

Article for *IBL*

**Automated Zone Measurement in Single Radial Immunodiffusion Assays
*Promises Rapid Quality Control of Influenza Vaccines***

By

**Simon Johns, International Product Manager, Synbiosis, Beacon House,
Nuffield Road, Cambridge, CB4 1TF, UK.**

Introduction

In the UK, people over 65 and those considered to be in high-risk groups are routinely offered an inoculation to protect them against the flu (influenza) virus. If the concentration of the inactivated viral vaccine is incorrect then those inoculated may be insufficiently protected, which could prove fatal among some of these vulnerable groups.

Since flu vaccines are used on a large number of people on a national basis their quality needs to be verified and to do this there are in place standard methods such as the Single Radial Immunodiffusion (SRD) assay. Across Europe there are a number of independent government institutes, which check the quality of a range of medicines including flu vaccines. They use accredited protocols to ensure vaccines comply with the Product Licence (Market Authorisation) before they receive a Release Certificate allowing their sale in Europe.

At one such European institute, its Virology Department is responsible for controlling the quality of viral flu vaccines. It samples each batch because for the vaccine to remain effective, the three strains of flu virus in the vaccine are changed every year. To assess the potency of each batch, the Department measures the serological responses the vaccine provokes using an SRD assay. This measures the haemagglutinin (HA) concentration of antigens by reaction with specific sheep antiserum in an agarose gel. The size of the reaction zone produced is directly proportional to the HA concentration, therefore measurement of the reaction zones provides an indirect measure of the potency of the vaccine.

To measure SRD reaction zones, the Virology Department currently uses a calibrated viewer to manually estimate the size of SRD reaction zones. Using this method, the Department estimates that reading an SRD plate consisting of 16 reaction zones takes approximately 1.5 hours. In addition, the printout from the calibrated viewer has to be re-keyed into another software programme for statistical

analysis. The Department recently reviewed this manual method of measuring reaction zones and concluded that it was extremely time consuming and with the data re-keying involved, was also very labour intensive.

Therefore, it decided to automate the process using a specially designed system. This consists of the ProtoCOL, automated zone reader and colony counter (Synbiosis, Cambridge, UK) integrated with a CCD camera on a macrostand over a light box. This system can read an entire SRD plate, measure reaction zones and transcribe the results into an Excel format in just under 10 minutes and therefore offers the opportunity to save many hours of highly repetitive work.

However, since changing part of a quality control process can have far reaching implications when testing flu vaccines, the Department had to ensure that any automated method it used was as accurate as its existing manual method. Therefore, the Department undertook a study, the results of which have been submitted to a National Accreditation Service, to compare the two methods before it started using the ProtoCOL system as part of its standard operating procedure.

Method

Preparation of an SRD Assay

To prepare agarose gel plates for an SRD assay, agarose (1 % w/v, 13ml) was melted and mixed with an in-house antiserum. This was poured into a 90mm diameter circular cast and left to set. A well template was used to cut out 16 wells of 4mm diameter and the agarose from these wells was removed with a vacuum pump.

To prepare the vaccine, an in-house antigen reagent control was diluted with PBSA (phosphate buffered saline pH 7.4 + 0.05% w/v sodium azide) to an initial concentration (30µg HA/ml). The viral vaccine (450µl) and antigen control (450µl) were treated with Zwittergent 3-14 (10 %v/v, 50µl) and incubated at room temperature (22°C, 30 minutes).

To load the agarose gel a series of four dilutions of 1, 0.75, 0.5 and 0.25 v/v dilutions using PBSA were made of both the detergent treated vaccine and antigen control. Each dilution was added to its assigned well in the agarose plate according to an in-house randomisation scheme, of which there are 10 and a different one was used for each SRD plate. When all 16 wells were filled the plate was incubated (18 hours, 20-

25°C). The agarose gel was rinsed in water and then pressed flat under filter paper layers and a weight (600g). The gel was dried in an incubator (35°C) and stained with Coomassie Blue (3% w/v) to make the SRD reaction zones visible.

Measuring Reaction Zones Using a Manual Method

The SRD plate was placed on a light box attached to a calibrated viewer with an assay data recorder. Operators in the Virology Department used the calibrated viewer to manually estimate the size of the reaction zones in two directions at 90° to each other. The print-out from the calibrated viewer was then re-keyed into a programme written by the Institute, which analysed the statistical validity of the test and calculated the HA concentration of the vaccine by comparison with the standard curve of the control antigen.

Measuring Reaction Zones Using an Automated Method

The SRD plate was placed on the ProtoCOL system's integrated light box. The ProtoCOL's CCD camera captured the plate image and showed it on the PC's screen. The ProtoCOL software has a template of eight circles that were placed over the on-screen image of the SRD plate. The circle templates were placed around each reaction zone and were adjusted by simple mouse clicks to fit around the zone image. Using a single click the software automatically measured the diameter of each zone and transferred the data into an Excel spreadsheet. It also flagged any areas of dispute such as fuzzy edges, so that operators could measure this manually with the on-screen ruler, if necessary. The data produced was transferred directly into a statistical analysis programme without the need to re-key it.

Comparison of Reaction Zone Measurement Methods

An SRD assay consisting of three vaccines (batch numbers 513/9, 757479A and 757479B) and one standard was analysed by measuring the reaction zones for each vaccine. The assay was measured in the Virology Department six times by operator one at weekly intervals using both the manual calibrated viewer method and the ProtoCOL system and was also read six times by operator two using only the ProtoCOL system.

