

# Manual versus Automated Readers for Hemagglutination Assays

Ives, J.T. and Woodward, C.  
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## SUMMARY

Fully automated hemagglutination assays will enable increased throughput, reliability, and consistency, and numerous laboratories have automated the liquid dispensing and plate handling aspects. However, assigning titer values to the processed samples remains the responsibility of expert human readers, in spite of the repetitive nature of the task, risks to data integrity and errors, and variability between readers. Automated readers like the Cypher One instrument provide a comprehensive system with accurate titer calls, efficient laboratory workflow, controlled data integrity, and overall quality assurance to improve the utility and efficiency of hemagglutination assays.

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## Introduction

Influenza hemagglutination assays have a prominent global role in vaccine manufacture and development, as well as in public and animal health surveillance. Many of these laboratories process hundreds to thousands of samples per day in a repetitive process involving conventional 96-well microtiter plates, standard biological reagents, and consistent process steps. These hemagglutination assays are therefore well matched to process automation with existing commercial plate handlers and liquid dispensing systems. In some of these laboratories, standalone instrumentation is integrated into a semi-automated laboratory workflow. In a few laboratories, almost the entire process is automated (1).

While automation has been applied to preparing hemagglutination assays, interpreting the visual patterns in the 96-well plates has primarily remained a manual task limited to trained, expert readers. The technical staff and automation equipment in hemagglutination laboratories follow prescribed steps to perform the hemagglutination assays, and then transfer stacks of plates to the resident expert who visually reviews each plate.

The expert writes the assigned titer call for each sample on paper, and works to remain within the time constraints of the assay and the load of hundreds of samples per day. Laboratory technicians often transcribe the written titer calls into a software worksheet for analysis. Both conceptually and operationally, the manual reading step is a potential bottleneck and point of risk for the entire process.

## Expert Human Readers

Manual plate reading by expert human readers is the most prevalent method of distinguishing agglutinated from non-agglutinated wells. In brief, the reader analyzes a hemagglutination inhibition (HAI) assay plate (**Figure 1**) by tracking the wells within each row and looking for a change from the dense red button in the center of non-agglutinated wells on the left side to the diffuse reddish appearance of agglutinated wells on the right side of the plate (Column 12 is a negative, non-agglutinated control.) The reader is primarily interested in noting the transition well, where the appearance changes from non-agglutinated to agglutinated. The sample's dilution factor for that well is used to determine the titer (titer is the



inverse of the dilution factor). The reader performs this visual analysis for every sample and plate. Hemagglutination assay (HA) plates have a similar appearance and reading process, although the left to right transition across the plate is reversed, i.e., agglutinated to non-agglutinated for HA.

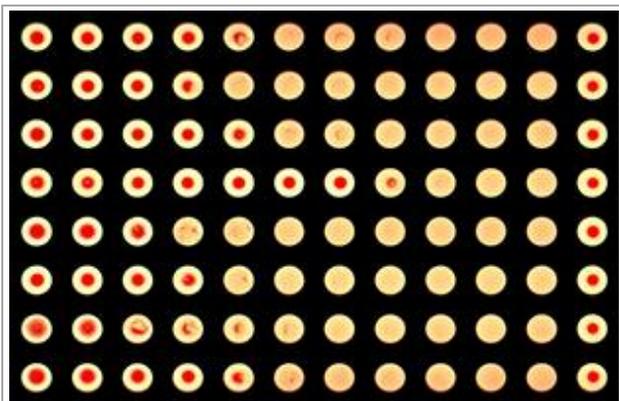


Figure 1. Hemagglutination Inhibition Assay Plate.

Human readers have demonstrated the ability to adjust for a variety of hemagglutination assay features, including:

- hemagglutination (HA) versus HAI assays,
- plate layout (row vs. column, placement of controls),
- erythrocytes (type, concentration, condition),
- non-specific inhibition (NSI).

NSI is caused by non-antibody proteins that inhibit agglutination and are present in the serum of some samples. Wells exhibiting NSI generally appear to have hazy, irregular center spots with relatively large diameters compared to the non-agglutinated negative control wells (**Figure 2**). A primary challenge with NSI samples is that the transition from non-agglutinated to agglutinated wells is obscured, which makes determining titer values difficult. Enzymatic treatments are commonly applied to reduce or eliminate NSI (2), but NSI remains a common obstacle.

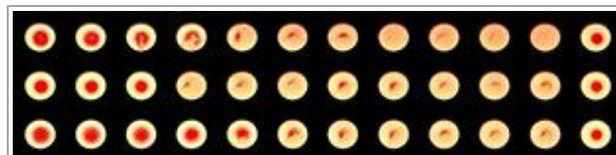


Figure 2. Example well images of sample with NSI.

### Limitations of Manual Readers

Although manual reading by an expert reader is the standard and compensations are made, limitations still remain.

