U.S. Food and Drug Administration

Center for Biologics Evaluation and Research Laboratory Quality System

Laboratory Quality System Laboratory Procedure, Test/Cal Method (TM)

Title: *Potency Determination of Inactivated Influenza Virus Vaccines and Testing of Influenza Reagents by the Single Radial Immunodiffusion (SRID) Method*

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1. Definitions

1.1 **Anti-HA Antibodies** are strain specific, produced by inoculation of sheep with purified HA antigen with Freund's adjuvant system. These antibodies are prepared and qualified for the optimal amount used in the SRID test (µl of antibodies per mL of agarose) in the Division of Biological Standards and Quality Control.

1.2 **HA:** Hemagglutinin, antigenic glycoprotein found on the surface of the influenza virus.

1.3 **OOS:** Out of Specifications

1.4 **Orthogonal diameters** are measurements taken perpendicular to each other.

1.5 **PBS:** Phosphate buffered saline

1.6 **Reference HA Antigen** is prepared by an influenza virus vaccine manufacturer under contract with CBER. The antigen is calibrated for the amount of HA antigen.

1.7 **SRID:** Single Radial Immunodiffusion assay, which is an antigen-antibody precipitation reaction used to quantify antigens.

1.8 **CV:** Coefficient of Variation

1.9 **% RSD:** Percent Relative Standard Deviation

2. Equipment

2.1 Calibrated single tip pipette devices capable of delivering 10-1000 µL volumes

2.2 Serological pipettes

2.3 Test tube racks of appropriate dimensions to accommodate tubes

2.4 De-capping device

2.5 Vortex mixer

2.6 Calibrated Time

2.7 Microwave for heating agarose

2.8 Scissors

2.9 Forceps

2.10 Water bath, 55±2°C.

2.11 Calibrated thermometer capable of measuring at least up to 60ºC

2.12 Humidity Chamber

2.13 Oven capable of maintaining 45ºC or lower temperature.

2.14 Weights or large books (approximately 2 kilograms) that are large enough to completely cover the gel bonds.

2.15 Leveling table of appropriate size to accommodate the GelBond Film

2.16 Spirit level to level the leveling table

2.17 Light box

2.18 An acrylic well punching template having holes in a 4 x 7 arrangement

2.19 Gel punch 4 mm diameter in size attached to a vacuum

2.20 Calibrated balance with 2 decimal places

2.21 Graduated Cylinders - 100mL, 500mL, 1000mL

2.22 Glass Bottles and Flasks - 250mL, 500mL, 1000mL

3. Consumables

3.1 Glacial Acetic Acid (Thermo Fisher Scientific, Cat. No. 42322) or equivalent

3.2 Agarose, SeaKem ME (Lonza Cat. No. 50010), or equivalent.

3.3 Coomassie Brilliant Blue R 250 (Thermo Fisher Scientific Cat. No. BP101) or equivalent

3.4 1X PBS (Phosphate Buffered Saline) Solution. (KD Medical Cat No. RGE-3190) or equivalent

3.5 Zwittergent 3-14 Detergent, CAS #14933-09-6, no exceptions (EMD Biosciences Cat# 693017 or equivalent.)

3.6 15 mL disposable plastic tubes with cap (Falcon or equivalent)

3.7 50 mL disposable plastic Tubes with cap (Falcon or equivalent)

3.8 10 × 75 mm, 12 x 75 mm or 13 x 75 mm Borosilicate Glass Tubes. Kimble Cat# 735001075 or equivalent.

3.9 Sterile disposable pipettes: 1 mL, 2 mL, 5 mL, 25 mL, 50 mL. Thermo Fisher brand or equivalent.

3.10 Sterile pipette tips: 10-1000 µL capacity. Thermo Fisher brand or equivalent.

3.11 Saline Solution, (0.9% sodium chloride in purified water made in house) or commercially available (e.g., KD medical Cat# RGE-5290 or equivalent).

3.12 GelBond Film, agarose support medium, 85 × 100 mm, 100 × 150 mm, 0.2 mm thickness. Lonza Rockland, Inc. Cat. # 53734, 53746 or equivalent.