Results

The SRD assays produced plates, which had reaction zones that could be measured by both the automated ProtoCOL system and the manual method of measurement.

The zone sizes obtained from both methods were input into a standard statistical slope-ratio model to produce the estimated potencies for vaccines, 513/9, 757479A and 757479B. The potency figures were then compared with the potency value stated by the manufacturer of the vaccine to produce an estimate of the precision of the assay.

The results produced by operator one for all three vaccines over a six-week period are shown in Table 1.

Table 1: Vaccine potencies and assay precision figures produced by operator one using a calibrated viewer and a Synbiosis ProtoCOL system.

Week number	Vaccine number	Manual Readings		Automated Readings using the ProtoCOL system.	
		Potency ($\mu\text{g}/0.5\text{ml}$)	Precision (%)	Potency ($\mu\text{g}/0.5\text{ml}$)	Precision (%)
1	513/9	11.305	93.6	11.697	89.9
	757479A	10.325	93.1	10.904	89.2
	757479B	15.955	94.9	15.798	91.7
2	513/9	11.513	93.8	11.654	89.6
	757479A	11.034	93.6	10.609	88.7
	757479B	16.531	95.1	15.975	91.6
3	513/9	11.513	92.1	11.753	89.5
	757479A	11.009	91.7	10.753	88.7
	757479B	16.706	93.7	15.58	91.4
4	513/9	11.417	92.2	11.617	89.3
	757479A	10.693	91.8	10.884	88.7
	757479B	16.31	93.9	15.653	91.3
5	513/9	11.667	92.8	11.812	91.6
	757479A	10.638	92.2	10.814	90.9
	757479B	16.122	94.2	15.707	93
6	513/9	11.389	90.7	11.792	91.7
	757479A	10.773	90.3	10.675	91.0
	757479B	15.992	92.7	15.741	93.2

The results produced by operator two using the ProtoCOL system automated method to measure the reaction zones for all three vaccines over a six-week period are shown in Table 2.

Table 2: Vaccine potencies and assay precision estimates produced by operator two using a ProtoCOL system.

Reading number	Vaccine number	Automated reading using the ProtoCOL system	
		Potency ($\mu\text{g}/0.5\text{ml}$)	Precision (%)
1	513/9	11.951	93.5
	757479A	10.909	93.0
	757479B	15.753	94.6
2	513/9	11.919	92.7
	757479A	10.964	92.2
	757479B	15.786	93.9
3	513/9	11.899	93.3
	757479A	10.882	92.8
	757479B	15.857	94.4
4	513/9	11.886	93.5
	757479A	10.958	93.0
	757479B	15.901	94.6
5	513/9	11.793	94.2
	757479A	10.967	93.9
	757479B	15.875	95.2
6	513/9	11.863	92.9
	757479A	10.949	92.4
	757479B	15.92	94.1

The results from Tables 1 and 2 were statistically analysed using a coefficient of variance (CV) calculation and the results are shown in Tables 3 and 4.

Table 3: CV analysis of vaccine potency measurements.

Vaccine	Operator 1 using manual calibrated viewer method (% CV)	Operator 1 using automated ProtoCOL method (% CV)	Operator 2 using automated ProtoCOL method (%CV)
513/9	1.1	0.7	0.5
757479A	2.4	1.1	0.3
757479B	1.9	0.9	0.5

Table 4: CV analysis of assay precision calculations.

Vaccine	Operator 1 using manual calibrated viewer method (% CV)	Operator 1 using automated ProtoCOL method (% CV)	Operator 2 using automated ProtoCOL method (%CV)
513/9	1.2	1.2	0.7
757479A	1.3	1.2	0.6
757479B	0.9	0.9	0.5

Discussion

The ProtoCOL system produces very consistent %CVs of approximately 1% for both potency and precision measurements, a figure that is well within the acceptable limits set by UKAS. The agreement between operators is also excellent, with a maximum difference of 0.16µg/0.5ml compared to a maximum difference of 0.53µg/0.5ml between the calibrated viewer and the ProtoCOL system for operator one in this study. Typically, the confidence interval set by UKAS on an estimated potency is +/- 1.5µg/0.5ml, so clearly the difference between operators using the Synbiosis ProtoCOL system is negligible for these assays.

Conclusions

The data from this study demonstrates the ProtoCOL system produces not only highly consistent SRD plate readings across a time period and between different operators but also provides more reproducible readings than the manual calibrated viewer method. The ProtoCOL system presents the results in a flexible Excel format so there they can be exported directly into any statistics programmes without the need to re-key them. This not only saves time but also prevents potential keying errors and therefore further enhances the accuracy of results.

In addition, the ProtoCOL offers the benefits of being much quicker, since it can read 16 wells from an SRD plate in 10 minutes compared to around 1.5 hours using the manual method. This study confirms that the ProtoCOL system is an excellent replacement for the manual method of reaction zone reading and because it produces accurate, rapid results could potentially speed up the official release of flu vaccines tested by any European regulatory organisation.

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For Further Information Contact:

Jayne Arthur, Synbiosis, Beacon House, Nuffield Road, Cambridge, CB4 1TF, UK.
Tel: +44(0) 1223-727125 Fax +44 (0) 1223-727101
Email: jayne.arthur@synbiosis.com Web site: www.synbiosis.com

Editor Contact:

Dr Sue Pearson, PO Box 170, Hitchin, Hertfordshire, SG5 3GD, UK.
Tel/Fax + 44 (0) 1462- 635327 Email: sue6.pearson@ntlworld.com