Research Article

Pharmaceutical BIOPROCESSING

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Automation of ELISAs & evaluation of emerging technologies for high-throughput quantitation of protein impurities

Background: Analysis of process-related protein impurities is critical to ensure process robustness and patient safety. Here, we report on automation of ELISA assays for quantitation of host cell protein and residual protein A. The automated assays were compared with three emerging technologies. **Results:** Data generated by the automated ELISA platform were comparable to manual results while the throughput was improved by three- to four-times and hands-on time reduced by six- to ten-times. The microfluidic assay enabled the broadest dynamic range and least sample consumption. The bead-based homogeneous assay was the least expensive. The automated ELISA platform demonstrated the highest throughput. **Conclusion:** Liquid-handler-based automation platform is determined to possess the maximum level of flexibility, adaptability and potential for improvement on assay throughput.

Keywords: automation • ELISA • emerging technologies • host cell protein • residual protein A

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Recombinant therapeutic proteins are produced from genetically modified bacterial, yeast or mammalian cell culture systems [1]. Residual host cell proteins (HCP) are therefore potential contaminants in the final drug product. These HCPs, even though often present in minute amounts in the final drug product, are always a concern for product safety [2] and there are regulatory recommendations to minimize HCPs to certain levels [3]. The guidelines for acceptable levels of HCPs by US FDA in 1997 were no more than 100 parts per million (ppm) in the final drug product [3]. Although reports of adverse effects due to HCP are rare, the clinical safety concerns still persist [1,2], making it essential to reduce HCP to the minimum level practical to prevent problems such as adverse immune reactions [4,5]. Reduction of HCPs, along with other impurities such as residual protein A ligand and residual host cell DNA, are achieved by downstream purification [1,6]. Clearance of HCP can serve as an indication of purification efficiency. It is therefore essential to analyze and monitor the intermediate samples during bioprocess development in order to guide development of a robust purification process and demonstrate clearance of impurities to an acceptable level [1,6].

The challenges of monitoring HCP impurities include their large population and variety, complex biological properties and wide concentration range of intermediate process samples. In order to achieve the dynamic range of an analytical method, it is often necessary to dilute samples at multiple dilutions to avoid multiple rounds of reanalysis [7,8]. On the other hand, the HCP levels in the final drug product are usually low, so the methods should therefore be very sensitive in order to detect low levels of HCP present in the final drug product [7,8].

Today, due to its high specificity and high sensitivity, ELISA remains the workhorse and gold standard for HCP quantitation [7,8]. However, ELISA assays usually have relatively high variability, high reagent and sample consumption, low throughput and lengthy assay time. In addition, due to the limited

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binding capacity on the microtiter plate, ELISA methods tend to have narrow dynamic range, and thus serial dilutions are required for most intermediate process samples to be measurable within the dynamic range of the methods. From serial dilution of samples and management of assay timing steps, to physically moving the assay plates between commonly used instruments such as plate washer and plate reader, manual ELISA assays are highly operator-dependent and require labor to be available at several fixed time intervals [7,8]. When many samples need to be processed routinely (e.g., from complex design of experiments studies in support of quality by design approach), manual operations are impractical and inefficient, which leads to a demand for automation [9,10]. However, automation of ELISA assays is often more complicated than a simple translation of manual pipetting steps; many factors of the liquid handling process and plate washer settings need to be optimized [11,12]. Proper validation is vital when converting a manual ELISA assay to an automated or semi-automated assay [11,12]. Therefore, seamless operation of robotics requires not only in-depth understanding of the underlying scientific principles of the analytical method, but also a high level of patience and time commitment in the process of script refinement and optimization.

To alleviate some of the abovementioned limitations with ELISA assays, in the past decade, several emerging technologies have been developed and proposed as viable alternatives to ELISA for HCP quantitation. This can be exemplified by Gyrolab™, AlphaLISA® and Octet®, each representing a different strategy for HCP quantitation. The Gyrolab platform is a fully automated system that employs microfluidics technology. Samples and reagents are mixed on a compact disc that consists of channels and microstructures for parallel sample processing [13,14]. Each microstructure contains a column packed with streptavidin-coated beads, which allows for affinity binding of proteins labeled with biotin. After passing the samples through the column, a fluorescently labeled detection antibody is used to monitor the amount of HCP bound to the capture reagent [13,14]. AlphaLISA is a homogeneous assay that employs the streptavidin-coated donor beads and antibody-conjugated AlphaLISA acceptor beads. In the presence of an analyte, the binding of the two antibodies to the analyte brings the donor beads and acceptor beads into proximity. Laser irradiation of donor beads at 680 nm generates a flow of singlet oxygen, triggering a cascade of chemical events in the nearby acceptor beads that leads to a chemiluminescent emission at 615 nm [14,15]. The Octet platform is based on bio-layer interferometry (BLI) technology. BLI monitors the interference pattern of white light reflected from two surfaces: a layer of immobilized anti-HCP antibody on the biosensor tip, and an internal reference layer. When dipped into the sample wells, changes in the number of HCP bound to the biosensor tip causes a shift in the interference pattern, which is used to determine the HCP levels [15].

Here, we report on the successful automation of the HCP ELISA and residual protein A ELISA assays on Tecan, a robotic liquid handling system. Data generated by the automated ELISA platform were comparable to those by the manual process while the assay throughput was improved by three- to five-times and hands-on time reduced by six- to ten-times. This automated ELISA platform was further compared with a microfluidic technology, a bead-based homogeneous assay, and BLI for HCP quantitation. The information presented will help laboratories in the pharmaceutical and biotechnology industries on selection, development and establishment of automation platforms for high-throughput analysis of immunoassays. In turn, automation will lead to the truncation of the development timeline for expedited delivery of therapeutic agents from the pipeline to clinical and market.

Experimental

Test samples

Test samples were either intermediate process samples or drug substance samples from different development projects at Bristol-Myers Squibb. Samples were analyzed neat or prediluted using the buffer recommended by the manufacturer to be within the assay's dynamic range.

Automation system & software

The robotic system used for ELISA assay automation was Freedom EVO® 200 from Tecan (Männedorf, Switzerland). It is composed of a robotic movement arm that is capable of robotic plate handling, an 8-channel liquid handling arm with disposable tips and a MultiChannel Arm™ with 96 channels. In addition, the Tecan is integrated with a HydroSpeed™ microplate washer (Tecan), an Infinite® M1000 PRO microplate reader (Tecan) and two 6-position tower plate incubators (Tecan) with shaking functions and temperature control.

The scripts that controlled the automation system were written using Freedom EVOware Standard software (Tecan). Communications with the plate washer, plate reader, and plate shaker can also be achieved through EVOware. The software that controls the HydroSpeed microplate washer is HydroControl™ (Tecan). The Infinite M1000 PRO microplate reader is controlled by i-control™ (Tecan) and Magellan™ (Tecan). ELISA data were collected, processed and exported using Magellan.

