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Automation of the mammalian CHO HCP ELISA using Beckman Coulter Biomek i7 Hybrid automated workstation

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Abstract

Chinese hamster ovary (CHO) cells are vital in the production of biotherapeutics. The process encompassing large scale synthesis of the therapeutics include culture of CHO cells to express recombinant biotherapeutic/drug product. The product, typically secreted into the host cell culture fluid (HCCF), undergoes multi-purification steps to eliminate flux of host impurities known as host cell proteins (HCP). This purification is a crucial step in the regulatory approval. Thus, various assays exist that quantify HCP concentration in a sample, where the gold standard is the HCP Enzyme-linked immunosorbent assay (HCP ELISA). HCP ELISA quantify the immunologic reaction between anti-HCP HRP antibody with HCP protein. Manual ELISA methods can benefit from refined automated protocols that can increase accuracy, rigor and throughput analysis.

The high deck capacity of the Biomek i7 hybrid automated workstation provides ample opportunities to automate the HCP ELISA protocol while allowing space to integrate useful devices for the ELISA (e.g., plate shakers, plate washers, and plate readers). The Mammalian CHO HCP ELISA 3G kit from Cygnus Technologies, Inc was automated on a Biomek i7 workstation. The monoclonal antibody producing cell line CHO-S cells were cultured for 198 hours, and HCCF was harvested at different time points. Automated ELISA was performed to quantify HCP in the HCCF and CHO-S cell lysates at baseline. Results were analyzed in comparison to manually acquired datasets. Despite a reduction in time to data acquisition in the automated experiments, there were striking similarities in the ELISA data acquired manually and through automation.

Introduction

The expression of a drug substance in vehicle cell lines provides unique and cost-effective means to generate large quantities of drugs for commercial application[1, 2]. One of the most commonly used vehicle cell line is the CHO cells, harvested from Chinese hamster ovary cells. A vital regulatory requirement of the manufacturing and purification processes is the capacity to demonstrate low to near zero levels of impurities by the host cell proteins (HCPs) from the CHO cells[3]. This is because these impurities can not only reduce the effectiveness of the drug substance but can also increase the unintended adverse effects/immunological reactions when applied to clinical subjects[4, 3]. Therefore, it is imperative to not only be able to reduce the contamination with impurities but to also have highly validated methods to ascertain the amount of such impurities in a given drug substance.

The conventional industry approach to establish the concentration of HCP in a sample is the ELISA method[1], which is a microtiter plate based immune enzymatic reaction that is semi-quantitative and provides opportunities for medium-to high-throughput analysis. The notion underlying a CHO assay is a two-site immune-enzymetric assay, where a CHO HCP containing sample is reacted to a horseradish peroxidase (HRP) enzyme labeled anti-CHO antibody in microtiter strips/plate **(Figure 1)**. These strips/plates are coated with an affinity purified capture anti-CHO antibody allowing for the immunological reaction that forms a sandwich-like complex of solid phase antibody-HCP-enzyme labeled antibody. Once the sandwich reaction has occurred, the microtiter strips can then be washed to remove

non-specifically bound or any unbound reactants. This is then followed by the addition of a substrate, tetramethylbenzidine (TMB), which hydrolyzes with the antibody-HCP-enzyme sandwich, where the amount of hydrolyzed substrate is proportional to the concentration of CHO HCP present in the sample[5]. The hydrolyzed product can thus be quantified using a microtiter plate to determine the unknown concentration of CHO HCPs present in a sample.

While ELISA methods can be highly sensitive and specific, their accuracy and reproducibility are dependent on the analyst experience and expertise[6]. Moreover, the multi-stage liquid transfer steps can also introduce pipetting errors that could yield differential data across large data sets. Developing automated CHO HCP ELISA procedures can eliminate the human error rate associated with ELISA protocols producing time efficient and highly reproducible results in a hands-free environment. The Beckman Coulter Biomek i7 hybrid liquid handling system **(Figure 2)** is uniquely suited for such automation, as it contains a large deck with ample space for direct integration of devices useful for the ELISA (e.g., plate shakers, plate washers, and plate readers). We performed an automated CHO-HCP ELISA on the Biomek i7 liquid handler using the mammalian ELISA 3G kit from Cygnus technologies Inc. Our results were highly reproducible and comparable to those acquired through manual experimentation and suggested a reduction in the total protocol time needed forfinal data acquisition.

