Application note 28-9771-57 AC Vaccines

Biacore™ biosensor assays for quantitation of influenza virus and HCP

Biacore biosensor technology is used for the quantitation of virus haemagglutinin (HA) and host cell proteins (HCPs) in influenza vaccines. Accurate quantitation of influenza virus is achieved using an inhibition assay, while the HCP concentration is determined in a direct binding assay. The HA method shows higher sensitivity, precision, and recovery as compared to single-radial immunodiffusion (SRID) which is the main method used today. In addition, the analysis time is shorter. The HCP method demonstrates higher sensitivity and is more specific compared with the Bradford total protein assay. Assays performed on Biacore systems have the potential to significantly improve vaccine development and manufacturing operations, as a complement to or replacement of existing methodologies.

Introduction

In vaccine production, the quantitation of virus concentration and HCP impurities is critical for the final formulation. Most influenza vaccines are produced in fertilized eggs, but the fear of a new pandemic influenza has speeded up the development of production methods based on cell culture systems. The need for shorter development cycles has also increased the demand for improved analytical tools. In this Application note, novel methods for quantitation of influenza virus (1) and HCP using biosensor technology are described.

The potency of influenza vaccines is predominantly determined by quantitation of HA using immunochemical methods. The most common method used for this purpose, approved by the Food and Drug Administration (FDA) and the European Agency for the Evaluation of Medicinal Products (EMEA), is SRID where vaccine dispersion is allowed to diffuse through a gel containing a specific antibody. SRID is a labor-intensive method and has several other disadvantages including low precision and sensitivity (2-5).

Host cell proteins are usually quantitated as total protein (i.e., Bradford protein assay) or with ELISAs using a mix of polyclonal antibodies against cellular proteins. Both methods exhibit specific limitations: Bradford total protein assay is not sensitive or specific enough to detect levels below the regulatory critical limits; ELISA methods using purified polyclonal antibodies increase the sensitivity and specificity, but are labor-intensive.

Biacore biosensor assays can be used to provide significant improvements in virus and HCP quantitations compared with the methods used today.

Materials and methods

Biacore T200, Typhoon™ variable mode imager, ImageQuant™ TL software, Sensor Chip CM5, Amine Coupling Kit, Surfactant P20, HBS-EP+ buffer (10 mM HEPES, 150 mM NaCl, 3 mM EDTA, 0.05% Surfactant P20), 50 mM NaOH, Cy™3 fluorescent dye, and agarose were from GE Healthcare. Zwittergent™ was from Merck and BSA from Sigma-Aldrich. Bradford solution was from BioRad. Plate reader was from Molecular Probes. Sera and virus antigens were from National Institute for Biological Standards and Control (NIBSC), except for the B/Brisbane/3/2007 serum and antigen, which were from Solvay Pharmaceuticals. Recombinant full-length HA proteins were from ProspecBio (A/H1N1/New Caledonia/20/99), Genway (A/H3N2/Wyoming/3/2002), and Protein Sciences (B/Jilin/20/2003).

Biacore T200 and Sensor Chip CM5 were used for all biosensor analyses. The process samples for human influenza were from GE Healthcare: influenza viruses were cultivated in Madine Darby Canine Kidney (MDCK) cells, except for the mono- and trivalent bulk vaccine (MBV and TBV) and the A/H1N1 TBV sample, which were provided by Solvay Pharmaceuticals. The trivalent vaccine was from a commercial manufacturer. Polyclonal antibodies against lysates of MDCK cells were produced in rabbits at the National Veterinary Institute, Sweden. The produced serum was affinity purified against MDCK cell lysate on HiTrap™ NHS-activated HP columns (GE Healthcare).

Biacore influenza virus quantitation assay

Recombinant HA antigen was immobilized on the dextran matrix to a level of 4 000 to 10 000 response units (RU) with standard amine coupling (Fig 1). Serum standards (from immunized sheep) containing antibodies with affinity for the specific influenza strains were diluted to give a response of about 1000 RU for a 400 s injection over the surface. This dilution factor resulted in a dynamic range of the standard curves of 0.5 to 8 µg virus/mL (Fig 1C). To stabilize the assay, 5 to 10 start-up cycles were run first with serum only, followed by regeneration.

