', 0.25% GPC', or 0.5% GPC' to the serum-virus neutralization reaction mixture increased the ED_{50} titers (Table 3B). However, the difference in the assigned IU/mL after normalization with the WHO-1 SRIG was within assay variability. On this basis, we chose not to include complement in the RFFIT.

3.1.4. Impact of BHK-21 cells on RFFIT performance

A sigmoid growth curve was observed and the viable cells peaked on Day 3 post-seeding but declined rapidly on Day 4 for all 4 passages evaluated (Fig. 1A). No differences (≤30%) were observed in RVNA titers (IU/mL) using cells from passages P61, P68, P77, and P88 (0, 8.3 and 0% vs P61 respectively). Nevertheless, BHK-21 cells at high passages (>P77) appeared excessively clumpy compared to low passage cells. The performance of RFFIT was reproducible and robust using cells up to 20 passages after initiating from the internal cell bank. Thus, we limited the usage of BHK-21 cells in RFFIT to 20 passages.

To increase the operational flexibility, the equivalency of using BHK-21 cells at Day 2 (5×10^6 cells/T75) vs. Day 3 (3×10^6 cells/T75) post-seeding was evaluated in the RFFIT using a panel of 33 RVNA positive human serum samples which included WHO-1 and WHO-2 SRIGs. A concordance analysis showed no differences in observed RVNA titers using Day 2 or Day 3 BHK-21 cells in terms of the concordance slope (1.01) and differences being within ±2-fold difference of each other (Fig. 1B). Therefore, we considered that BHK-21 cells at both Day 2 or Day 3 post-seeding were suitable for RFFIT.

3.1.5. Effect of DEAE on rabies virus infectivity of BHK-21 cells

Treatment of BHK-21 cells with high concentrations of DEAE (25 and 50 µg/mL) 15 min before adding to the serum-virus mixture resulted in cell death. BHK-21 cells treated with low concentrations of DEAE (10 and 15 µg/mL) facilitated rabies virus entry into these cells, thereby enhancing virus infectivity. The ED_{50} geometric mean titer (GMT) percentage difference in DEAE-treated versus untreated BHK-21 cells was greater using 15 µg/mL of DEAE (−18.1%; 10 samples) than 10 µg/mL (−8.5%; 5 samples). However, after normalization and calibration with the WHO SRIG, no significant differences were observed in geometric mean concentration (GMC) IU/mL between 10 and 15 µg/mL DEAE-treated and untreated samples (−1.4% and 1.1% respectively). The concentration of DEAE selected to be used in the RFFIT was 10 µg/mL.

3.1.6. Effect of incubation time post-infection before immunostaining

An even distribution of the individual fluorescent viral foci was observed at 22 h post-infection with 50 $TCID_{50}$/100 µL of CVS-11 challenge virus dose (Fig. 1C). In contrast, overlapped fluorescent foci were observed at 46 h post-infection with 50 $TCID_{50}$/100 µL of CVS-11 challenge dose (Fig. 1C). When the virus challenge dose was decreased

Table 3
The effect of exogenous complement on: A) non-specific killing of rabies virus; and B) RFFIT sensitivity enhancement.

A	% Exogenous Complement							
	20%	10%	5%	2.50%	1.25%	0.60%	0.30%	0.15%
Complement [Lot#]	% of Non-Specific Virus Killing							
BRC' [Lot #1]	100%	100%	100%	90%	40%	10%	0%	0%
BRC' [Lot #2]	100%	100%	100%	90%	50%	10%	0%	0%
GPC' [Lot #1]	100%	100%	90%	40%	20%	10%	0%	0%
GPC' [Lot #2]	100%	100%	90%	60%	20%	0%	0%	0%
No Complement	0%	0%	0%	0%	0%	0%	0%	0%