3.13 Whatman Qualitative Filter Paper, Cat# 1001-240, Cat#1003-240 or equivalent.

3.14 Purified water (commercially available or generated in house)

3.15 Miscellaneous glassware, such as beakers, flasks, measuring cylinders, etc.

3.16 Methanol (Thermo Fisher Scientific Cat. No. A452) or equivalent.

3.17 Ethanol (Thermo Fisher Scientific Cat. No. S73985) or equivalent.

3.18 Sodium chloride (Fisher Scientific Cat No. BP358-1) or equivalent.

Critical Reagents

3.19 Strain Specific Reference Antigen with product circular: CBER Reference antigens for various influenza virus strains used in SRID assay are developed and calibrated in collaboration with World Health Organization's collaborating laboratories. These reference antigens are obtained from the Standards section of DBSQC and are used as per recommendations in the product circular. No further qualification is required.

3.20 Strain Specific Anti-HA Serum with product circular: Antibodies for various influenza virus strains used in SRID assay are developed and calibrated in collaboration with World Health Organization's collaborating laboratories. These antibodies are obtained from the Standards section of DBSQC and are used as per recommendations in the product circular. Depending upon the characteristics of the antibodies, the concentration used in the SRID test may be optimized. No further qualification is required.

4. Process Description

4.1 Preparation of Solutions

4.1.1 Destain Solution:

Note: *Two formulations are listed below, use either the methanol or ethanol formula.*

4.1.1.1 Under a fume hood, add 100 mL of Acetic acid to 500 mL of methanol or 450 mL ethanol in a polystyrene or glass container.

4.1.1.2 Add 450 mL purified water and mix gently.

4.1.1.3 Label the bottle with "SRID Destain Solution", date of preparation, expiration date and preparer's initials. This solution can be used for 6 months.

4.1.1.4 Larger or smaller volumes of the solution may be prepared by adjusting the ingredient amounts accordingly as long as the composition of the final solution remains the same.

4.1.2 Stain Solution:

4.1.2.1 Weigh 0.5 g of Coomassie Brilliant Blue and mix into 500 mL of Destain solution in a bottle with a magnetic stir bar.

4.1.2.2 Place bottle on a magnetic stirrer and mix thoroughly for approximately one hour.

4.1.2.3 Label the bottle with "SRID Stain Solution", date of preparation, expiration date and preparer's initials. This solution can be used for 3 months.

4.1.2.4 Larger or smaller volumes may be prepared by adjusting the ingredient amounts accordingly.

Note: *A higher concentration of Coomassie Brilliant Blue in staining solution may be needed to get distinct SRID rings observed with certain influenza virus strains, which may depend upon the affinity of the antibodies and characteristics of the HA antigen. In such instances, if after staining with 0.1% stain solution SRID rings are faint, use 0.2% Coomassie solution. If the SRID rings are still faint, use 0.5% stain solution. These solutions can be prepared by proportionally increasing the amount of Coomassie Brilliant Blue.*

4.1.3 10% and 1% solution of Zwittergent:

4.1.3.1 Weigh 1 g of Zwittergent and mix in 10 mL purified water in a conical tube to prepare a 10% solution.

Vortex to make sure detergent is completely dissolved. You may place in warm water bath to aid in this process.

4.1.3.2 Label the bottles with "10% Zwittergent Solution", as appropriate, date of preparation, expiration date, lot number and preparer's initials. This solution can be used for 6 months.

4.1.3.3 Mix 1 mL of 10% Zwittergent Solution (from above) with 9 mL of 1 x PBS to prepare "1% Zwittergent Solution". Prepare fresh daily.

4.1.3.4 Larger or smaller volumes of each of the solutions may be prepared by adjusting the component amounts accordingly.

4.1.4 Saline Solution (0.9% Sodium Chloride)

4.1.4.1 Weigh 9.0 g of Sodium Chloride and mix in 1000 mL of purified water.

4.1.4.2 Label the bottles with "Saline: 0.9% Sodium Chloride" as date of preparation, expiration date and preparer's initials. This solution can be used for a week.