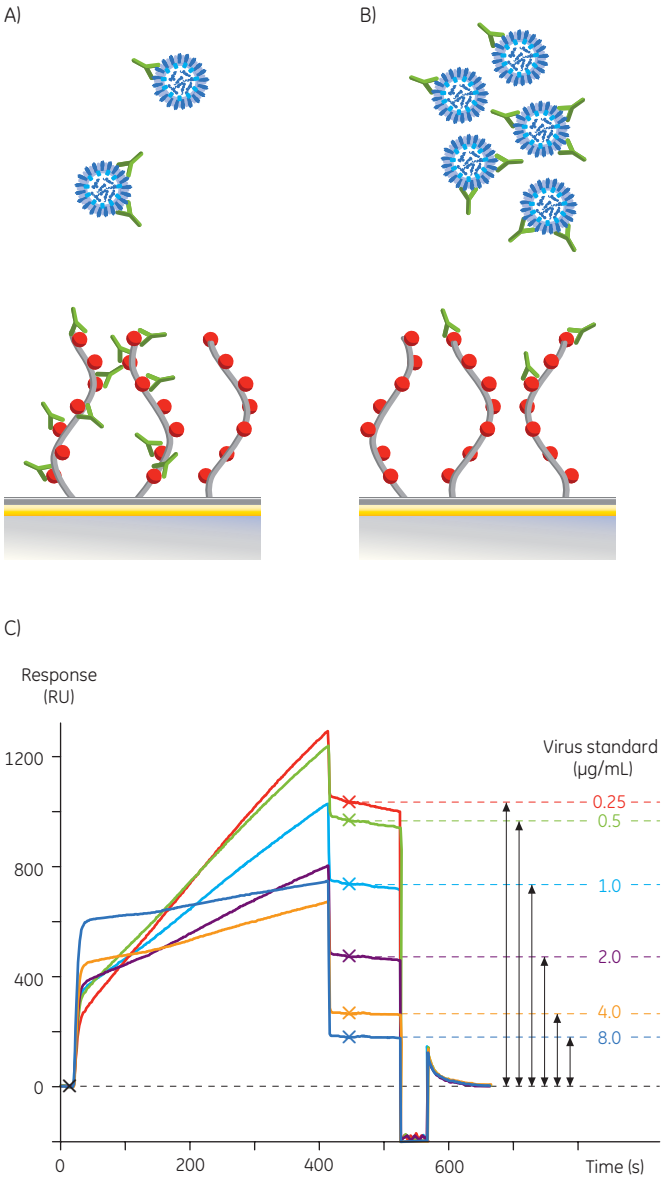

Fig 1. Inhibition assay principle **(A, B)**. HA is first immobilized on the dextran matrix (red circles). Virus is then mixed with a fixed concentration of serum and injected over the surface. Free antibodies (not bound to virus at equilibrium) bind to the surface HA, giving a response. Low concentration of virus in the sample **(A)** gives high antibody binding, while high virus concentration **(B)** results in low binding level. **(C)** Overlay plot showing sensorgrams of injected serum mixed with a concentration series of virus standard. Report points (marked as X) were taken before and after injection to measure response levels. The surface was regenerated after each injection to prepare for a new sample.

Samples were analyzed without pretreatment. Mixtures of virus reference antigen or a virus sample with unknown concentration and a fixed dilution of anti-influenza serum were injected for 400 s (Fig 1A and 1B), followed by regeneration of the surface using freshly prepared 50 mM HCl, 0.05% Surfactant P20 for 30 s. Process samples were diluted so that at least two dilutions per sample reached a concentration within the standard curve range.

Standard curves were run first, in the middle, and last in the assays. An interpolated calibration, performed by the software, was used to ensure high precision in calculated concentrations.

In the trivalent assay, three recombinant HA proteins (A/H1N1, A/H3N2, and B) were immobilized in three different flow cells on the same sensor chip. The three corresponding reference antigens and sera were mixed to form a trivalent standard solution. The standards and samples were allowed to pass over the surfaces, analyzing all three strains simultaneously.

Biacore biosensor HCP assay

HCP from MDCK cells were quantitated using a direct binding assay (6).