ELISA protocols

The Chinese Hamster Ovary (CHO) host cell protein third generation kit from Cygnus Technologies (NC, USA) was used to determine the HCP levels from test samples. The HCP standards were provided at 100, 40, 12, 3, 1 and 0 ng/ml. The Cygnus HCP ELISA assays were performed according to the manufacturer's protocol. In addition, two quality control solutions at 50 and 20 ng/ml were prepared and included on each plate. The protein A ELISA kits for the detection of MabSelect SuRe™ ligand from RepliGen (MA, USA) was used to determine the residual protein A levels from the test samples. The standard curve was composed of standards at 1.6, 0.8, 0.4, 0.2, 0.1 and 0.05 ng/ml. The residual protein A ELISA assays were performed according to the manufacturer's protocol. In addition, two quality control solutions at concentrations of 0.8 and 0.4 ng/ml were prepared and included on each plate.

During manual execution of an ELISA assay, all of the pipetting steps were performed using a handheld manual pipette. The plate incubation time was tracked manually. The ELISA plates were washed manually using the conditions recommended by the manufacturer. During each wash cycle, the wash buffer was pipetted into each well and the plate was inverted to decant. For shaking incubation during an HCP ELISA assay, the plate was incubated at 180 rpm using a bench top plate shaker (VWR, PA, USA). During automated execution of an ELISA assay, after the Tecan deck was set up according to a predefined deck layout, the assay was performed by the Tecan instrument without manual intervention (Supplementary Figure 1), including all sample dilutions, ELISA procedures, data capture, data calculation and data validity check.

ELISA data processing & reporting

The optical density of the plate was measured by an Infinite M1000 PRO microplate reader. The percentage recovery of standard solutions and quality control solutions was calculated as:

$$\frac{\text{Observed concentration}}{\text{Expected concentration}} \times 100\%$$

The average and percentage CV of each sample from serial dilutions were calculated. The spike recovery of samples was calculated as:

$$\frac{\text{Observed concentration - inherent concentration}}{\text{Spiked concentration}} \times 100\%$$

When comparing the automation results with manual results, the percentage difference was calculated as:

$$\frac{\text{Automation data - manual data}}{\text{Average of automation and manual data}} \times 100\%$$

An ELISA assay was deemed acceptable if the standard curve had a coefficient of determination (R^2) equal to or greater than 0.99; the percentage recoveries of the standard solutions were equal to or within ±20% of their expected values; the percentage recoveries of the quality control solutions were equal to or within ±20% of their expected values; and the CV percentage of replicate preparations for standards and quality control solutions were equal to or within 20%. A test sample result was considered valid when the determined impurity level was within the range of the standard curve and the sequential dilutions of the same sample had a percentage CV of less than or equal to 20%. If a spiked control sample was tested, the spike recovery must be within 80–120% of the expected values. If the sample results were higher than the highest point of the standard curve or failed to meet the dilution linearity criteria, the samples must be retested at a higher dilution. If the values determined from all dilutions were below the range of the standard curve and further dilution was not possible, the impurity level would be reported as < LOQ × dilution factor.

Quantitation of HCP levels by emerging technologies

The HCP levels from the test samples were determined using Gyrolab CHO-HCP kit 1 on a Gyrolab xPlore™ workstation (Gyros, Uppsala, Sweden), anti-CHO HCP detection kit on an Octet HTX system (Pall Fortebio, CA, USA) and AlphaLISA CHO HCP broad reactivity kit (Perkin Elmer, MA, USA), according to manufacturers' protocols.

Results & discussion

Automation of host cell proteins ELISA

Conversion of manual ELISA protocols into automation scripts

When adapting a manual ELISA assay on a liquid handler, modifications to the manual protocol are often required in order to fit liquid handler's configuration and robotic arm movement. For instance, the following modifications were made to the Cygnus HCP ELISA protocol when adapting this assay on liquid handler: the ELISA plate layout (Supplementary Figure 2) was modified to align with the direction of liquid handling arm movement. The plate sealer used in a manual ELISA method was replaced with a plate lid in an automated assay. Light-sensitive reagents such as tetramethyl benzidine (TMB) were stored in a reagent trough that was covered with a black film and a black lid. Robotic handling of the plate lid was performed by robotic movement arm.

Table 1. Validation of automated host cell proteins ELISA platform using Chinese hamster ovary host cell proteins standards.

HCP results (n = 5)	CHO HCP standard (ng/ml)†						
	100	80	60	40	20	12	3
Mean HCP values determined (ng/ml)	99	78	57	36	18	11	3
Mean percentage recovery	99	97	96	91	90	90	89
Intra-assay percentage RSD	2.3	1.9	6.9	6.0	5.9	7.9	8.5

CHO: Chinese hamster ovary; HCP: Host cell protein.

Adjustment of plate washer settings

Similar to automation of any analytical assays that involve liquid handling, adjustment and optimization of the liquid handling steps (e.g., aspiration/dispense speed and position) is required for each pipetting step of the assay [12]. In addition, optimization of the plate washer settings is critical for full automation of ELISA assays. Compared with manual plate wash procedures in which the residual liquid left after each wash cycle was emptied by tapping the plate inverted on paper