4.1.4.3 Larger or smaller volumes may be prepared by adjusting the ingredient amounts accordingly.

4.2 Preparation of Agarose

4.2.1 Turn on water bath and allow it to reach to 55±2ºC. Confirm temperature setting using a calibrated thermometer.

4.2.2 Make 1% agarose with 1× PBS (1 g in 100 mL 1× PBS) in a bottle or a flask. Total volume required for a test is calculated based on the number of samples to be tested and the number of gels needed. Record the details of preparation.

Note: *The capacity of bottle or flask used to prepare agarose should be at least 2 times the volume of agarose prepared.*

4.2.3 Bring the solution to the verge of boiling in a microwave a few times (approximately 3 times, stirring or swirling in-between heating) until the agarose is completely in solution (i.e., there should be no granules or flakes).

Note: *Ensure the solution is not excessively boiled as evaporation may offset the results.*

4.2.4 Place melted agarose in the hot water bath and allow it to equilibrate to 55±2ºC (approximately 30 minutes) before adding anti-HA antibodies. Record start time and end time of agarose incubation.

Note: *Melted Agarose should be used on the day of preparation.*

4.2.5 Antibodies used in SRID assay are developed and are supplied by appropriate regulatory agencies. These antibody reagents are used as per supplier's recommendations.

4.2.6 Gels are normally poured to a thickness of approximately 3 mm, requiring 45 mL of Agarose for 100 x 150 mm gel bonds and 25 mL for 85 x 100 mm gel bonds. Preparation of 'thin' gels is sometimes required to replicate conditions used by some manufacturers. 'Thin' gels are poured to a thickness of approximately 2 mm, requiring 33 mL of Agarose for 100 x 150 gel bonds and 18 mL for 85 x 100 mm gel bonds. Table 1 shows the configuration of 'normal' and 'thin' gels and the required agarose volumes.

Note: *Gel thickness and/or volume of agarose used to make gels may be adjusted if needed When preparing thin gels concentration of antibody may need to be adjusted to get clear precipitation rings*

4.2.7 Calculate the volume of anti-HA antibody required to add to each agarose gel (refer to the product circular). Add the required volume of antibody to a 50 mL tubes or appropriate size flask (label tubes if using multiple anti-HA antibodies for multiple gels). To ensure proper mixing, if the volume of antibody added is less than 0.5 mL,

make up to 0.5 mL with PBS. Record the volume of anti-HA antibody.

4.3 Pouring of Agarose Gels

4.3.1 Prepare gel bonds by labeling on the bottom, hydrophobic side.

4.3.2 Place gel bonds on a leveling table. Ensure table is leveled using a spirit level. Make sure the hydrophilic side of the gel bond is facing upwards.

Note: *On the hydrophilic side of the gel water will spread out more when compared to the other side where water has a greater tendency to bead-up on the surface.*

4.3.3 Using Table 1 below pipette the appropriate amount of agarose into the 50 mL tube containing anti-HA antibody. Cap the tube and slowly invert several times (avoid forming air bubbles). Then slowly pour the agarose containing anti-HA antibody onto a gel bond.

Table 1 Agarose Allocations per Gel Bond Size

Size of Gel Bond	Gel Thickness	Agarose (mL)	Well configuration	Maximum Samples (includes reference)
85 × 100 mm	Thin	18	4 x 2; 4 x 3; 4 × 4, 4 × 5	5 (depending on configuration)
85 × 100 mm	Normal	25	4 x 2; 4 x 3; 4 × 4; 4 × 5	5 (depending on configuration)
100 × 150 mm	Thin	33	4 x 2; 4 x 3; 4 × 4; 4 × 6; 4 × 7	7 (depending on configuration)
100 × 150 mm	Normal	45	4 x 2; 4 x 3; 4 × 4; 4 × 6; 4 × 7	7 (depending on configuration)

4.3.4 Wait approximately 5 minutes for gels to solidify before moving them. Gels may be placed in a refrigerator for approximately 15 minutes before punching the holes.

4.3.5 If the gels are not going to be used with in ~2 hours, keep the prepared gels in the humidified chambers to avoid drying of agarose.

4.4 Preparation of Reference Antigen and Test Samples

4.4.1 CBER reference antigens for various influenza virus strains used in SRID assay are developed and calibrated in collaboration with World Health Organization's collaborating laboratories. These reference antigens are obtained from the Standards section of DBSQC and are used as per recommendations in the product circular. Working solutions of reference antigen and test samples (monovalent bulk samples) for the SRID test should contain approximately 25 – 40 µg HA/mL.