Affinity-purified rabbit antibodies against MDCK cell lysate were immobilized using standard amine coupling to a response of 7 000 RU. The samples were diluted in HBS-EP+ with 1% BSA and injected over the surface for 300 s. The surface was regenerated with 50 mM NaOH. Standard curves using MDCK cell lysate were run first, in the middle, and last in measurement series. An interpolated calibration, performed by the software, was used to ensure high precision in calculated concentrations.

SRID assay, human influenza virus

A small portion of each serum was labeled with Cy3 (<0.5% of the total serum amount). Unlabeled serum and Cy3-labeled serum were mixed with molten agarose (1%) and cast in a mould creating holes in the gel. Samples and reference antigens were treated with Zwittergent™ (1%) for 30 min at room temperature and added to the holes in the gel. The gels were incubated for 15 to 18 h at room temperature and subsequently dried and scanned at 450 nm in Typhoon 9200.

Bradford total protein assay

The method was performed according to the manufacturer's instructions. Bradford solution (200 µL) was mixed with BSA standard or diluted samples (20 µL) in a 96-well plate format. The plate was incubated for ~1 h, and measured in a plate reader at 595 nm. The evaluation was performed in the Softmax™ Pro v5 software.

Results and discussion

Influenza vaccine quantitation, human influenza virus

The seasonal influenza vaccine for 2006/2007 consisted of the three viral strains A/H1N1 (A/Solomon Islands/3/2006), A/H3N2 (A/Wisconsin/67/2005), and B (Malaysia/2506/2004). Quantitation using these strains was performed either one by one or in combination. Calibration curves for the three virus subtypes are shown in Figure 2.