B			GMT without C′		GMT with C′		Percent Difference	
C′ Source	C′ Conc.	N	ED_{50} Titer ($TCID_{50}$)	IU/mL	ED_{50} Titer ($TCID_{50}$)	IU/mL	ED_{50} Titer	IU/mL
BRC'	0.25%	33 [a]	74.3 (56)	1.26	85.9 (55)	1.28	13.50%	1.40%
GPC'	0.25%	18 [b]	35.2 (91)	1.15	147.5 (67)	1.26	319.40%	10.30%
GPC'	0.50%	18 [b]	35.2 (91)	1.15	160.4 (61)	1.17	356.00%	2.10%

BRC', Baby Rabbit Complement; C′, complement; Conc., concentration; ED_{50}, 50% neutralization endpoint; GPC', Guinea Pig Complement; $TCID_{50}$, 50% Tissue Culture Infectious Dose.

[a] 11 RVNA positive samples were tested in singleton by 3 analysts.

[b] 9 RVNA positive samples were tested in singleton by 2 analysts.

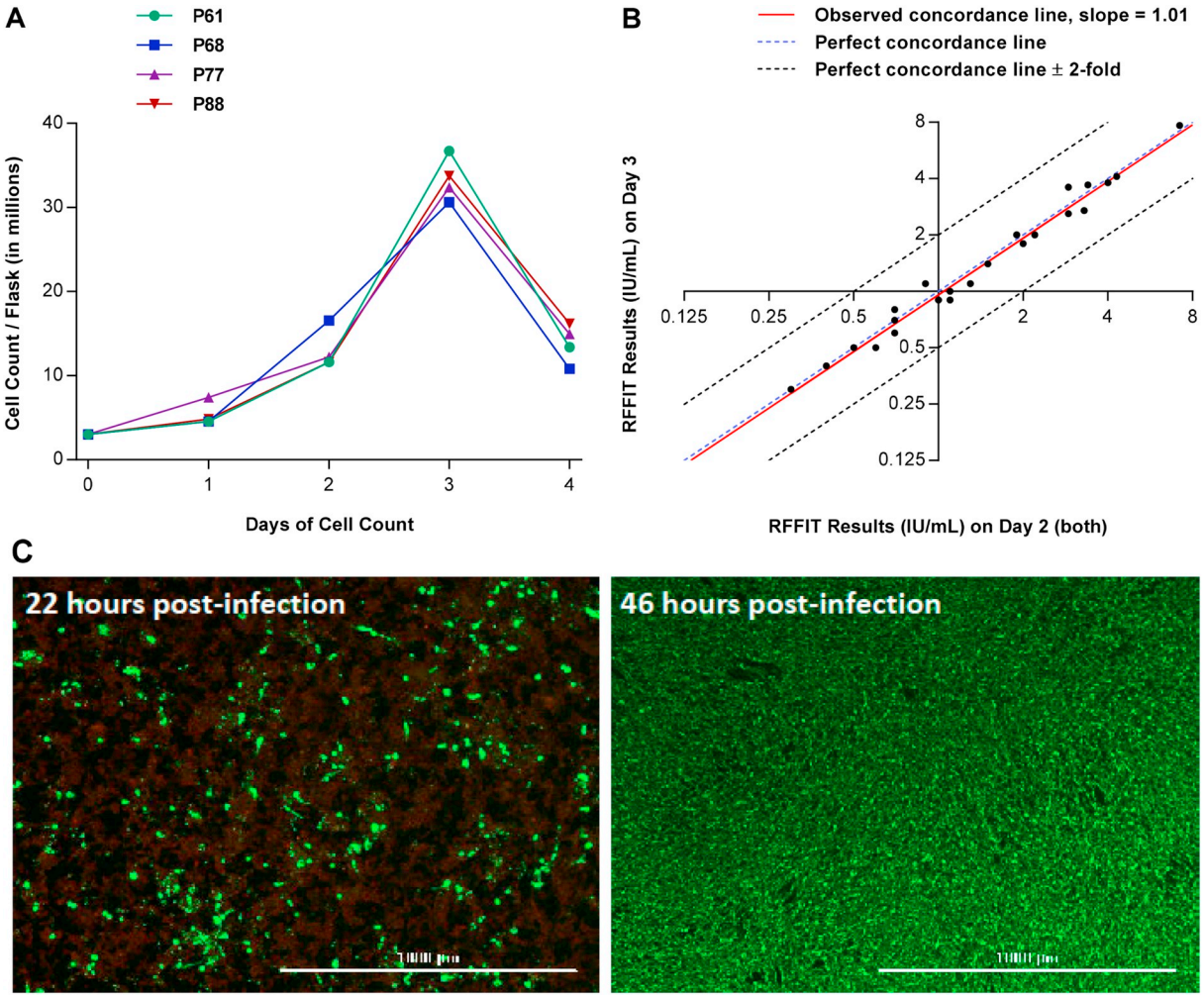

Fig. 1. A) BHK-21 cell growth kinetics for four passages (P61, P68, P77, and P88); B) Concordance analysis RFFIT GMT of 29 samples in IU/mL by using cells at Day 2 and Day 3 post-seeding; and C) Rabies virus control wells at 22 h and 46 h post-infection (BHK-21 cells infected with CVS-11 were immunostained using FITC-conjugated anti-rabies virus mAb and are represented by the green fluorescent spots). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
RFFIT, Rapid Fluorescent Focus Inhibition Test.

to 5 $TCID_{50}$/100 µL, patchy but still overlapped fluorescent viral foci were observed at 46 h post-infection and the sample ED_{50} titers were inconsistent and fluctuated (data not shown). Thus, we retained a 22 h post-infection time in RFFIT.

3.1.7. Comparison between counting methods of fluorescent fields