4.4.2 Reconstitute the reference antigen vial as per instruction provided in the appropriate "Product Circular". Ensure all material is dissolved before use by mixing or vortexing the vial.

4.4.3 For monovalent references, dilute the reconstituted reference antigen to the desired starting concentration using 1× PBS depending on the initial concentration found in the strain specific product circular. Record volume of reference antigen and PBS.

4.4.4 For bivalent references (required when testing quadrivalent vaccines), both B-Strain reference antigens should be combined at the time of use. (Note: Dilute each reference antigen appropriately using 1X PBS in a way that there is an equal concentration of each B-reference antigen in the final Bivalent mix).

4.4.5 Based on the potency values provided by the manufacturer, dilute monovalent bulk samples with 1× PBS to the desired starting concentration. Record volume of sample and PBS added.

Note: *Final formulated seasonal influenza vaccines are formulated at approximately 30 - 50 µg HA/mL. Therefore, no dilution is required at this step. Final formulated vaccines for pandemic flu are formulated at 90 µg HA/mL, therefore an appropriate dilution is required. Additionally, flu vaccines with higher hemagglutinin contents (e.g High Dose and Intradermal vaccines) will require an appropriate dilution before use in the assay as determined by the manufacturer's formulation target.*

4.5 Punching Wells in Agarose Gels

4.5.1 Place one gel in a gel well punching jig and punch 4 mm wells equally spaced using a gel punch attached to a vacuum. You may use various row configurations depending on the size of gel bond used.

4.5.2 Inspect the wells to ensure they are free of excess gel and moisture, which may be removed, if necessary, with a Pasteur pipette/pipette tip attached to an aspirator.

4.5.3 Gels must be used on the day of preparation.

4.6 Dilution of Reference Antigen and Test Samples

4.6.1 Label sets of 10 × 75, 12 x 75 or 13 x 75 mm Borosilicate glass tubes (4 tubes for each reference and sample) with the dilution value listed in Table 2.
When testing 5 samples, 6 sets of tubes are required. When testing multiple lots of samples in a single test, the same dilutions of reference HA antigen can be added to different gels.

Table 2 - Reference Antigen and Test Sample Dilution

Dilution	Reference Antigen or Test Sample (Ll)	10% Zwittergent in (µL)	1% Zwittergent in (µL)
Neat	450	50	-
1.5	100 (of Neat)	-	50
2.0	100 (of Neat)	-	100
4.0	50 (of Neat)	-	150

4.6.2 For the Neat sample take 450 µL of reconstituted reference antigen or test sample and add 50 µL of 10% Zwittergent detergent to each tube.

4.6.3 Vortex mixture of sample and detergent on low speed and incubate for at least 30 minutes at room temperature. Record start time and end time of incubation.

4.6.4 After incubation, complete the subsequent dilutions of the reference antigen and test samples using the table above

4.6.5 First add diluent (1% Zwittergent in PBS) into each tube as specified in the table.

4.6.6 Transfer the detergent treated sample (Neat) to each of 3 subsequent dilutions in accordance with the amounts specified within the table

4.6.7 Vortex each dilution on low speed prior to loading the gel.

Note: *The volume of reference antigen or test sample (see 11.7.2) can be scaled up or down if needed. On such occasions, the volume of 10% Zwittergent detergent is adjusted accordingly so that, after dilution, the final concentration of Zwittergent is 1%.*

Similarly, for preparing various dilutions (in Table 2 above) the volumes of samples and the 1% Zwittergent can be appropriately scaled up/down as needed).

Details of the preparation of non-standard volumes should be recorded.

4.7 Loading SRID Gels

4.7.1 Each test will consist of two replicate gels with identical sample loadings, *e.g* of 4 x6 well configuration is illustrated in Table 3.

Table 3 - Gel Loading Layout

Lane 1 Ref Antigen	Lane 2 Sample 1	Lane 3 Sample 2	Lane 4 Sample 3	Lane 5 Sample 4	Lane 6 Sample 5
Neat	Neat	Neat	Neat	Neat	Neat
1.5	1.5	1.5	1.5	1.5	1.5
2	2	2	2	2	2
4	4	4	4	4	4

4.7.2 Load each well with 20 µL of the appropriate dilution as indicated in the table above using a micropipette. As mentioned above 2 gels are used for each set of samples.