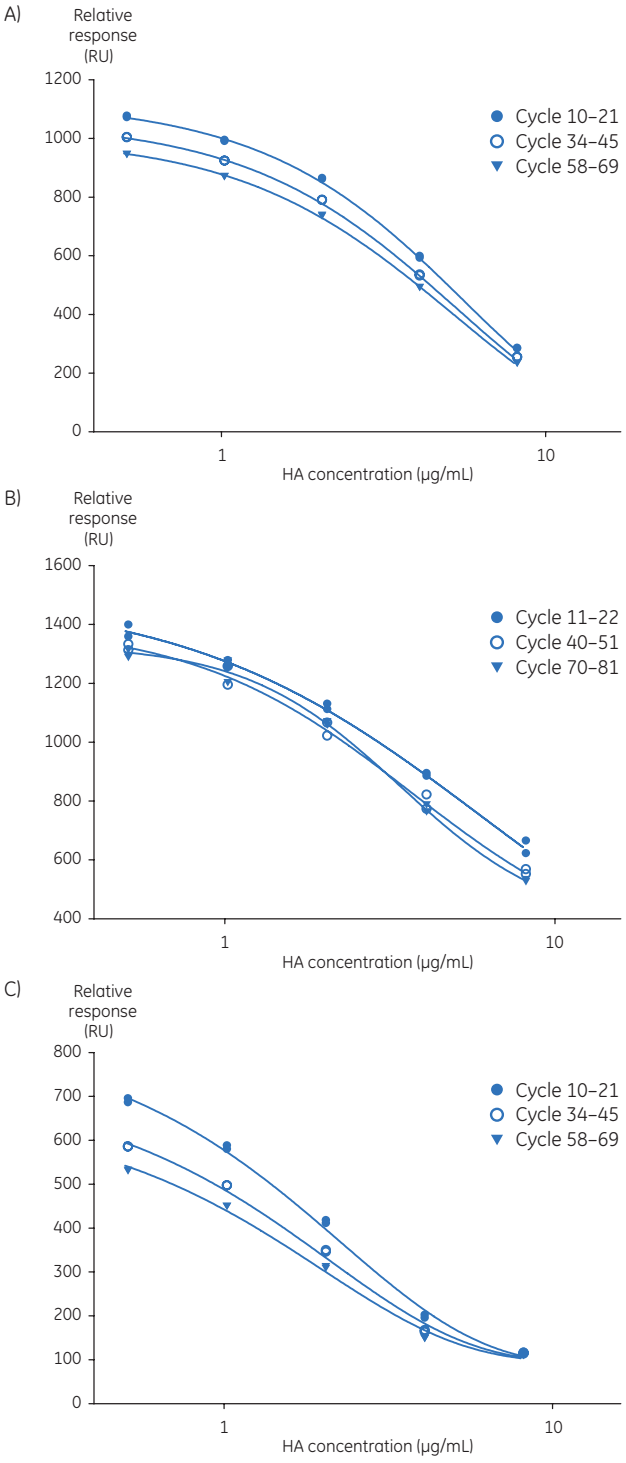

Fig 2. Standard curves for **(A)** A/H1N1 Solomon Islands/3/2006, **(B)** B/Malaysia/2506/2004, and **(C)** A/H3N2/Wisconsin/67/2005, with a concentration range between 0.5 and 8 µg/mL of each standard antigen reagent in corresponding serum.

A slight downward drift of serum response levels was found and therefore calibration samples were measured three times during the analysis as controls. The evaluation software has a built-in compensation for drift, an interpolated calibration, enabling high precision of the calculated sample concentrations. The concentrations of the standards calculated in this way are shown in Table 1.

Table 1. The recovery of the standard concentrations calculated with the Biacore software for A/H1N1/Solomon Islands/3/2006 from Figure 2

Standard HA (µg/mL)	Biacore HA (µg/mL)	CV (%) n=3
1.0	1.0	4
2.0	2.0	5
4.0	3.9	3
6.0	6.1	2
8.0	8.1	2

The assay was verified for native samples by analyzing samples from different steps in an influenza vaccine purification process, from harvest of cell culture supernatants to filtered end-products. For comparison, the same samples were also analyzed with SRID (Table 2).

Table 2. Quantitation of process samples; results from Biacore biosensor assay and SRID

Strain	Sample Type	Biacore HA (µg/mL)	CV (%) n=2	SRID HA (µg/mL)	CV (%) n=2
B					
Brisbane/3/2007	TBV[1]	31	0.4	33	7
	TBV[1]	43	0.6	36	5
	TBV[1]	43	2	30	8
	MBV[1]	260	0.02	244	16
A/H1N1					
Solomon Islands/3/2006	Harvest	8.3	10	8.0	7
	UFD	78	4	76	5
	TBV[1]	50	4	40	5
New Caledonia/20/99	Start 1	47	2	58	11
	Start 2	18	1	23	na
	Eluate 2	63	2	61	6
	Start 3	20	2	22	31
	Eluate 3	47	1	45	17
PR/8/34	Harvest	7.4	1	<LOD	na
	UFD	20	9	19	21
A/H3N2					
Wisconsin/67/2005	Harvest	1.3	4	<LOD	na
	NFF	1.0	2	<LOD	na
	DF	4.3	1	<LOD	na
	TBV	28	0.2	27	3
	TBV	38	0.3	35	8

TBV = trivalent bulk vaccine, MBV = monovalent bulk vaccine, Harvest = supernatant from infected MDCK cell culture, UFD = ultra- and diafiltration, LOD = limit of detection, Start = UFD filtrated virus diluted 10× in 10 mM NaP pH 7.4, Eluate 2 and Eluate 3 = fractions from chromatography of Start 2 and Start 3 in ~0.5 M NaCl, NFF = normal-flow filtration, DF = diafiltration, na = not analyzed in replicates.

1) from Solvay Pharmaceuticals

Biacore measurements show significantly lower coefficient of variance (CV) between replicates, than SRID (Table 2 and 3).

Table 3. A comparison of the precision of SRID and Biacore biosensor assay. Purified A/H1N1/Solomon Islands were analyzed on the same sensor chip (intra CV) and on different chips (inter CV)

	Intra CV (%)	No. of samples	No. of operators
SRID	9	12	1
Biacore	2	5	1

	Inter CV (%)	No. of samples	No. of operators
SRID	18	5	2
Biacore	5	3	2

In addition, the Biacore biosensor assay shows lower hands-on time and higher sensitivity with a limit of quantitation (LOQ) of 1 µg/mL compared with SRID (Table 4).