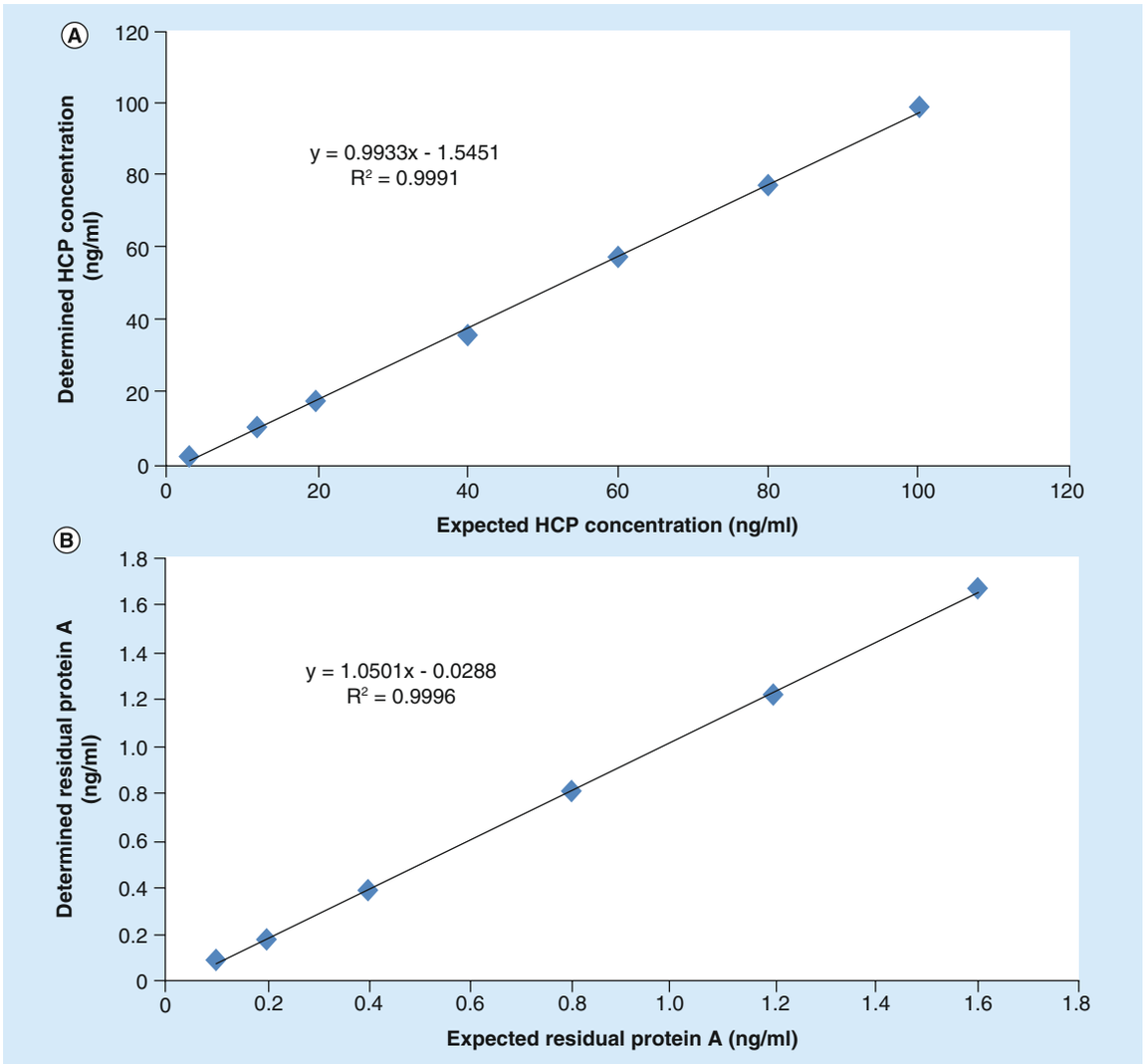

Figure 1. Linear regression analysis of expected versus determined concentrations for HCP and protein A. (A) HCP standards were treated as test samples; **(B)** protein A standards were treated as test samples; **(C)** results on HCP standards from 53 sets of HCP experiments were used; and (**D**) results on protein A standards from 24 sets of residual protein A experiments. HCP: Host cell proteins.

towel by an operator, the automatic plate wash steps fully relied on the plate washer to aspirate the residual liquid. Any remaining liquid left in the wells will result in nonuniform ELISA results.

To optimize the plate washer for effective aspiration, different wash head movement patterns and aspiration Z height positions were tested. A crosswise movement pattern, which involved movement of the aspiration needle from one side of the wall to the center of the well, was determined to be effective in aspirating the residual liquid. To determine the optimal aspiration Z height position of the wash head, sequential plates at various plunge depths were empirically tested. Aspiration at the deep end of the well resulted in well scratching and nonuniform results, presumably caused by the disturbance of the coated surface, whereas aspirating high up in the well left residual liquid behind. The optimal aspiration Z height settings for the same plate could be slightly different for different washers, and thus optimization of each plate washer is often required.

Flexible sample dilution

The HCP levels from the intermediate process samples often range from several million ppm in harvest to single digit ppm in final drug product. Due to the narrow dynamic range of ELISA methods, samples need to be diluted at multiple dilutions in order to achieve the dynamic range of the assay and avoid multiple rounds of reanalysis. These sample dilution steps are labor-intensive, time-consuming and error-prone during manual process.

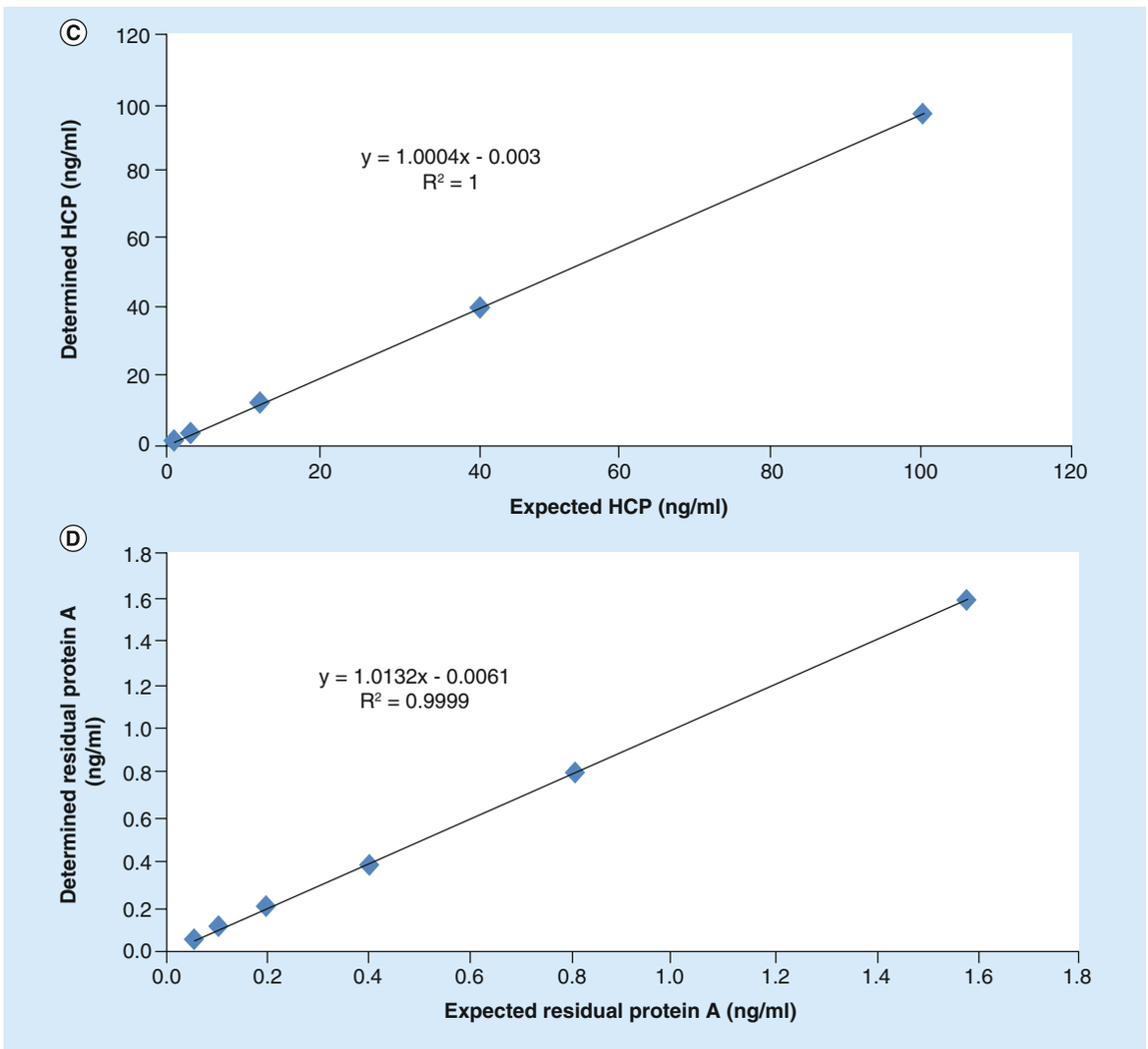

Figure 1. Linear regression analysis of expected versus determined concentrations for HCP and protein A (cont.). **(A)** HCP standards were treated as test samples; **(B)** protein A standards were treated as test samples; **(C)** results on HCP standards from 53 sets of HCP experiments were used; and (**D**) results on protein A standards from 24 sets of residual protein A experiments. HCP: Host cell proteins.