Fluorescent fields from 480 chambers (8 chambers/slide × 60 slides) were counted both under the microscope and read off the scanned images generated by the imaging reader. The data analysis demonstrated 333/480 chambers had either (0, 0) or (20, 20) fluorescent positive fields by both reading methods, confirming the consistency in reading between the methods. The two counting methods did not give the exact same counts. However, these differences in counts did not introduce systematic bias, as shown by an equal distribution around the line of agreement (Fig. 2A and B).

In addition, we demonstrated that ED_{50} titers calculated using the Reed and Muench method (Reed and Muench, 1938) were equivalent between data obtained using the microscope and cell imaging reader (Fig. 2C); concordance slope was 1.00 with 90% confidence interval

Table 4
Percent differences in RVNA titers (IU/mL) for each varied assay condition versus the optimal target control condition.

Condition	SVN time (min)	PI time (hours)	FITC time (min)	DEAE time (min)	Difference (%)
1	Low (75)	Low (20)	Low (30)	Target (15)	8.8
2	Low (75)	Low (20)	High (50)	Target (15)	−7.3
3	Low (75)	High (24)	Low (30)	Target (15)	−14.8
4	Low (75)	High (24)	High (50)	Target (15)	−8.8
5	High (105)	Low (20)	Low (30)	Target (15)	−0.1
6	High (105)	Low (20)	High (50)	Target (15)	4.2
7	High (105)	High (24)	Low (30)	Target (15)	−5.7
8	High (105)	High (24)	High (50)	Target (15)	−10.8
9	Target (90)	Target (22)	Target (40)	High (30)	6.3
Control	Target (90)	Target (22)	Target (40)	Target (15)	N/A

DEAE, diethylaminoethyl; FITC, fluorescein isothiocyanate; NA, not applicable. PI, post-infection; SVN, serum-virus neutralization.