#### Variability of the Read

A significant problem with human readers is variability in titer calls and procedures. Titer calls are often accepted as equivalent if they are within  $\pm 1$  well in a 2-fold dilution series (3, 4). For example, titer calls of 20, 40, and 80 for the same sample would be accepted as equivalent yet there is a wide delta in those values. Many factors likely contribute to this variability ranging from human bias, to differences in training at each facility. Drift can also be attributed to the repetition of reading 100's of samples and thousands of wells every day. This mundane task can be influenced by the reader's emotional and physiological state, time of day and other variables affecting human cognitive performance. Overall, numerous procedural and training factors contribute to variability.

#### Lack of standardization

Compounding variability in titer calls is the lack of standardization. A lack of standardization is far reaching throughout the industry (3).

Labs also handle manual interpretation protocols differently. Some laboratories rely on the judgement of a single human expert as being correct, while other laboratories have multiple readers for every plate and use consensus calls. Tilt angles and read times can also vary for different operators and laboratories. These have a direct effect on the accuracy of the plate read.

Nonspecific inhibition is a challenge for any lab. One approach often used by human readers to

manage NSI is to tilt the plates at an angle of roughly 45° and monitor movement of the erythrocytes within the well. With agglutinated wells, the diffuse reddish appearance does not change upon tilting as the erythrocytes are held in their agglutinated network. Erythrocytes in non-agglutinated wells move from the central button and flow toward a low position creating a smear or teardrop pattern when tilted. But, the “pseudo-buttons” of wells with NSI act more like agglutinated wells with little or no smearing when tilted. Very few labs use a jig or other mechanism to standardize the tilt angle. Steeper versus shallower angles can affect the rate of run. Additionally, manual interpretation of tilted plates takes an expertly trained eye to catch often subtle changes the rate of the pellet run.

#### Push for Increased Quality Controls

The use of human readers also has disadvantages in terms of quality lab management and overall quality assurance. The plates are visually examined and then discarded (the hemagglutination process has a limited read window), which leaves no permanent record of the actual plate for review and confirmation. The risk of errors in writing and then transcribing titer values for each sample is significant, particularly when the tasks are repeated hundreds of times per day. Ensuring data integrity and traceability through these manual processes is a difficult challenge, particularly for laboratories certified for ISO operation.

#### Cost Disadvantages

The operational costs of manual reading are another disadvantage and an opportunity for improvement. Significant labor costs are associated with reading, recording, and transcribing results, especially when the most senior and well trained personnel are often responsible for reading plates and assigning titer values. By offering a way to free up resources that can be used on higher value tasks, the labs can better manage resource loading and work output.

## Automated Readers

Digitizing the process of hemagglutination plate reading has the potential to significantly improve titer call consistency and provide operational benefits. A few companies are working to provide more rigorous solutions utilizing software to provide consistency and reliability in these measurements. Cypher One (InDevR, Boulder, USA), FluHema (SciRobotics, Israel) and Sanofi Pasteur VaxDesign (Florida USA) (5) are three instruments designed for reading HA and HAI assay plates. Little is known on FluHema and the Sanofi VaxDesign platforms' broad commercial readiness, and performance information is limited or lacking in the scientific literature. Cost and availability are also unclear, with the Sanofi VaxDesign instrument being reported as an internal project without commercial availability or intent.

Conversely, the commercial Cypher One Automated Hemagglutination Analyzer (InDevR, Colorado USA) is a new instrument for imaging and analyzing hemagglutination assays in 96-well microtiter plates (**Figure 3**). The instrument has been designed to operate in a variety of laboratories analyzing agglutination assays and automatically provide titer values or numeric well values based on the laboratory's preference.



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

Cypher One provides a comprehensive management tool for hemagglutination assays, in addition to reading 96-well microtiter plates. User access is restricted, the system can be interfaced to the laboratory's information management system, and experimental information, such as the

operator's name, time/date stamp, sample names, and dilution factors are recorded and linked to the plate image and associated titer calls. All the data and information is stored in a user-accessible database. These capabilities help ensure data integrity and compliance with 21 CFR Part 11 and EU Annex 11, and are more efficiently performed than with a manual paper-based method. In addition, an audit trail provides an automated system of tracking changes in personnel, analysis, and approvals.

Cypher One's Plate Setup view is a user interface (Figure 4) that allows the user to enter experimental information, such as sample names, dilution factors, type of assay (HA, HAI, or other), and plate orientation (sample dilution along row or column). Information can be entered manually for each plate, or files with information about multiple plates can be imported. After entering the relevant information, the user can instruct the Cypher One system to record a digital image of the plate and perform image analysis.