In this study, a flexible sample dilution scheme was developed for dilution of HCP samples. The scheme imported the dilution factors from an Excel spreadsheet into the script. The dilution factors were then transcribed into different pipetting volumes of the dilution buffer for each well of the dilution plate. This flexible dilution scheme allowed for different initial dilutions and serial dilutions to be applied to each individual sample. With the combination of the two dilution factors, this flexible dilution scheme provided a dilution range of 2–33,000-fold for each sample, broad enough to cover the HCP range of the vast majority of intermediate process samples.

Performance assessment of automated host cell proteins ELISA platform

The performance of the automated HCP ELISA platform was initially assessed by treating HCP standard solutions as test samples (Table 1) and tested following a plate map shown in Supplementary Figure 2A. Cygnus CHO HCP standards at 100, 80, 60, 40, 20, 12 and 3 ng/ml were serially diluted and tested. A total of five independent experiments were performed over five different days. As shown in Table 1, the percentage recovery for the standard solutions evaluated ranged from 89–99%. The intra-assay RSD ranged from 1.9–8.5%. In addition, the automated HCP ELISA platform demonstrated a linear response between the determined values and the expected HCP concentrations in the range of 3–100 ng/ml, with a slope of 0.9933 and an R^2 of 0.9991 (Figure 1).

Next, eight representative intermediate process samples from different purification steps over a broad range of HCP levels (~10 to ~10^6 ng/ml) were tested by manual process and the automated ELISA platform (Table 2). The percentage difference between the automation and manual results ranged from 0.1–25.0%. In addition, the manual and automated processes showed comparable level of percentage RSD between replicate preparations (Table 2).

To assess the accuracy of the automated ELISA platform, two drug substance samples (from two different biologics programs) spiked with 20 ng/ml of HCP standard were tested in parallel with their unspiked control samples using the automated ELISA platform. The plate setup for the spike recovery experiment is shown in Supplementary Figure 2B. Both the spiked (+) and unspiked control samples (-) were tested in quadruplicate. The spike recovery of the samples determined by the automated ELISA platform was 99%, indicating that the automated ELISA platform possessed great accuracy for HCP quantitation.

Parallel processing of multiple HCP ELISA plates

After the Cygnus HCP ELISA assay was automated for one plate per run, approaches for increasing the assay throughput was explored. One approach was to update the instrument to physically accommodate an increased number of samples and allow their simultaneous processing (e.g., accommodate 384-well ELISA plates instead of 96-well plates). The other approach was through parallelization, which allowed for multiple ELISA plates to be processed in parallel. In this study, due to the popularity of the 96-well microtiter plate format and unavailability of miniaturized plate format from Cygnus, the parallelization approach was used. However, it is to be emphasized that the former approach has the potential to become a stronger feature in high-throughput analytics and should be implemented whenever possible, such as in-house ELISA assays where different plate formats could be used.

The schedule for processing five ELISA plates in parallel is shown in Figure 2A. Generally, parallel processing of two ELISA plates manually is straightforward. However, as the number of plates increases, due to the more complex logistics, this parallelization becomes

Table 2. Comparison of automated and manual host cell proteins ELISA results.

Samples	Automation results (n = 10)		Manual results (n = 2)		% difference
	Mean HCP (ng/ml)	% RSD	Mean HCP (ng/ml)	% RSD	
HCP1	1040387	6.2	1059062	6.7	1.8
HCP2	30249	6.5	30285	5.8	0.1
HCP3	29880	5.5	28384	4.3	5.1
HCP4	20078	5.7	21213	2.6	5.5
HCP5	59	1.9	68	1.8	14.2
HCP6	9	5.9	7	8.1	25.0
HCP7	455	2.5	500	3.3	9.4
HCP8	729	4.7	773	1.7	5.9

HCP: Host cell protein.

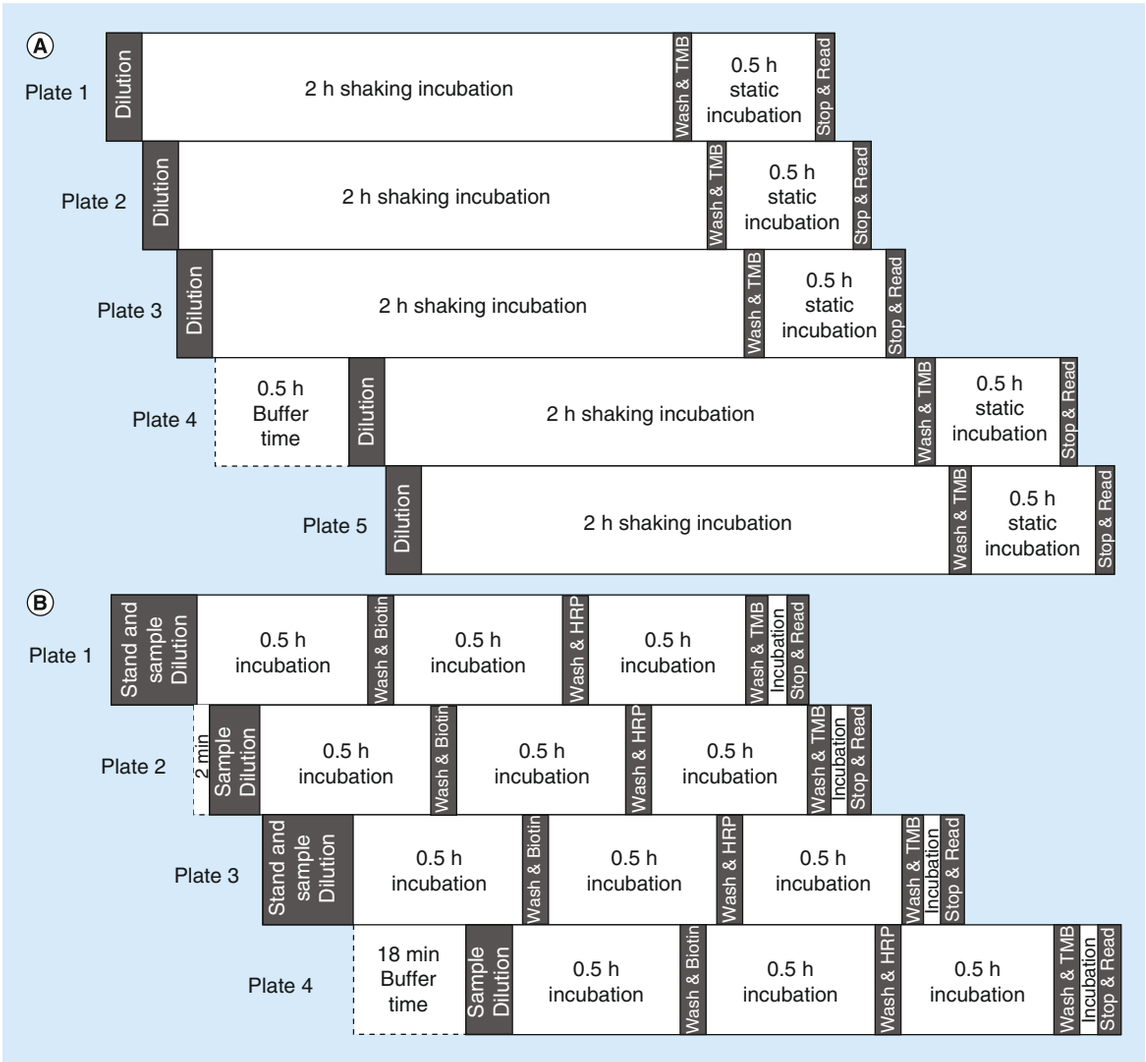

Figure 2. Schedule for parallel processing of multiple plates. Steps that do not involve Tecan movement are shown in white. Steps that require Tecan movement are shown in gray. **(A)** Parallel processing of five HCP ELISA plates. **(B)** Parallel processing of four residual protein A ELISA plates.
HCP: Host cell proteins.