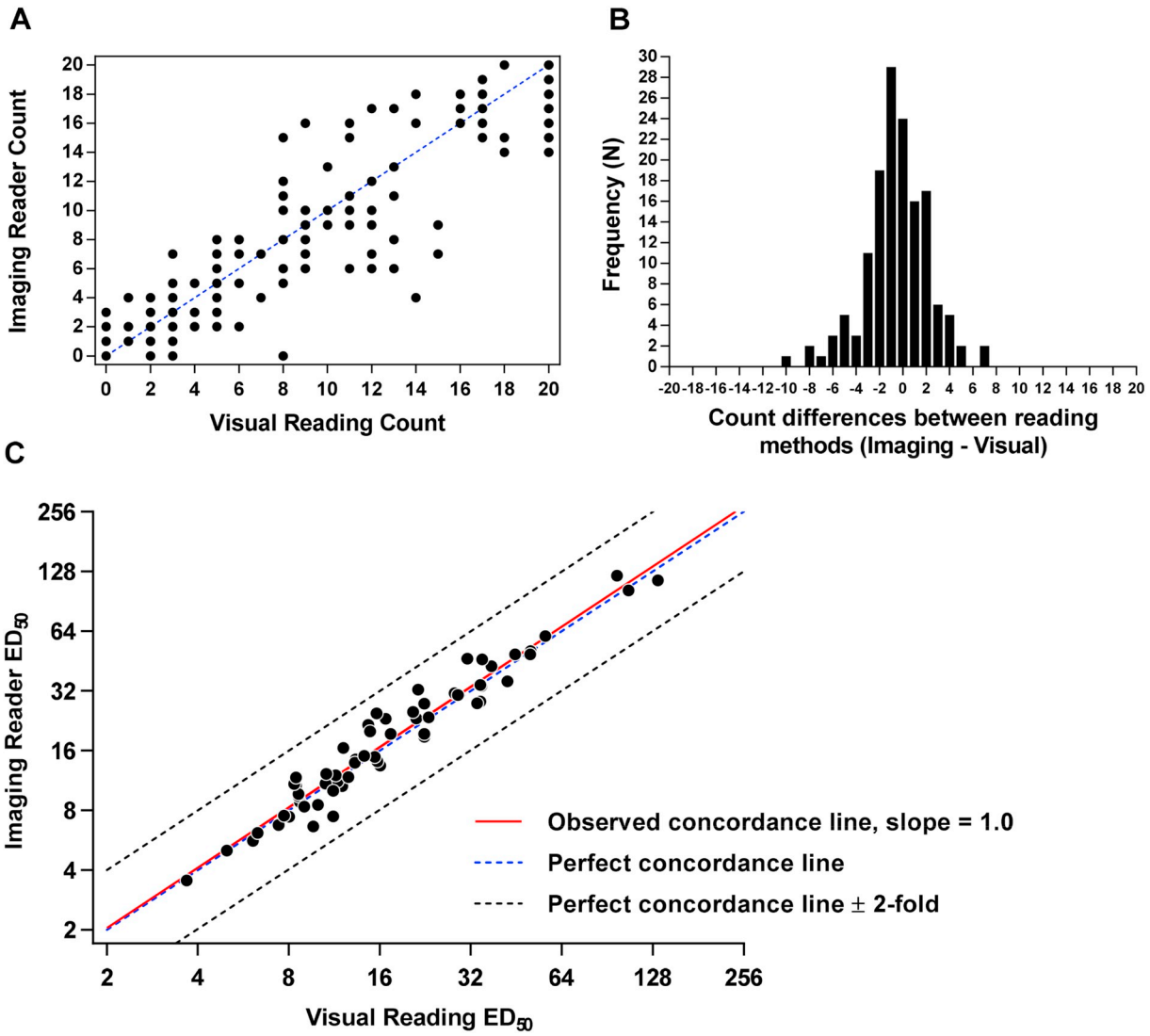

Fig. 2. A) Scatter plot of cell reader vs visual reading of fluorescent positive viral fields count; B) Histogram showing distribution of the cell reader vs visual reading difference on the fluorescent positive viral fields count; and C) Concordance of cell reader vs. visual reading on ED_{50} titers.
ED_{50}, 50% neutralization endpoint.
RFFIT, Rapid Fluorescent Focus Inhibition Test.

(CI) (0.94, 1.07)). Percent difference between the GMT of ED_{50} results of two reading methods was 4.6%. Percent agreement of the ED_{50} titers within ±2-fold (variability of the ED_{50}) of each other was 100% (60/60 slides tested).