4.7.3 Place gels in a sealed chamber containing a damp sponge (to provide humidification) at room temperature for 18-24 hours. Record incubation start and stop times.

4.8 Processing SRID Gels

4.8.1 After incubation, place gels in a tray and submerge in 0.9% saline solution and place on a rocker for approximately 20 minutes.

4.8.2 Rinse gels by submerging them in a tray of purified water and placing on a rocker for approximately 10 minutes.

4.8.3 Place gel face-up on a flat surface (for example, glass) and cover with three layers of filter paper soaked in water (two 1 mm sheets then one 3 mm sheet); ensure there are no air bubbles between the gel and the filter paper; then place 1-2 absorbent pads on top of the filter paper. Ensure there are no creases and press with another flat plate that is weighed down with the large books or weights. After approximately 30 minutes, place the gel back in the tray and wash with 0.9% saline for approximately 10 minutes and rinse with purified water for 10 minutes.

4.8.4 Press again for approximately 40 minutes. Absorbent pad may be changed after ~20 minutes.

Note: *If gels contain a titer of antibody that is known to give low background levels when processed, the 2nd washing and pressing step may be eliminated.*

4.8.5 Place the gels in a drying oven until they are dry. One sheet of damp 1 mm filter paper may be left on the gels during drying. Ensure that the oven temperature does not exceed 45°C. Depending on the oven (e.g., convection, venting, etc.) and temperature, drying time may very between 1 – 6 hours.

Note: *Lower range of oven temperature is not important. At a lower temperature or at room temperature, the gels take longer period to dry without any impact on test results.*

Note: *Ensure gels are completely dry before staining to prevent a background that would interfere with measuring precipitation rings.*

4.8.6 Place gels in a tray of stain solution for approximately 7-10 minutes.

4.8.7 Place gels in a tray of destain solution until they are destained sufficiently for reading. Ensure that precipitation rings have stained dark enough to provide accurate measurements.

Note: *If staining and destaining multiple gels, the destain solution can be changed halfway through the process. Destaining too much will result in precipitation rings that are not visible, and gels will have to be stained again.*

4.9 Reading Gels

5. Assay Validity

5.1 Criteria for Individual Tests

5.1.1 To evaluate linearity of the reference and test curves correlation coefficient (r) must be ≥0.95. If r for the reference curve is <0.95, results for that gel are invalid and potency of the samples on that gel should not be calculated. If the r of one or more of the samples in a gel is <0.95, results of that particular sample(s) in that gel are invalid.

5.1.2 To evaluate parallelism between reference and sample curves, equality of slopes between test and reference antigens is determined by Student's 't' test, which must be less than 4.604.

5.2 Criteria for Results of Monovalent Bulks

5.2.1 Five tests are performed on monovalent bulk samples. Of the 5 tests performed, if the "r" of a sample from one test is <0.95 for only one of the 2 gels, results of that particular sample(s) should be calculated using the data obtained from the (one) gel with r value ≥0.95. If both gels show r value <0.95, the test for that particular sample is invalid.

5.2.2 Five tests are performed on monovalent bulk samples. Of the 5 tests performed, if the "t" of a sample from one test is >4.604 for only one of the 2 gels, results of that particular sample(s) should be calculated using the data obtained from the (one) gel with 't' value less than 4.604. If both gels show 't' value >4.604, the test for that particular

sample is invalid.

5.2.3 Geometric mean and standard deviation of log potency values of 5 valid tests are calculated. Geometric mean of 5 tests is reported if the standard deviation for the 5 tests is ≤0.049.

5.2.4 If the standard deviation of 5 tests is >0.049, the tests are invalidated, unless there is a known laboratory error or cause for one or more tests. If there is a known laboratory error for one or more tests, that test or those tests are repeated. When all 5 tests are invalidated, another 5 tests are performed. If these 5 tests meet validity criteria for standard deviation, results from these tests are reported. If these 5 tests fail specification for standard deviation again, the tests are invalidated unless there is a known laboratory error or cause for one or more tests. If there is a known laboratory error for one or more tests, that test or those tests are repeated. When these 5 tests from the second set are invalidated, 10 additional tests are performed. Standard deviation from 10 tests must be ≤ 0.042. If the standard deviation from 10 tests is > 0.042, an investigation must be performed before proceeding with additional testing.