less easily achievable. By writing this logistics into the script, the liquid handler performs the assay precisely according to the predefined schedule, allowing scientists to walk-away to perform other tasks. In this study, liquid handler performed additional operations during plate incubations. However, a 30 min buffer time was required to avoid conflict between the reading of plate III and the washing of plate IV. In addition, the timing for each step was precisely controlled by a series of timers written within the script. A total of five ELISA plates could be analyzed in approximately 4 h, a significant improvement on assay throughput compared with 2 h and 45 min for one plate. On a daily basis, the assay throughput of the automated platform was improved by approximately four-times and hands-on time reduced by approximately ten-times.

The plate-to-plate variability within a five-plate run was tested using eight representative intermediate samples from different purification steps. Each sample was tested in duplicate on each plate for a total of ten replicates. The plate-to-plate percentage RSD for the samples analyzed ranged from 1.9 to 6.5% (Figure 3). This is similar to the intra-assay variations determined when treating HCP standards as test samples (Table 1). This consistency between different plates within a multi-plate run was expected because each plate was handled exactly with the same conditions during the analysis, including reagent addition, incubation time, wash and shaking conditions, thereby reducing some of the inherent variability seen with manual ELISA assays.

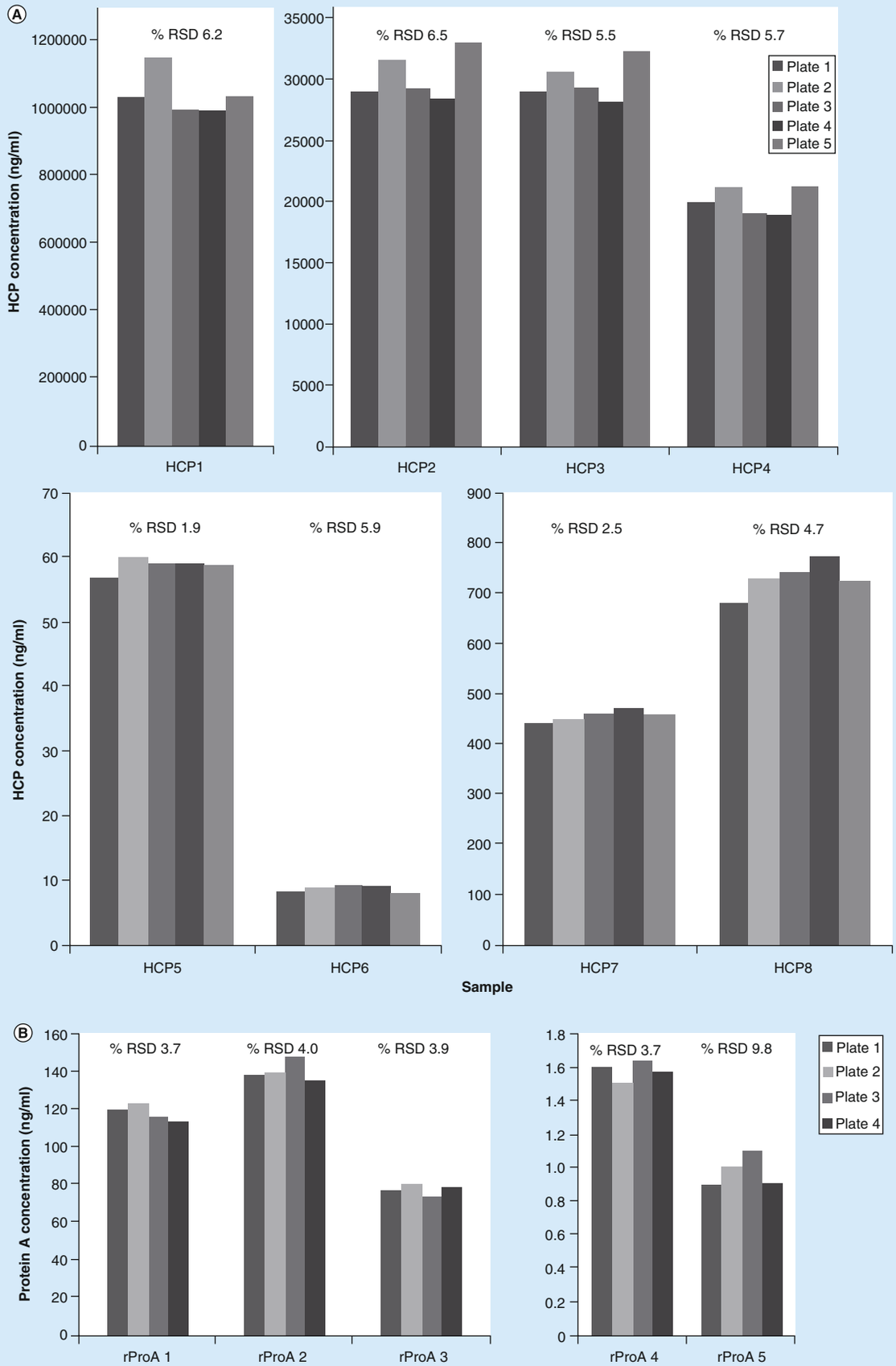

Figure 3. Plate-to-plate variations from a multiple plate run. (A) HCP ELISA and **(B)** residual protein A ELISA. HCP: Host cell proteins.

Table 3. Day-to-day variability of automated host cell protein ELISA platform (n = 53).

Day-to-day variability of HCP results	CHO HCP standard (ng/ml)					Quality control (ng/ml)	
	100	40	12	3	1	50	20
Mean HCP values determined (ng/ml)	100	40	12	3	1	52	20
Mean percentage recovery	100	100	100	101	98	108	101
Percentage RSD	0.1	0.1	0.4	3.4	7.0	4.0	4.3

CHO: Chinese hamster ovary; HCP: Host cell proteins.

