# Standardizing Hemagglutination Titer Readings

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**SUMMARY**

Hemagglutination assays have a critical role in the surveillance and diagnostics of influenza and other infectious microorganisms, but reproducibility between different laboratories is a recognized limitation. Ongoing programs to standardize reagents and processes have demonstrated improved consistency, and further improvements may be limited by inconsistencies between expert human readers. Automated reading instruments have the potential to reproducibly determine titer values, along with other benefits of workflow efficiency, laboratory control, and quality assurance.

## Introduction

Hemagglutination (HA) and hemagglutination inhibition (HAI) assays were first developed over 70 years ago for characterizing influenza viruses and antisera produced in response to influenza vaccines (1). Today HA and HAI assays are used for characterizing a wide range of viruses, such as arboviruses, flaviviruses, and paramyxoviruses, and the associated immune response. The WHO Global Influenza Program considers HAI as the test of choice for global influenza surveillance (2,3). The committee also recognized several significant weaknesses of the assay, including poor reproducibility between different laboratories. HAI titer determinations between different laboratories often have large variations, with CV's of approximately 100% being common and sometimes greater than 300% (4,5).

One step toward reducing the variability has been efforts to standardize reagents and procedures. Antibody standards specific to individual influenza subtypes have been investigated (6), and Zacour and colleagues enforced consistent use of HAI reagents and procedures across five different laboratories (7). Titer agreement across the five laboratories was approximately 95%, based on the guideline that titer values within 1 dilution are equivalent. Treating titer values within ±1 dilution factor as equivalent is common practice (7,8). Standardizing influenza assays is also the primary mission of the Consortium for Standardization of Influenza Seroepidemiology (CONSISE) (http://consise.tghn.org/). A report from CONSISE collaborators illustrates the reduced variability achieved by standardizing procedures for influenza microneutralization (9). Microneutralization is a closely related alternative to HAI assays.

### Variability in Titer Interpretation

Interpretation of the agglutinated/non-agglutinated well patterns can also contribute to variability. A report from the WHO (1) indicated that current methods of manually interpreting HAI plates have several limitations, including i) the requirement of specialized expertise in reading the results of the test, ii) poor lab-to-lab consistency in training and interpretation of titer, and iii) interpretation challenges when nonspecific inhibition occurs. Each of these factors contributes to variability. Long tenured expert readers can develop patterns or biases that differ between experts and contribute to variations between laboratories. Groups that train additional staff as readers can have the potential

biases of the lead trainer transferred to the new trainees, and the performance of the various readers may not be tracked or consistent. Nonspecific inhibition (NSI) is a reagent-related phenomenon that complicates the reading of the hemagglutination well patterns by inhibiting agglutination and altering the plate appearance (**Figure 1**).

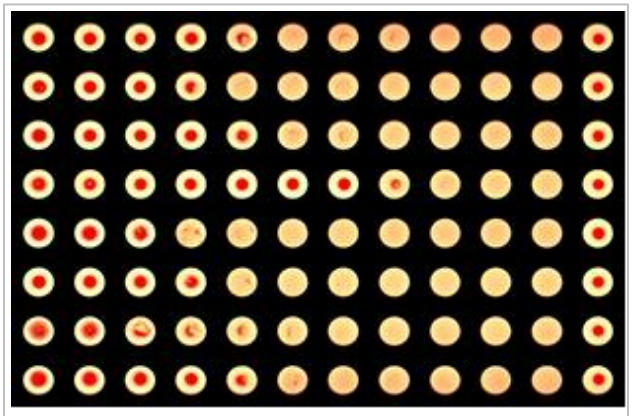

**Figure 1. Hemagglutination Inhibition Assay Plate.**

In an effort to assess variability in titer determination across different experts, InDevR conducted an online survey. Each respondent was pre-qualified through a series of questions that addressed their experience with HA/HAI assays. For those with significant experience, each human reader was asked to view online images of HA assays in microtiter plates and identify the wells showing the transition from non-agglutinated to agglutinated. A total of 390 unique `reads` (interpretations) were generated from 13 responders each reading 30 different images. The distribution of interpreted titer values is summarized in **Figure 2** where the "correct" titer value is assumed to be the consensus value for each sample by the various human readers. Approximately 57%, or 223, of the titer determinations were exact matches, and 82% of the titer calls were within the ±1 dilution guideline. In addition, the survey included triplicate images of hemagglutination assays, and 16% of the time, a given reader would choose a different titer assignment. The changes were usually small, only plus or minus 1 dilution, but the inconsistency contributes to overall variability. Combining the survey results with the variability observed when reagents and assay processes are carefully controlled (7) indicates that variation in human expert reads is a significant factor in variability between laboratories.