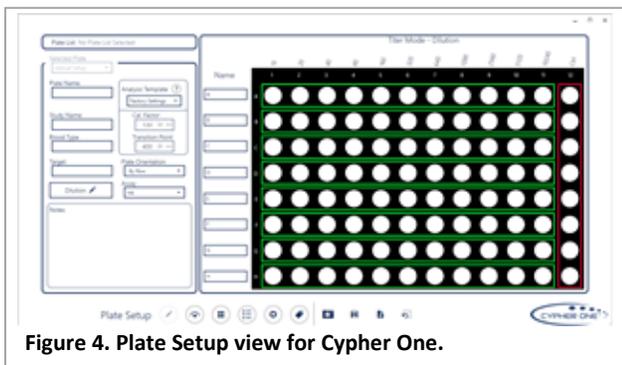


Figure 4. Plate Setup view for Cypher One.

Another benefit of automated reading is the recorded, digital image of the plate. In contrast to manual readers where the time varying appearance of the plate introduces variation among readers and quality system challenges, the digital image provides a traceable record that is identical for all current and future observations. A potential cGMP scenario would allow many plates to be imaged and analyzed during routine processing, and the laboratory manager or other quality professional could then review the batch of results and images at a later time for approval.

With Cypher One, the plate image and analyzed results are displayed in the Plate View user interface (Figure 5). The titer value for each sample is displayed both in the plate image (yellow circles around the transition wells) and as a text box adjacent to each sample listing. The digital image and results are saved, and appropriate links between setup information, image, and results are stored in a secure database. Select personnel, such as the laboratory manager, can also edit, re-analyze and approve previous results, and the changes are stored in an audit trail.

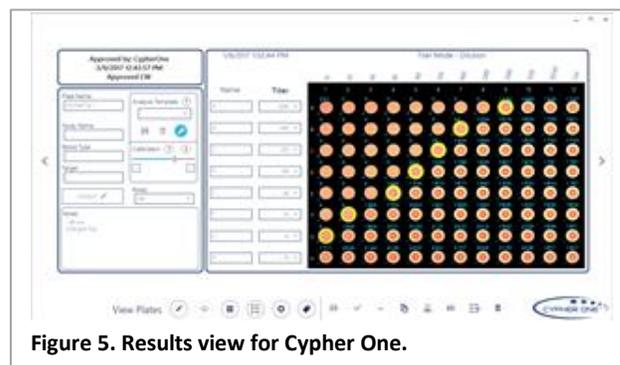


Figure 5. Results view for Cypher One.

The most critical feature of an automated reader is probably the accuracy of the titer calls, demonstrated by correlation with expert human readers. Cypher One was used in a study of 896 HAI samples (turkey red blood cells), and the titer values assigned by an expert human reader and Cypher One are compared in Figure 6. The histogram displays how many times the Cypher One call was an exact match to the expert reader's call (0 dilutions separating the manual and Cypher One call) and how many times the calls differed by 1, 2, or more dilutions. In total, an exact match occurred for 69.5% of the samples, and the Cypher One and human reader calls were within 1 dilution of agreement for 90.7% of the samples. Given that multiple humans are considered in agreement if their calls are within 1 dilution, then the Cypher One's performance is well matched to human determinations. Cypher One has been used in other laboratories with comparable accuracies, and an article describing a larger data set has been submitted for publication.

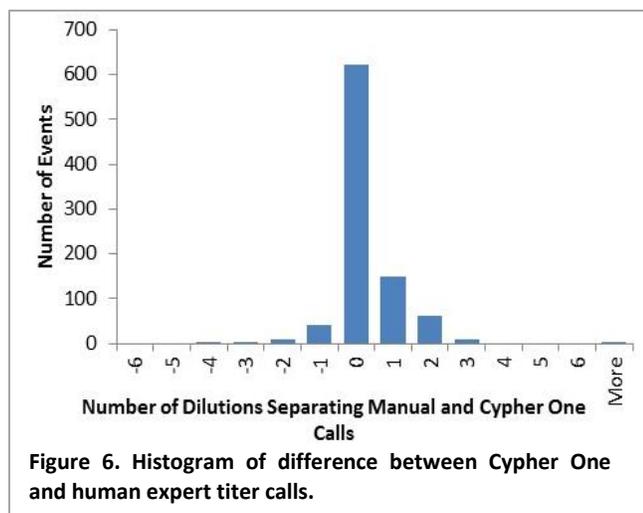


Figure 6. Histogram of difference between Cypher One and human expert titer calls.

**Conclusions**

Automated readers have the potential to significantly improve laboratory workflow and consistency of results across different laboratories performing HA and HAI assays. Table 1 compares the use of manual, i.e., expert human readers, and automated reading with Cypher One for several key capabilities.

Capability	Reading Method	
	Manual	Cypher One
Accurate	Yes	Yes
Flexible Plate Format	Yes	Yes
21 CFR Part 11 Compatible	Not Applicable	Yes
Digital Record of Image	No	Yes
Digital Record of Experiment	Not Applicable	Yes
Consistent Performance	No	Yes

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