Data trending for automated HCP ELISA platform

The day-to-day automation data from a total of 53 sets of independent experiments are summarized in Table 3. The percentage recovery of average standard concentration was in the range of 98–101% with an RSD of 0.1–7.0%. As expected, the variability of the results increased as the HCP level approached the low end of the dynamic range (1 ng/ml). The recovery of the 20 and 50 ng/ml quality control solutions were 101 and 108% with an RSD of 4.0 and 4.3%, respectively. In addition, the average of the HCP standard solutions from 53 sets of experiments showed a good linear response between the determined results versus the expected HCP concentrations in the range of 1–100 ng/ml, with a slope of 1.004 and R^2 of 1 (Figure 1C). Therefore, the automated HCP ELISA assay was determined to be suitable for routine support of process development, delivering high quality results in terms of accuracy, precision and linearity in the range of 1–100 ng/ml.

Automation of residual protein A ELISA

Besides HCP, one of the other commonly present process-related impurities is residual protein A. Protein A affinity chromatography is commonly utilized during downstream purification to facilitate the purification of monoclonal antibodies. While these resins provide a high capacity and selectivity for the target protein, trace amounts of the protein A ligands have been found to leach from the column, contaminating the final drug product. It is therefore necessary to monitor and demonstrate clearance of residual protein A ligand during bioprocess development [4]. Compared with Cygnus HCP ELISA method, the Repligen residual protein A ELISA method involves two additional cycles of plate wash, reagent addition and incubation, rendering challenges for parallelization of the assay. In addition, the last incubation step of the assay was as short as 4 min, which had led to data discrepancy as described below:

Troubleshooting during automation of residual protein A ELISA

The manual residual protein A ELISA method was converted into an automated assay format as described for HCP ELISA assay above in *'Conversion of manual ELISA protocols into automation scripts'*. However, when initially tested on liquid handler, the automated assay showed faulty reduced protein A responses across the microtiter plate (left to right). This issue was identified when the same protein A sample was tested at two different horizontal locations on the same ELISA plate. Moreover, the amount of residual protein A determined from the same sample went down along with serial dilutions. Initial investigations revealed a couple of possible causes for the abovementioned observations: First, the last incubation step prior to reading of the ELISA plates was as short as 4 min. This step involved addition of TMB solution into each well, incubation for 4 min, followed by addition of stop solution. Reagents were multidispensed into the plates by the single-channel dispenser. Given the short incubation time, the time difference in receiving the reagents between the initial column and the last column of the ELISA plate could be significant. After replacing the single-channel dispenser with multichannel dispenser, data discrepancy from different horizontal locations of the plate was then resolved (data not shown). To ensure data uniformity, all critical reagent addition steps were later performed by the multichannel dispenser. However, the issue with decreasing in protein A responses along with serial dilutions still existed after the replacement of reagent dispenser. It was then suspected that some of the protein A ligands were absorbed by the surfaces of 96-well plates during analysis, and as the samples became more diluted, the impact from the absorbance became more prominent and could not be neglected. This issue was circumvented by replacing the polypropylene dilution plate (from VWR) with a low-protein-binding plate (Eppendorf, Hauppauge, NY, USA). Consistent responses along with dilutions was then achieved (data not shown).

Performance assessment of automated residual protein A ELISA platform

Similar to Cygnus HCP ELISA, the initial platform evaluation was carried out by testing residual protein A standards as test samples. The residual protein A standards at 1.6, 0.8, 0.4, 0.2 and 0.1 ng/ml were serially diluted and tested on a liquid handler. A total of five independent experiments were performed over five dif-

Table 4. Validation of automated residual protein A ELISA platform using protein A standards.

Protein A results (n = 5)	Protein A standard (ng/ml)					
	1.6	1.2	0.8	0.4	0.2	0.1
Mean protein A values determined (ng/ml)	1.7	1.2	0.8	0.4	0.2	0.1
Mean percentage recovery	104	101	101	96	91	88
Intra-assay percentage RSD	3.4	8.8	3.8	7.3	2.5	13.5

ferent days (Table 4). The percentage recovery for the standard solutions evaluated ranged from 88–104%. The intra-assay RSD ranged from 2.5–13.5%. Data generated were closely correlated with the theoretical values (R^2 of 0.9996, slope 1.0501, Figure 1B).

Next, six intermediate process samples were tested by both the manual process and the automated residual protein A ELISA platform (Table 5). The percentage difference between the automation and manual results ranged from 1.5–13.7%. In addition, the manual and automated processes showed comparable level of percentage RSD between replicate preparations (Table 5).

Parallel processing of multiple residual protein A ELISA plates

Parallel processing of multiple ELISA plates on a liquid handler was developed for the residual protein A assay. Due to the additional reagents used for this assay, which occupied more deck space, up to four ELISA plates could be processed in parallel (Supplementary Figure 3B). The schedule for parallel processing of four assay plates is shown in Figure 2B. To avoid conflict and alleviate the time space issue, an initial buffer time was added prior to initiation of plate II and plate IV. Assay run time for processing four plates is approximately 4 h, which is a substantial improvement compare with 2 h and 15 min of processing time for one plate. On a daily basis, the assay throughput was improved by approximately three-times and hands-on time reduced by approximately six-times.

The platform was optimized and evaluated using a set of five representative intermediate samples from different purification steps. The samples used for evaluation covered a broad range of residual protein A impurity levels (~1 to ~150 ng/ml). Each sample was tested in duplicate on each plate for a total of eight replicates. As shown in Figure 3B, the plate-to-plate variations observed (3.7–9.8%) were comparable with the intra-assay variability (percentage RSD ranged from 3.4 to 13.5, Table 4).

Data trending for residual protein A ELISA automation platform

The day-to-day automation data from 24 sets of independent automated residual protein A experiments were summarized in Table 6. The recovery of the standard solutions was between 98 and 107%, with the RSD between 0.1 and 9.2%. The recovery of the 0.8 and 0.4 ng/ml quality control solutions was 103 and 107% with an RSD of 5.0 and 9.0%, respectively. In addition, the average values determined from 24 sets of independent experiments showed a linear response between the determined values and the theoretical protein A concentration in the range of 0.05–1.6 ng/ml with a slope of 1.0132 and an R^2 of 0.9999 (Figure 1D). The automated residual protein A assay was shown to be suitable for routine support of process development delivering high quality data.

Evaluation of emerging technologies for HCP quantitation

The automated ELISA platform was further compared with three emerging technologies, including microfluidic immunoassays of Gyrolab [13,16], wash-free AlphaLISA technology [15,17] and the biosensor-based technology of Octet [15], each representing a different strategy

Table 5. Comparison of automated and manual residual protein A ELISA results.

Samples	Automation data (n = 2)		Manual data (n = 2)		% difference
	Mean protein A (ng/ml)	% RSD	Mean protein A (ng/ml)	% RSD	
rProA 1	16.4	0.9	17.4	2.5	5.9
rProA 2	6.8	2.7	6.4	2.3	6.1
rProA 3	18.8	2.3	17.7	3.1	6.0
rProA 4	16.4	0.9	14.3	3.2	13.7
rProA 5	11.5	2.0	11.0	5.5	4.4
rProA 6	6.5	2.3	6.6	3.1	1.5

Table 6. Day-to-day variability of automated residual protein A ELISA assay (n = 24)

Protein A results (ng/ml)	Protein A standard (ng/ml)						Quality control (ng/ml)	
	1.6	0.8	0.4	0.2	0.1	0.05	0.8	0.4
Mean protein A (ng/ml)	1.6	0.8	0.4	0.2	0.1	0.05	0.8	0.4
Mean percentage recovery	100	101	98	100	107	100	103	107
Percentage RSD	0.1	0.6	2.0	2.5	5.6	9.2	5.0	9.0

for HCP quantitation. The assay throughput, run time, sample volume requirement, dynamic range, cost and data comparability with ELISA were evaluated.