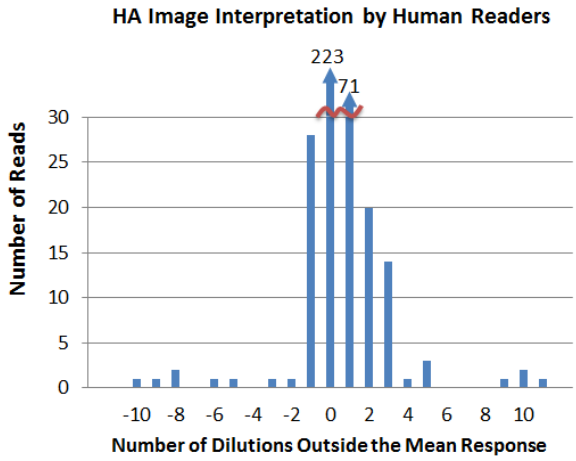


**Figure 2. Variation of HA interpretation among expert readers; 13 readers interpreted the titer value for 30 samples each. The mean response is the consensus titer value of the 13 readers for each sample. 223 titer calls were an exact match to the mean titer. The other bins display how often the titer calls were 1 or more dilutions from the mean titer.**

## Automated Titer Determinations

One approach to reducing and potentially eliminating the variability in interpreting HAI titer determinations is to use automated imaging and analysis instruments. As a digital instrument, an automated reader would avoid the drift and bias associated with human readers and provides consistent results regardless of the location or monotony of the task. Automated reading would also include the benefit of reduced operational costs compared to the labor and documentation costs associated with manual reading.

Automated HAI readers have been described in the scientific, and patent literature, however, commercial availability is limited for some. Researchers at Sanofi Pasteur and its subsidiary, VaxDesign, have described surface-activated plates, algorithms, and a high throughput instrument to address hemagglutination titer determinations (US patent 8,962,256) (10); although, it has been reported that the project may be an internally developed instrument without commercial intent. SciRobotics (Kfar Saba, Israel) has developed the FluHema instrument that images hemagglutination assays in microtiter plates to report whether the hemagglutination pattern in each well is positive or negative. Reportedly, commercial availability has been limited. Both platforms have limited performance in the literature, while cost, maintenance and availability are also unclear.

**Figure 3. Cypher One Automated Hemagglutination Analyzer.**

InDevR has developed the commercially available Cypher One Automated Hemagglutination Analyzer (**Figure 3**) to record images and determine titers of HA and HAI assays in 96-well plates (http://indevr.com/products/cypher-one/). The Cypher One system provides a digital record of the plate image, associated experimental information (operator, RBC type and concentration, dilutions, etc.), and analyzed results, as well as a 21 CFR Part 11 compatible user interface and options for automated analyses of well values independent of specific titer determinations.

Cypher One titer calls closely match the titer calls of expert human readers (see "Manual versus Automated Readers for Hemagglutination Assays" white paper), and are therefore accurate relative to the gold standard. In addition, Cypher One instruments are manufactured and adjusted with calibrated standards and quality control procedures to ensure reproducibility when different instruments are used in different facilities. The diversity among human readers makes similar reproducible performance unlikely. More extensive comparisons between an automated reader like Cypher One and manual reading are necessary to fully validate the systems, but automated readers have the potential to significantly improve standardization.

**Conclusions**

- Titer determinations based on hemagglutination and hemagglutination inhibition assays can be highly variable across different laboratories
- Standardization of reagents and procedures can significantly reduce variability
- Automated reading is another option for standardizing HA and HAI titer determinations to reduce variability

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