The cell imaging reader used for automated slide scanning provides traceability and permanent records of the raw data meeting good clinical laboratory practice (GCLP) requirements, and allows the slides to be read more easily from the scanned images. On this basis, we selected the cell imaging reader for use in RFFIT.

3.1.8. RFFIT robustness

The overall percent differences in RVNA titers (IU/mL) between the results generated using various assay incubation conditions versus the target control condition ranged from −14.8% to +8.8%, which were within the expected variability of a cell-based neutralization assay of ±30% (Table 4).

On this basis we considered the SP RFFIT method to be robust within the tested incubation time ranges of the DEAE, SVN, post-infection incubation time, and FITC-conjugated mAb.

3.1.9. Stability rabies virus and RVNA in RFFIT serum samples

The short-term stability of RVNA in human serum samples was demonstrated for all conditions tested. For 5 freeze-thaw cycles or storage for 4 weeks at 4 °C, 100% of test samples remained within a ± 30% difference. For storage for 2 weeks at 4 °C or incubation at ambient temperature for 4 h, 80% of test samples remained within a ± 30% difference.

The suitable short-term stability of rabies virus after keeping for 15 min at ambient temperature before adding to serum-virus mixture was demonstrated by having results of 80% of test samples within a ± 30% difference.

3.2. RFFIT validation

3.2.1. RFFIT precision

The calculated % GCV of the intra-assay precision (repeatability) was 15.7% (95% CI: 14.5, 17.2) and the overall % GCV of the intermediate precision was 19.8% (95% CI: 18.2, 21.6), which were within the acceptable limit of ≤30%. All four negative samples assessed remained negative in all assay runs. The profiles of the intra-assay and intermediate precision using the results of 150 and 50 samples, respectively, are shown in Fig. 3A and B. No discernable trend was observed, showing that the GMC values were evenly distributed for all RVNA positive human serum samples covering the dynamic range of the assay. The acceptance criteria for precision were met.

3.2.2. RFFIT dilutability

Using 25 undiluted and 1:10 diluted paired RVNA positive human serum samples (one pair of the 26 was removed from statistical analysis as per a validation protocol deviation) 100% (25/25) of the samples tested had an absolute value of the percent difference ≤ 30% between the value for each 1:10 diluted and undiluted serum sample. Linear regression using the GMCs of 25 paired samples (undiluted and 1:10 diluted) demonstrated an estimated R^2 of 0.96 and slope of 0.97, which were within the acceptable limits of ≥0.95 and 0.80–1.25, respectively (Fig. 3C). The acceptance criteria for dilutability were met.

3.2.3. RFFIT LLOQ

Within the subset of samples meeting the criteria for LLOQ assessment (12 RVNA positive samples tested in 3 independent assay runs), the % GCV for intra-assay precision was 20.3% (95% CI: 17.2, 24.7) and for intermediate precision was 23.1% (95% CI: 19.4, 28.6). Additionally, the 4 RVNA negative human serum samples remained negative (<0.2 IU/mL) in all assay runs. The acceptance criteria for LLOQ were met, and an LLOQ of 0.2 IU/mL was confirmed.

3.2.4. RFFIT accuracy/linearity

Of the various concentrations of spiked WHO-1 and WHO-2 SRIG samples assessed (with ≥0 to 2.0 IU/mL), 100% (5/5) and 83.3% (5/6), respectively, had a percent recovery of the observed GMC vs. the expected GMC within the acceptable range of 70–130%. Linear regressions demonstrated estimated R^2 values of 1.00 and 0.99 for WHO-1 and WHO-2 SRIGs, respectively, which were within the acceptable limits of ≥0.95. Linear regressions demonstrated estimated slope values of 1.10 and 1.08 for WHO-1 and WHO-2 SRIGs respectively, which were within the acceptable limits 0.80–1.25 (Fig. 3D and E). The acceptance criteria for accuracy/linearity were met.