Data Comparability between ELISA & emerging technologies on bioprocess samples

To compare the automated Cygnus HCP ELISA platform with three emerging technologies, bioprocess samples from two different monoclonal antibodies were analyzed. Each set contained samples from harvest, protein A column, a polishing step and the final drug substance. As shown in Figure 4, although the absolute HCP results differed, all four methods showed the same level of log reduction along with the bioprocess steps. This similarity on the HCP results determined was likely due to the same source of antibodies used by these technologies, which was the anti-CHO HCP antibody from Cygnus. In particular, Gyrolab and Octet (seven out of eight samples evaluated) showed the best correlation with ELISA. Three out of eight HCP samples analyzed by AlphaLISA had results much higher than ELISA. This overestimation of HCP levels by AlphaLISA was likely caused by increased potential for interference inherent to this assay format. Matrix effects in ELISAs are mainly caused by factors other than those intended to interact with the analyte of interest, including ionic strength, pH, cations, viscosity and proteins and so on [18]. Comparing with ELISA, Gyrolab and Octet, which involve several cycles of wash steps to remove unbound molecules and background signals, the test compounds in an AlphaLISA assay are present at the final readout step, causing increased potential for interference. In contrast, the 'dip and wash' steps were included during each separation cycle of Octet. Similarly, the microfluidics or 'flow through' principle of Gyrolab allows only a brief contact time between the capture antibody and sample, which reduces matrix effects and enhances the signal to noise ratio [13,19].

Comparison of assay dynamic range

Method dynamic range is one of the features that can affect the fit of analytical methods for high-throughput applications. The number of dilutions required during HCP analysis is dependent upon how the expected HCP level would fit into the dynamic range of the assay. Due to the limited binding capacity on the microtiter plate, ELISA methods tend to have narrow dynamic range. The dynamic range of Cygnus HCP ELISA is 1–100 ng/ml. In contrast, the dynamic range of Gyrolab is 1–8000 ng/ml, the broadest among the three technologies evaluated. This is followed by AlphaLISA (1–1000 ng/ml) and Octet (0.5–200 ng/ml). The broad dynamic range from Gyrolab is due to the streptavidin-coated bead columns used in their technology, which increases the surface area for interaction between HCP and antibodies [20]. Less sample dilutions are therefore needed to achieve the dynamic range of the method, which can shorten the testing time during analysis.

Comparison of assay throughput, run time, sample volume requirement & cost

The three emerging technologies/platforms were further compared with the automated HCP ELISA platform on assay run time and throughput (Table 7). Gyrolab™ xP workstation and Octet® HTX, which are models that offer the highest throughput from Gyros and Octet, respectively, were used for comparison. Both the automated ELISA platform and Gyrolab xP workstation allow for processing of multiple plates/compact discs (CDs), offering the maximum walk-away time. Both platforms also offer the highest throughput of up to 960 data points per day. In contrast, Octet HTX system can generate up to 96 data points at a time, but will require operator to feed the instrument during the day and thus not have quite as much walk-away time as the former systems. AlphaLISA can be automated on a liquid handler like Tecan, and once automated, the assay throughput and walk-away time will be increased.

When it comes to sample volume requirements, Gyrolab has the lowest sample volume requirement (Table 7), which is beneficial for high-throughput studies when sample volume limits the number of analyses per sample. As to the cost, on a per sample basis, AlphaLISA is the least expensive, and Octet is the most expensive due to consumption of both the HCP reagents and the biosensors. The automated ELISA platform and Gyrolab xP workstation were

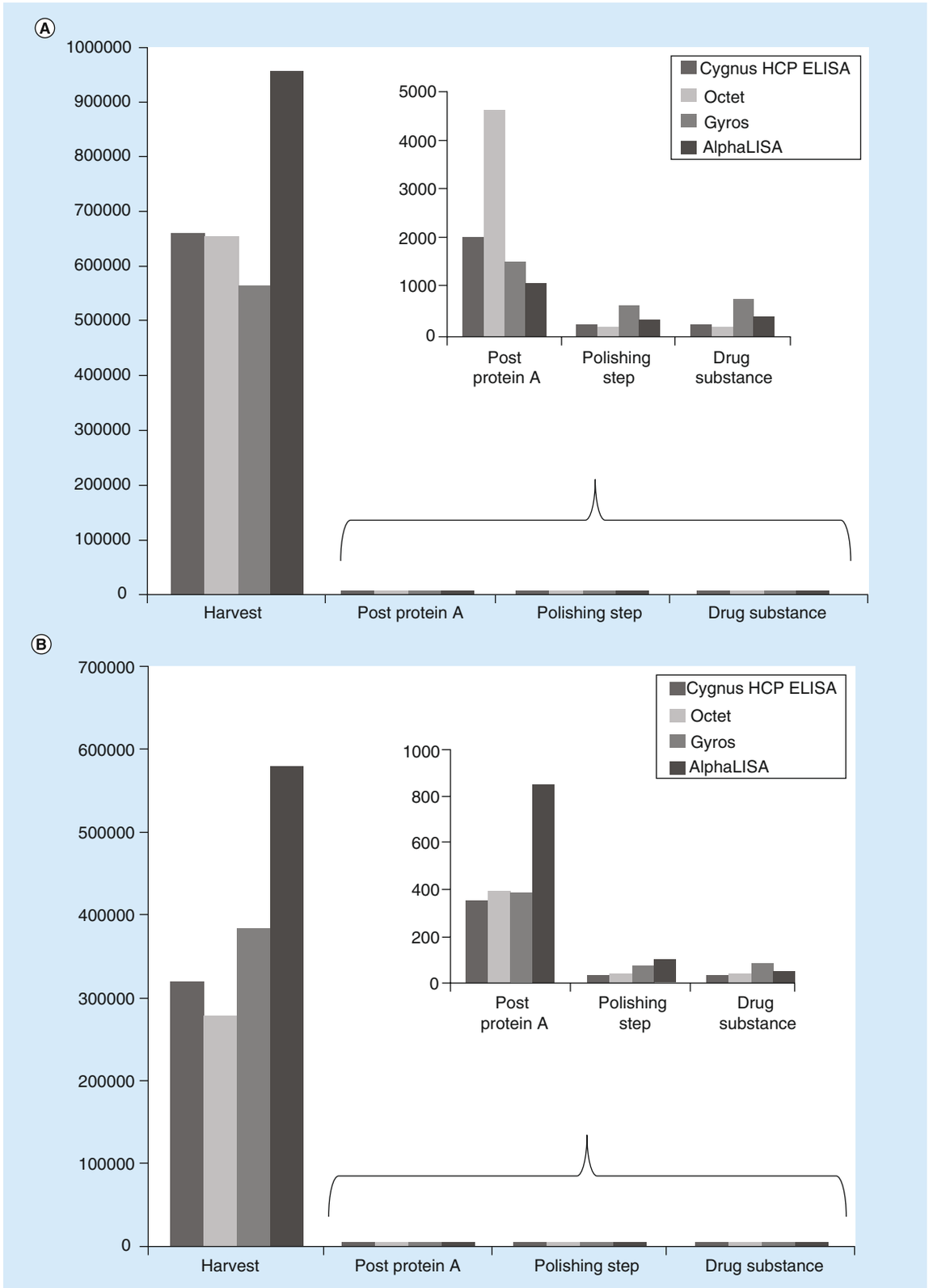

Figure 4. HCP quantitation by Cygnus HCP ELISA on Tecan, Gyrolab™, AlphaLISA® and Octet® for different mAbs.
(A) mAb1 and **(B)** mAb2
HCP: Host cell protein.

both approximately three-times more expensive than AlphaLISA (Table 7).