Table 4. Comparison of Biacore biosensor and SRID assays

	Biacore	SRID
Standard curve range	0.5 to 10 µg HA/mL	5 to 30 µg HA/mL
Sensitivity:		
LOD[1]	0.5 µg HA/mL	5 µg HA/mL
LOQ	1.0 µg HA/mL	10 µg HA/mL
Precision (no. of samples CV < 5%)	97	18
Recovery	95% to 105%	90% to 110%
Time for 100 samples:		
Hands-on	1 to 2 h	6 to 8 h
Total	~ 17 to 18 h	~ 20 to 22 h

1) LOD = limit of detection

Biacore batch release assay for trivalent vaccines

The seasonal influenza vaccine consists of three viral strains (A/H1N1, A/H3N2, and B), and therefore batch release with SRID requires analysis on three different gels. In Biacore T200, each sample can be analyzed on up to four different surfaces on the same sensor chip. Trivalent samples can thus be determined in a single experiment by mixing with three specific sera and injection of the samples over the three strain-specific surfaces simultaneously. Thus, one analysis gives three results, see Figure 3 for a schematic comparison with SRID.

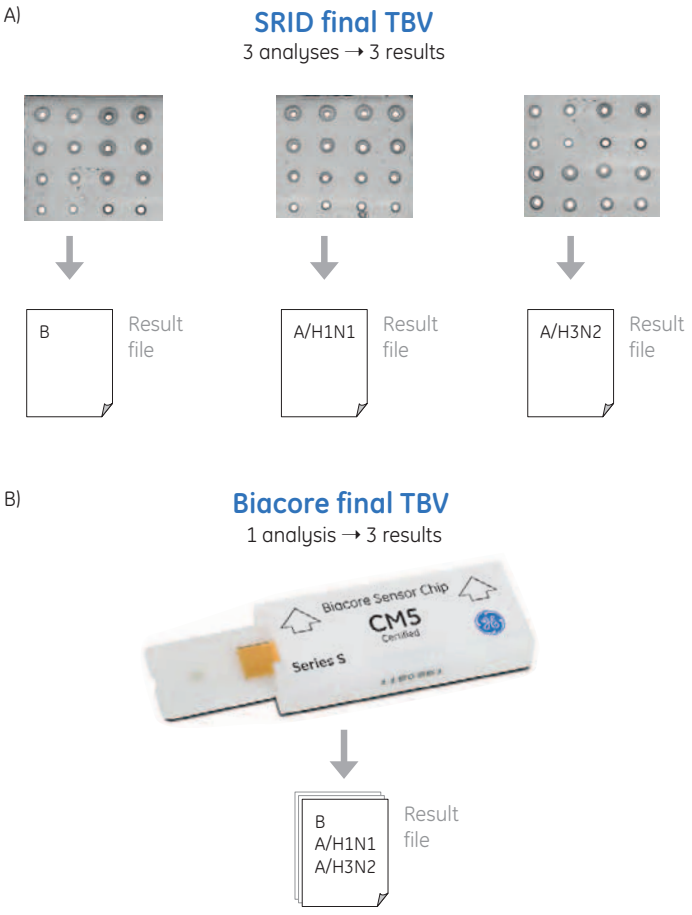

Fig 3. Schematic diagram of analysis of trivalent formulations using **(A)** SRID and **(B)** Biacore biosensor.

The results from analysis of an egg-derived trivalent commercial vaccine with SRID and Biacore biosensor are shown in Figure 4.

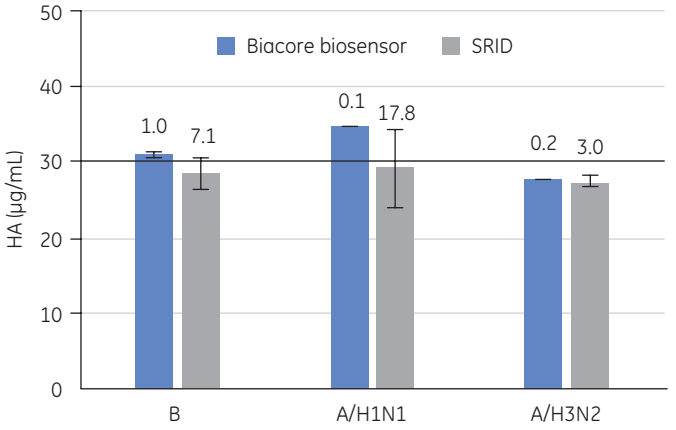

Fig 4. Analyses of a commercial trivalent vaccine. The vaccine was quantitated using SRID and Biacore biosensor. Coefficients of variation (CV [%]) of duplicates are presented over each column. The thick black line represents the specified concentration from the manufacturer of 30 µg/mL of each strain in the vaccine (15 µg per dose).