3.2.5. RFFIT specificity

For the homologous competitor (PM-IN rabies virus) analysis, a dose-dependent inhibition in the ED_{50} titers was observed for 100% (7/7) of samples when various amounts of homologous competitor were used (Fig. 3F). Following competition with the highest concentration of homologous competitor, reductions in RVNA ED_{50} titers were ≥ 93.1% for samples with high titers, and the low titer samples had values < LLOQ.

For the heterologous competitor (VSV-IN) analysis, 100% (7/7) of samples had a change in the ED_{50} titers from 0% to ≤22.8% that was within the assay variability of ≤30%, indicating that the heterologous antigen did not have significant impact on the RFFIT titer. The results of the competition studies demonstrate suitable specificity of the RFFIT in measuring specific RVNA in human serum samples.

For the matrix effect analysis, 85.7% (6/7), 85.7% (6/7), and 100% (7/7) samples spiked into hemolytic, icteric, and lipemic matrices, respectively, had an absolute percentage difference ≤ 30% in RVNA titers compared to those determined in 'normal' human serum matrix (baseline control). This indicates the RFFIT can accurately measure RVNA-specific antibodies in the presence of hemolytic, icteric and lipemic serum matrices. The acceptance criteria for specificity using competition studies and matrix studies were met.

4. Discussion

Clinical trials of rabies vaccine candidates require reliable methods to evaluate vaccine immunogenicity and efficacy. The rabies RFFIT has been used for over 50 years and is considered the gold standard for the detection of RVNA in human serum samples (Smith et al., 1973; Moore and Hanlon, 2010; World Health Organisation/Department of Control of Neglected Tropical Diseases, 2018). However, because RFFIT performance can be affected by a number of factors and lab to lab variations were frequently observed in assay specifications (SAGE Working Group on Rabies Vaccines and Immunoglobulins/World Health Organization, 2017), there is a need to assess the impact of varying these specifications, to select optimal assay parameters, and to validate the performance of the optimized assay. Moreover, the inherent subjectivity of manual counting of the fluorescent fields under the microscope may introduce additional analyst to analyst variation.

We optimized and validated the in-house RFFIT to improve assay sensitivity and robustness, and to reduce assay subjectivity. Of the assay variations evaluated, the following parameters benefitted from optimization as compared to the standard RFFIT (World Health Organisation/Department of Control of Neglected Tropical Diseases, 2018). Two-fold serial dilutions were associated with slightly improved precision versus 5-fold serial dilutions (Table 2).

Analysis of growth kinetics of BHK-21 cells post-seeding determined an optimal cell passage range of 20 (P58 to P78) at densities of 5×10^6 cells/T75 on Day 2 post-seeding or 2×10^6 cells/T75 on Day 3 post-seeding. We found that adding 10 μg/mL of DEAE facilitated virus infectivity of BHK-21 cells, whereas a higher dose (50 μg/mL), which is recommended for use in the RFFIT (Kaplan et al., 1967) was associated with increased cell death. The use of DEAE in rabies virus neutralization assays was also evaluated by Nie (Nie et al., 2017) and 10 μg/mL was