Comparison between liquid handlers & automated technology platforms

The technologies/platforms evaluated in this study can be generally divided into two groups: liquid handler robotics and commercial platforms. Systems like Gyrolab and Octet represent a group of commercial platforms developed by vendors that operate on unique and proprietary assay technologies. They are designed to complete all assay steps after sample dilution, including reagent addition, washes and data collection in an enclosed system. The biggest benefit of such platforms is no or minimal investment of time and resources in programming of these platforms prior to their operations. In addition, systems like Gyrolab and Octet are often multifunctional, and can be used for other applications such as protein A titer determination. However, sample preparations, which are often labor-intensive and time-consuming, still need to be performed outside of the platform. In order to achieve the maximum efficiency, partnering with a liquid handler is often required. In addition, these platforms are tied to one unique proprietary technology, making them not as flexible as the liquid handler robotics. The initial cost of some of these instruments may be prohibitively expensive. Furthermore, the use of dedicated instruments and assay systems can be under the risk that they can be withdrawn, changed or not supported at the whim of the provider, which can cause considerable problems for users.

The automated ELISA platform described in this study represents automation platforms based on liquid handlers. Liquid handlers can range from specialized bench-top 8-channel robot to customized-for-process automated liquid handling systems, such as the Tecan Freedom EVO and Janus from Perkin Elmer. Different liquid handlers (e.g., the ones from Tecan, Hamilton, Perkin Elmer or Beckman Coulter) differ in their flexibility, functionality, adaptability to other technologies and future expandability. With proper design, they can be set up to run complete ELISAs, including sample dilutions, reagent additions, washes and instrument reads, providing the precision needed for handling large number of sample dilutions and maximum walk-away time. Such platforms also provide the maximum flexibility and adaptability. In essence, any analytical assay that involves liquid handling steps can be automated to certain degrees on a liquid handler. As new technologies and platforms become available, these instruments can be integrated with liquid handlers. Strategic integration of parallelization and miniaturization approaches into the automation of ELISA assays can maximize assay throughput. For instance, by incorporating miniaturized plate format (e.g., 384-well plates) into the HCP ELISA described in this study, the throughput can be increased by fourfold, far exceeding the throughput of any automation platforms evaluated here. However, liquid handler robotics can be capital intense, especially when integrating with peripheral components such as plate readers and washers. Implementation of liquid handler robotics also requires sophisticated knowledge of the programming language and a significant amount of dedicated time.

Conclusion

While each technology has its pros and cons, liquid handler based automation platform is determined to possess the maximum level of flexibility and adaptability, making them indispensable for high throughput analytics. The commercial platforms such as GyrolabTM are attractive because programming is not required. This is balanced by the fact that this invest-

Table 7. Technology comparison.

HCP assay	Manual ELISA	Automated ELISA	Gyrolab™ xP	Octet® HTX	AlphaLISA®
Assay time (run)	4 h	4 h	5 h	1 h	1.5 h
Hands-on time (run)	3 h with frequent intermission	1 h setup	1 h setup	1 h setup	1.5 h with frequent intermission
Dynamic range (ng/ml)	1–100	1–100	1–8000	0.5–200	1–1000
Minimum sample required (µl)	50	50	8	40	20
Max throughput (run)	192 data points	480 data points	480 data points	96 data points	192 data points
Max throughput (day)	384 data points (based on two runs)	1344 data points (based on three runs)	960 data points (based on two runs)	672 data points (based on six runs)	576 data points (based on three runs)
Cost (data point)	2.5×	3×	2.5×	5×	1×

ment is a commitment to only one assay technology, and thus they are not as flexible as liquid handler based automation platforms. Whether to apply liquid handlers alone or in combination with commercial platform is a task of weighing disadvantages against the advantages by each organization.

Future perspective

The acceleration in bioprocess development has driven the demands for fast and cost–effective analytical tools. This requires us to continually examine both high- and low-end improvements so that analytics can deliver higher quality data faster. In the next 3–5 years, more and more laboratories will move away from manual preparation and embrace automation in order to stay competitive. Much like mechanization in the industrial revolution, nothing can block technological progress. For us in the rapidly developing pharmaceutical industry, robotics have already started operating in our laboratories to aid in developing new drug candidates and processes in a truncated time space. It is anticipated that the role of laboratory automation will continue to increase and will eventually be introduced into QC laboratories for drug substance release testing. In turn, automation will lead to the truncation of the development timeline for expedited delivery of therapeutic agents from the pipeline to clinical trials and commercial markets.

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Executive summary

Background

- Analysis of protein impurities such as host cell protein (HCP) and residual protein A during bioprocess development is critical to guide development of a robust purification process and demonstrates clearance of impurities to an acceptable level.
- Today, ELISA remains the workhorse and gold standard for HCP quantitation. However, ELISA assays usually have relatively high variability, high reagent and sample consumption, low throughput and lengthy assay time.

Automation of ELISA assays

- Optimization of the plate washer settings (wash head movement patterns, aspiration Z height positions and wash rate) is critical for full automation of ELISA assays.
- A flexible sample dilution scheme that allowed for different initial dilutions and serial dilutions to be applied to each individual sample was developed, providing a dilution range of 2–33,000-fold for each sample.
- Parallel processing of multiple ELISA plates significantly increased the assay throughput.
- The automated HCP ELISA assay was capable of delivering high quality results in terms of accuracy, precision and linearity.
- Data generated by the automated ELISA platform were comparable to those by the manual process while the assay throughput was improved by three to four-times and hands-on time reduced by six to ten-times.

Platform comparison

- Four assay platforms, automated ELISA, Gyrolab, AlphaLISA and Octet were compared using intermediate samples from a series of purification processes of two monoclonal antibodies.
- All four methods showed the same level of log reduction along with the bioprocess steps. Gyrolab and Octet showed the best correlation with ELISA results.
- An overestimation of HCP levels was observed for AlphaLISA, which was likely caused by increased potential for interference inherent to this assay format.
- Gyrolab enabled the broadest dynamic range and least sample consumption.
- AlphaLISA was the least expensive platform evaluated.
- The automated ELISA platform demonstrated the highest throughput.

Conclusion & future perspective

- While each technology has its pros and cons, liquidhandler-based automation platform is determined to possess the maximum level of flexibility, adaptability and potential for improvement on assay throughput.

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