Biacore biosensor HCP assay

In this assay, anti-HCP antibodies were immobilized on the surface of Sensor Chip CM5 using amine coupling and the process samples were injected over the surface. Specific HCP proteins in the sample bound to the surface to give a direct response (Fig 5). Antibodies on the surface did not bind to the viral protein HA (data not shown). MDCK cell lysate used for production of the HCP antibodies were used to create a standard curve (Fig 6). To increase the precision of the HCP assay, three calibration curves were run, (first, in the middle, and at the end of the measurement series) and the sample concentrations were determined from interpolated calibration curves. The recovery of the proteins in the standard curves was between 96% and 108% (Table 5).

The HCP assay was compared to the total protein assay described by Bradford (7). In the Bradford assay, BSA in concentrations ranging from 50 to 600 µg/mL were used as a reference as recommended by the manufacturer.

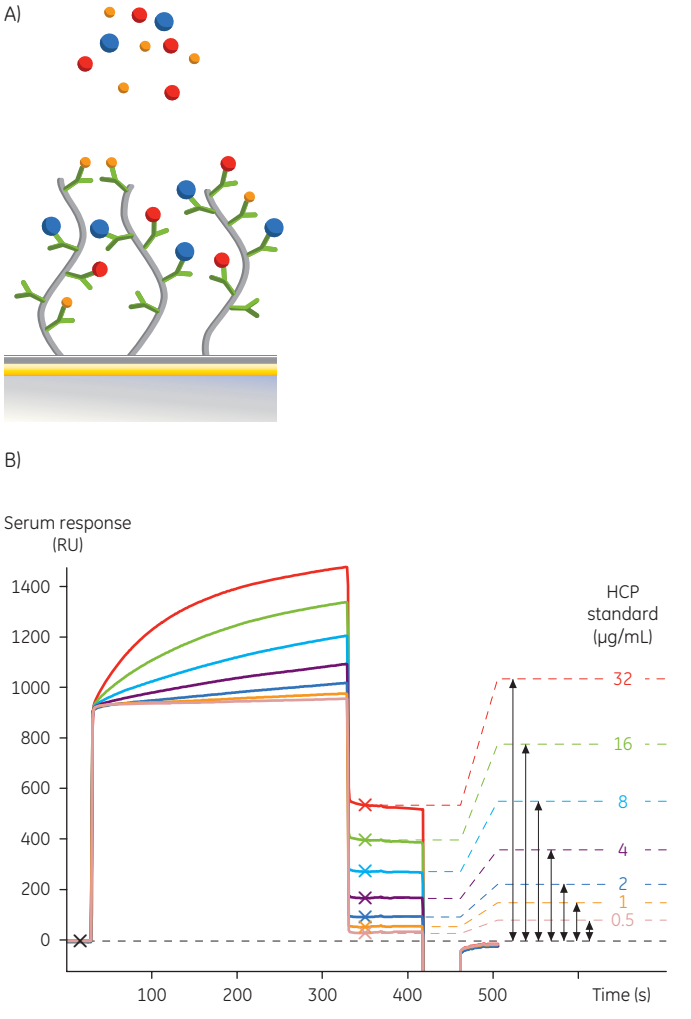

Fig 5. Direct binding assay principle **(A)** Anti-HCP antibodies were immobilized on the surface and HCP proteins in the sample bind directly to the antibodies. **(B)** Overlay plot showing sensorgrams of injected HCP standards. Report points (marked as X) were taken immediately before and after injection and the difference in level between each pair was measured (arrow). After each injection, the surface was regenerated in preparation for a new sample.