5.3 Criteria for Potency Results of Formulated Bulks

5.3.1 No. of Tests to perform:

a) For all vaccines bulk and final containers (with exception of High Dose and Intradermal vaccines): Arithmetic mean and standard deviation of 3 valid tests are calculated.

b) For High Dose and Intradermal formulated bulks: Arithmetic mean and standard deviation of 6 valid tests are calculated.

5.3.2 Mean potency of formulated final bulks from 3 valid tests must not be less than 27.0 with the upper limit for standard deviation as 3.704 or % RSD of ≤ 12.3%.

5.3.3 If a sample does not meet the specification of standard deviation (or % RSD), the tests are invalidated, unless there is a known laboratory error or cause for one or more tests. If there is a known laboratory error for one or more tests, that test or those tests are repeated. When all 3 tests are invalidated, another 3 tests are performed. If these 3 tests meet validity criteria for standard deviation (or % RSD), results from these tests are reported. If these 3 tests fail specification for standard deviation (or % RSD) again, the tests are invalidated unless there is a known laboratory error or cause for one or more tests. If there is a known laboratory error for one or more tests, that test or those tests are repeated. When these 3 tests from the second set are invalidated, 6 additional tests are performed. Standard deviation from 6 tests must be ≤ 2.998 or % RSD should be ≤10.0% (and the potency specification

must now be not less than 28.0). If the standard deviation from 6 tests is > 2.998 or % RSD is >10.0%, an investigation must be performed before proceeding with additional testing.

5.3.4 If a sample meets standard deviation (or % RSD) validity criteria but does not meet the specification of potency from the original 3 tests, an additional three tests are performed and data from the 6 tests are pooled. Mean potency of formulated final bulks from 6 valid tests must not be less than 28.0 with the upper limit for standard deviation as 2.998 (or % RSD be ≤10.0%). If the results are not within specifications after 6 valid tests, an OOS investigation must be performed.

5.3.5. Mean potency from 6 valid tests for (i) HD Trivalent formulated bulks must not be less than 108 µg HA per mL and HD-QIV formulated bulks must not be less than 77 µg HA per mL and (ii) for Intradermal formulated bulks must not be less than 81 µg HA per mL. For each of these vaccines the upper limit for % RSD is ≤ 10.0 %. If a sample does not meet the specification of % RSD, the tests are invalidated, unless there is a known laboratory error or cause for one or more tests. If there is a known laboratory error for one or more tests, that test or those tests are repeated. When all 6 tests are invalidated, another 6 tests are performed. If these 6 tests meet validity criteria for % RSD, results from these tests are reported. If these 6 tests fail specification for % RSD again, the tests are invalidated unless there is a known laboratory error or cause for one or more tests. If there is a known laboratory error for one or more tests, that test or those tests are repeated. If the %RSD from second set of 6 tests is > 10.0 %, an investigation must be performed before proceeding with additional testing.

5.3.6 If a sample does not meet the specifications of potency additional 6 tests are performed. Data from second set of 6 tests are used to calculate the arithmetic mean and standard deviation and to confirm the observations made in the first set of tests. Mean potency of formulated final bulks from 6 valid tests must not be less than 108 µg HA per mL (for HD-Tri), not less than 77 µg HA per mL (for HD-QIV) and not less than 81 µg HA per mL (for Intradermal) with the upper limit for %RSD as ≤ 10%. If the results are not within specifications after repeat testing, an OOS investigation must be performed.

5.4 Criteria for Identity of Formulated Bulks

5.4.1 Ring diameters for formulated bulk samples tested for identity are evaluated by naked eye and observed for a decrease in ring size as the dilution increases.

5.4.2 If the decrease in ring size corresponding to increasing dilutions for the reference and test samples appears comparable by the naked eye, the test is valid.

5.4.3 If the ring diameters for the reference and test samples at similar concentrations/dilution appear comparable by naked eye, the sample passes the identity test.