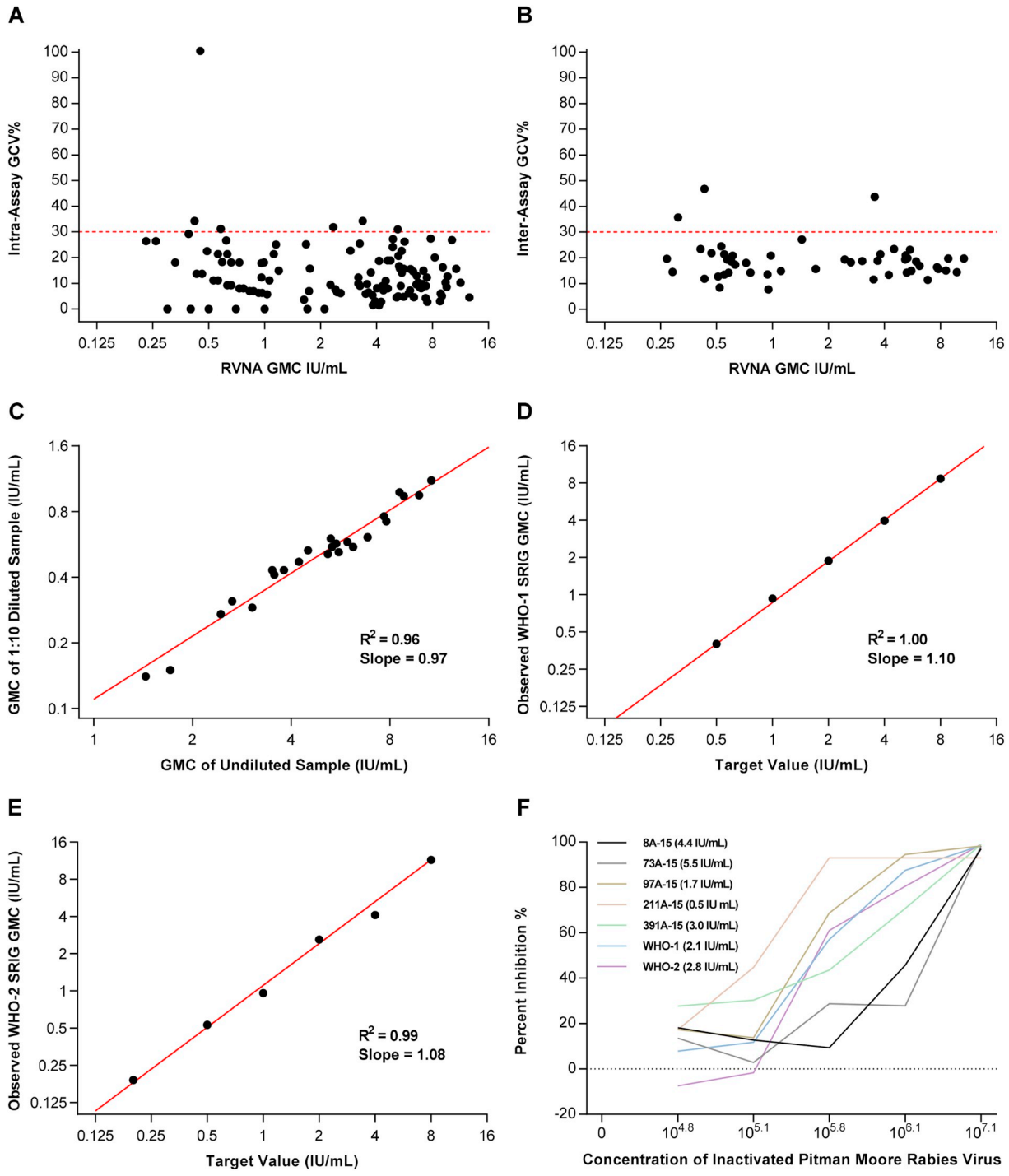

Fig. 3. A) Summary of profile of the RFFIT intra-assay precision; B) Summary of profile of the RFFIT and intermediate precision; C) Dilutability regression plot for the RFFIT using 25 paired RVNA positive human serum samples; D) Dilutional Linearity of WHO-1 SRIG; E) Dilutional Linearity of WHO-2 SRIG; and F) RFFIT specificity: dose-dependent inhibition with inactivated homologous competitor (inactivated Pitman-Moore rabies virus). The 1:10 dilution of two samples resulted in GMCs below the LLOQ of <0.2 IU/mL, therefore, these samples were excluded in Figs. A and B.

