Hemagglutination Assays for Titer Determinations

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March 21, 2017

Summary
Hemagglutination refers to the binding interaction between some microorganisms, like influenza A viruses, and erythrocytes (red blood cells). Hemagglutination assays apply the binding interactions to create visible networks indicative of relative viral and anti-viral antibody concentrations, and these assays have been broadly applied to vaccine development, diagnostics, and surveillance in human and animal health. Assay design and operation are relatively simple and routinely provide critical results in laboratories worldwide, but various factors, such as reagent type and condition and non-specific inhibition, must be recognized and managed in order to provide reliable viral and antibody titers.

Introduction
Hemagglutination assays are well established methods (1) with broad applications throughout the influenza vaccine, diagnostic, and surveillance communities to measure virus and antibody titers and monitor influenza subtypes. Hemagglutination inhibition assays are recommended by the Global Influenza Surveillance Network (GISN) of the World Health Organization (WHO) for serological diagnosis and surveillance of influenza (2), and many laboratories use hemagglutination inhibition assays to assess immunogenicity and determine vaccine antigens.

Hemagglutination occurs when the hemagglutinin proteins on the surface of influenza virus bind to sialic acid on red blood cells (RBC) (3). With sufficient numbers of virus and RBC’s, the virus particles act as bridging agents to create a network of linked RBC’s. Although assays with influenza virus are probably the most commonly performed hemagglutination assays, adenovirus, rabies virus, Newcastle disease virus, and many other microorganisms also agglutinate with RBC’s and are the basis for a wide range of veterinary and life science diagnostic assays. Hemagglutination assays are similar in design to agglutination assays with latex microparticles and other particulates.

Hemagglutination Assay Design
Influenza hemagglutination assays (HA) are often used as a high throughput method to determine relative virus concentration. Assays are prepared in U- and V-bottom 96-well microtiter plates, and a model layout has a high concentration of each sample’s influenza virus placed in a different row of Column 1, i.e., sample 1 in Row A, sample 2 in Row B, etc. A serial dilution of the virus samples is then performed across the plate. A fixed concentration of RBC’s is added to all wells to allow the RBC’s and virus to interact. Agglutination will occur in the wells with high concentrations of virus, and these wells
will have a diffuse red appearance. Wells with low concentrations of virus will have limited agglutination, if any, and non-agglutinated RBC’s will sink to the bottom of the well. These non-agglutinated wells appear to have a dark red dot, or button, in the well center. A typical progression in a HA plate is therefore diffuse red agglutinated wells in the low number columns and a transition to button wells in the higher number columns (Figure 1). Depending on the initial virus concentration of each sample, the transition from agglutinated to non-agglutinated will happen at a different dilution. The transition from fully agglutinated to clearly non-agglutinated wells typically happens over 2 to 3 wells when performing a conventional 2-fold viral dilution series (Figure 2).

The image shows an HA assay performed in a U bottom, 96-well microtiter plate. Eight individual samples were placed in rows A through H, and a series of 2-fold dilutions were performed across the plate from Column 1 through 11. Column 12 is reserved as a non-agglutinated negative control.

An important complement to the HA assay is the hemagglutination inhibition assay (HAI). HAI measures the presence of anti-hemagglutinin antibodies in serum by titrating the serum concentration against a fixed concentration of influenza virus and RBC’s. The antibodies bind to the viral hemagglutinin proteins which are then unable to bind to sialic acid receptors on RBC’s. As a consequence, hemagglutination is inhibited by antibodies, and the degree of agglutination depends on the antibody concentration. At high serum, and therefore antibody, concentrations, the antibodies prevent agglutination, and as the serum is diluted, agglutination can occur. The HAI assay shown in Figure 3 appears similar to an HA plate, but the transition from high to low concentration (low to high column numbers) moves from non-agglutinated to agglutinated.
Titer Determination

The analytical goal of HA and HAI assays is to determine the relative concentration, or titer, of the virus or antibody, respectively. The titer value is commonly based on the transition well that marks the boundary when moving from agglutinated to non-agglutinated. Therefore, the transition well in HA assays is the last fully agglutinated well within the series, and, for HAI assays, the transition well is last non-agglutinated well within the series. The sample in the transition well has been diluted by a specific factor, such as 1/128, and the sample’s titer value is the inverse of the dilution factor, or 128 in this example.

Influenza HA/HAI Variations

Several common factors vary the appearance and interpretation of HA and HAI assays. Plate orientation can vary, as some laboratories dilute across the columns, and others dilute along the rows. Different laboratories also choose different controls (positive and negative agglutination, serum controls, etc.), and the wells where controls are placed. Reagents also vary between assays and laboratories. For example, RBC’s from chicken, turkey, horse, guinea pig, and human are commonly used, and the specific type of RBC can alter the appearance of agglutinated and non-agglutinated wells. The RBC concentration and its age or condition also impact the appearance. The choice of U or V-bottom wells is another factor that has an impact on appearance.

A major factor in HAI assays is the presence of non-specific inhibition (NSI) caused by biomolecules in the serum that are not antibodies, but bind to the virus and interfere with virus-RBC agglutination (1). Recommended protocols with receptor destroying enzyme (RDE) have been published to address NSI (1, 4, 5), but NSI remains a common occurrence in HAI assays (Figure 4). Note the difference in appearance between the negative, non-agglutinated control and wells A1-A2, and C1-C4 with non-specific inhibition.

Alternative Hemagglutination Assays

In addition to HA and HAI assays for influenza virus characterization, laboratories apply hemagglutination to non-influenza assays such as mycoplasma, Newcastle disease, parvovirus, and Brucellosis. A plate image for Mycoplasma gallisepticum is shown in Figure 6, and an image for parvovirus is shown in Figure 5.
References

4. https://consise.tghn.org/site_media/media/articles/160713_Modified_Hemagglutination_Inhibition_Assay_Using_Horse_RBCs.pdf