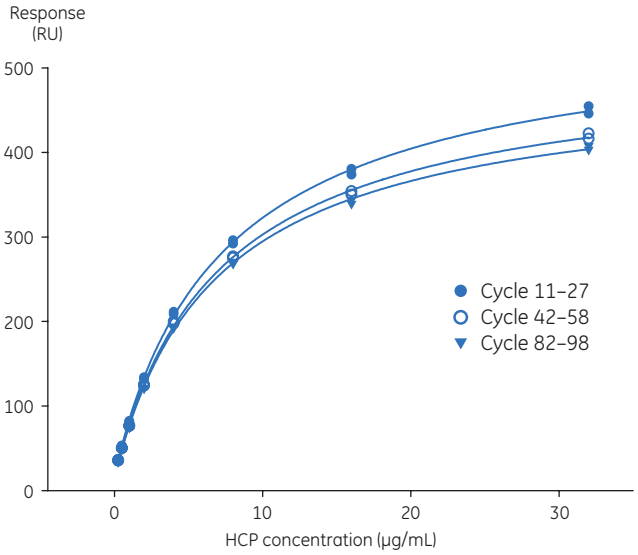

Fig 6. Standard curves for Biacore biosensor HCP assay with a concentration range of 0.25 to 32 µg/mL.

Table 5. The calculated concentrations and recovery from the Biacore biosensor HCP assay

Standard[1] HCP (µg/mL)	Biacore HCP (µg/mL)	CV (%) n=6	Recovery (%)
0.25	0.27	2	108
0.5	0.5	2	100
1	1.0	0.3	96
2	2.0	1	98
4	4.1	0.2	103
8	8.0	7	100
16	15.5	0.1	97
32	32.7	4	102

1) MDCK cell lysate

Samples from various steps of the purification process were quantitated with both Bradford protein assay (6) and Biacore biosensor HCP assay (Table 6). The samples were diluted so that at least two dilutions per sample reached a concentration within the standard curve range.

Samples analyzed by the Bradford protein assay show higher protein concentration values compared with analysis using Biacore biosensor HCP assay. This can be expected since Bradford detects the total protein content, including viral proteins, which are not detected by the Biacore biosensor HCP assay.

For the process samples, the Biacore biosensor HCP assay shows over 100 times higher sensitivity than Bradford protein assay, with a LOQ of 0.3 µg/mL compared with 60 µg/mL for Bradford (Table 6). With this LOQ, it is possible to quantitate a much wider range of samples in process development.

Table 6. Quantitation of HCP in samples from various steps of the purification process with Bradford protein and Biacore biosensor HCP assays

Sample type	Bradford protein assay (µg/mL)	CV (%) n=2	Biacore biosensor HCP assay (µg/mL)	CV (%) n=2
Harvest[1]	307	10	214	10
Normal-flow filtration step 1	267	8	100	5
Normal-flow filtration step 2	176	9	105	5
Ultra- and diafiltration step 1	<LOQ	na[2]	67	3
Ultra- and diafiltration step 2	<LOQ	na	28	20
Start[3]	358	30	274	na
Flowthrough 1	204	20	106	10
Eluate 1	<LOQ	na	46	4
Flowthrough 2	208	5	126	5
Eluate 2	<LOQ	na	48	5
Pooled flowthroughs	209	8	127	5

1) Supernatant from infected MDCK cell culture
2) na = not analyzed
3) Ultra- and diafiltrated virus in 10 mM sodium phosphate pH 7.4

Conclusions

Biacore biosensor assays allow significant improvement in vaccine development and manufacturing operations, as a replacement or complement to existing methodologies. The vaccine quantitation assay has higher sensitivity and considerably higher precision compared with the commonly used SRID assay, as well as significantly lower analysis and hands-on time. The HA concentration values obtained show high similarity between Biacore and SRID methods for different purification process samples, including samples from both human and bird influenza. Biacore biosensor assay is simple, does not need any sample pretreatment, and the HA concentration results are available within 24 h. In addition, the specific Biacore HCP assay has notably higher sensitivity compared with the total protein assay Bradford.

References

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Ordering information

Product	Code no.
Biacore T200 Processing Unit	28-9750-01
Series S Sensor Chip CM5	BR-1006-68
Amine Coupling Kit	BR-1000-50
HBS-EP+	BR-1006-69
Typhoon FLA 9000 Biomolecular Imager	28-9558-08
Image Quant TL	28-9236-62
Cy3 Ab Labeling Kit	PA33000
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