GMC, Geometric Mean Concentration; GCV, Geometric Coefficient of Variation; RVNA, Rabies Virus Neutralizing Antibody; SRIG, Standard Rabies Immune Globulin; WHO, World Health Organization.

found to be optimal. In accordance with an earlier report by Kaplan (Kaplan et al., 1967), DEAE had an enhancing effect on the initial interaction between virus and cell increasing the uptake of rabies virus in BHK-21 cells and thus rabies virus infectivity. It was shown by Wunner (Wunner et al., 1984) that viral attachment kinetics to BHK-21 cells were enhanced by DEAE-dextran, an effect which in turn enhanced the apparent infectivity of the virus. They also observed that cells pretreated with DEAE before virus attachment exhibited a similar enhancing binding. The use of the cell imaging reader to scan slides produced comparable results to those obtained by the experienced, qualified RFFIT reader using the microscope, which was consistent with a previous report (Peharpre et al., 1999). The added benefits of this approach include reading slides more easily from the scanned images by accessing from any location with the appropriate software and to obtain consistent results on the same test, by multiple analysts. In addition, permanent records of the raw data meeting GCLP requirements can be used for auditing purposes. It should be noted that other cell imagers/readers can be used for scanning and analyzing images. We also conducted a series of experiments demonstrating that the SP RFFIT method was robust to various incubation times for DEAE treatment of BHK-21 cells, SVN, post-infection, and FITC-conjugated mAb. The optimized SP RFFIT procedure has been defined and is summarized in full in the supplementary material.

In addition to optimization, we validated the RFFIT method and demonstrated it to be precise, accurate, linear, and specific with an LLOQ of 0.2 IU/mL. The calculated % GCV of the intra-assay precision was 15.7% and the overall % GCV of the intermediate precision was 19.8%, which were within the acceptable limit of $<30\%$. It expected that cell-based neutralization assays have high variability with an acceptable precision of 30%, and up to 50% in some cases (Chaloner-Larsson et al., 1999; Centre for Drug Evaluation and Research, 2001). For serological titration assays, it is generally accepted that a 2-fold difference in replicate measurements is considered to represent the upper limit of reproducibility (Wood and Durham, 1980). Thus, the SP RFFIT is validated and suitable for its intended use to quantify specific RVNA in human serum samples.

While the semi-automated 2-fold serial dilutions and use of the cell imaging reader represent major advances, the SP RFFIT method is not fully automated and still requires the use of live rabies virus with the attendant requirement for high biosafety laboratory containment, and remains a time-consuming and complex assay to perform. To circumvent these limitations, modifications of the RFFIT have been attempted to increase automation of the detection of infected cells, notably by the use of flow cytometry (Bordignon et al., 2002) and recombinant virus CV-11-eGFP strain that stably expresses enhanced green fluorescent protein (Xue et al., 2014). Pseudovirus-based neutralization assays have also been developed to obviate the need for live rabies virus (Wright et al., 2009; Nie et al., 2017). Non-cell based methods that are faster than either RFFIT or ELISA, such as the rapid neutralizing antibody detection test based upon immunochromatography, which uses inactivated rabies virus have also been developed (Shiota et al., 2009; Nishizono et al., 2012). Although modifications and new developments in rabies assays will continue to emerge, the equivalency of these modified assays and their acceptance by the Regulatory Agencies still remain to be demonstrated, hence the RFFIT is likely to remain as the gold standard for some time to come.

In conclusion, the optimal rabies RFFIT method conditions were selected to accurately and precisely measure specific RVNA in human serum samples, and the SP RFFIT method was validated as precise, accurate, linear, specific, and robust. In addition, the cell imaging reader used for scanning slides provided a consistent method of digital image capture, traceability, and permanent records of the raw data meeting GCLP requirements.

Financial disclosure

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Role of funding source

All authors at the time the study was undertaken were employees of Sanofi Pasteur, except Susan Moore. All authors were responsible for the study design, the collection, analysis and interpretation of data, the writing of the report, and in the decision to submit the article for publication.

Declaration of Competing Interest

Branda Hu was an employee of Sanofi Pasteur at the time the study was undertaken. Tatyana Timiryasova, Ping Luo, Lingyi Zheng, Amy Singer, Rebecca Zedar, Sanjay Garg, Celine Petit, and Monique Brown are currently employees of Sanofi Pasteur. Susan Moore is currently employed by Kansas State University.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jim.2019.06.017.

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