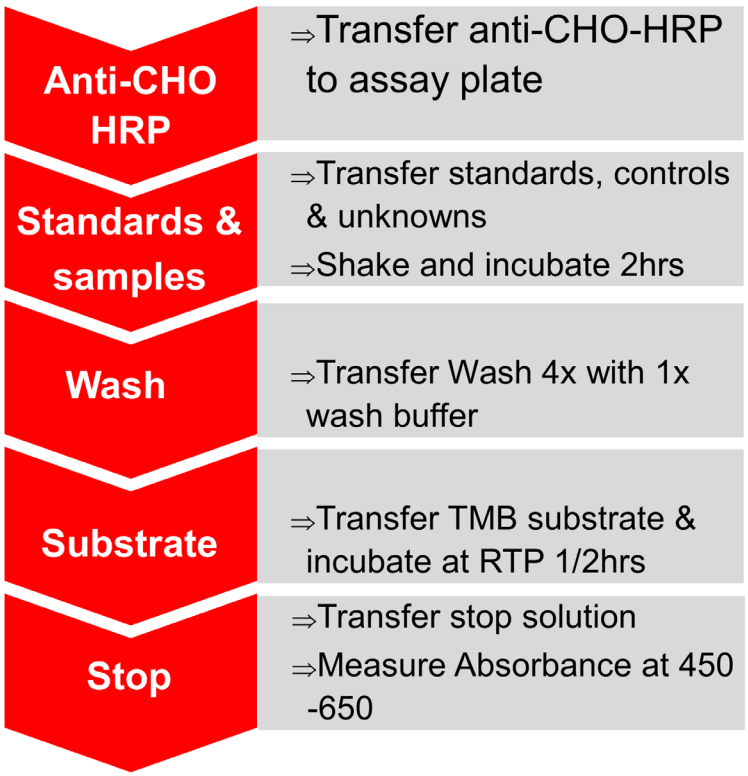

Figure 1. CHO HCP ELISA Workflow

Methods

ELISA

The ELISA manual experiments were performed following the recommended protocol of the mammalian CHO HCP ELISA 3G kit from Cygnus Technologies, Inc (Cat #F550-1). The kit was manually validated using the standards provided to generate a standard curve (Figure 3A). Additional validation was performed using a secondary standard, designed by spiking varying concentrations (0 – 1000 ng/mL) of the mammalian CHO HCP protein concentrate (Cygnus Cat# no. F553H) spiked into deionized water (diH_2O). All experiments were performed in triplicates. The concentration of protein from unknown samples was determined using the standard curve slope intercept equation.
Key equipment, reagents and resources used are listed in the material section. For automation, manual ELISA protocol was converted into Biomek methods using Biomek software.

Biomek methods

The automated experiments were performed by converting the liquid movement and transfer steps of the protocol **(Figure 1)** as recommended in the kit into Biomek methods using the Biomek i5 software (**Figure 2**-right panel). Minor adjustments and modifications were added to enhance the automated experiments. Biomek i7 instruments have a high-density deck that can accommodate enough labware to use fresh tips for each transfer (**Figure 2**-bottom panel) yet retain the flexibility to integrate devices useful for the ELISA such as shakers, plate washers, and plate readers. The current experiments were performed on a Biomek i7 containing an integrated orbital plate shaker and a SpectraMax i3x plate reader (Molecular Devices). Shaking and incubations were performed using an on-deck orbital shaker. The Biomek i5 software provides efficient ability to control liquid movement and transfer making, it possible to accommodate removal of all samples, reagents, and washes from the wells in the absence of a plate washer. Aspiration of the large volume (340 µL) of the wash buffer was followed by small volume (5 µL) aspirations on edge of the plate to ensure complete removal of media and automate the step in the manual protocol that requires inversion of the plate on absorbent pads.

CHO-S cell culture

CHO-S Cells (cGMP Banked) were ordered from Fisher Scientific (cat. No. A11557-01) and were cultured in CD CHO Medium (Fisher Scientific, Cat No. 10743-029) supplemented with 8mM L-Glutamine (Fisher Scientific, Cat. No A2916801). The cells were Incubated at 37°C in a humidified atmosphere of 5% CO_2 (VWR Scientific Products) in air on an orbital shaker (Orbi-shaker, Benchmark Scientific) platform rotating at 125 rpm. The culture flask caps were Loosened to allow for gas exchange. Cell confluency was measure at 48, 120, and 198 hours using Beckman Coulter Vi-CELL automated cell viability analyzer. Both HCCF and cell pellets were collected by centrifugation on an Allegra X-15R centrifuge (Beckman Coulter) and the protein was isolated from cell pellets using the mammalian M-PER protein extraction reagent from Thermo Scientific (Cat. No 78501).

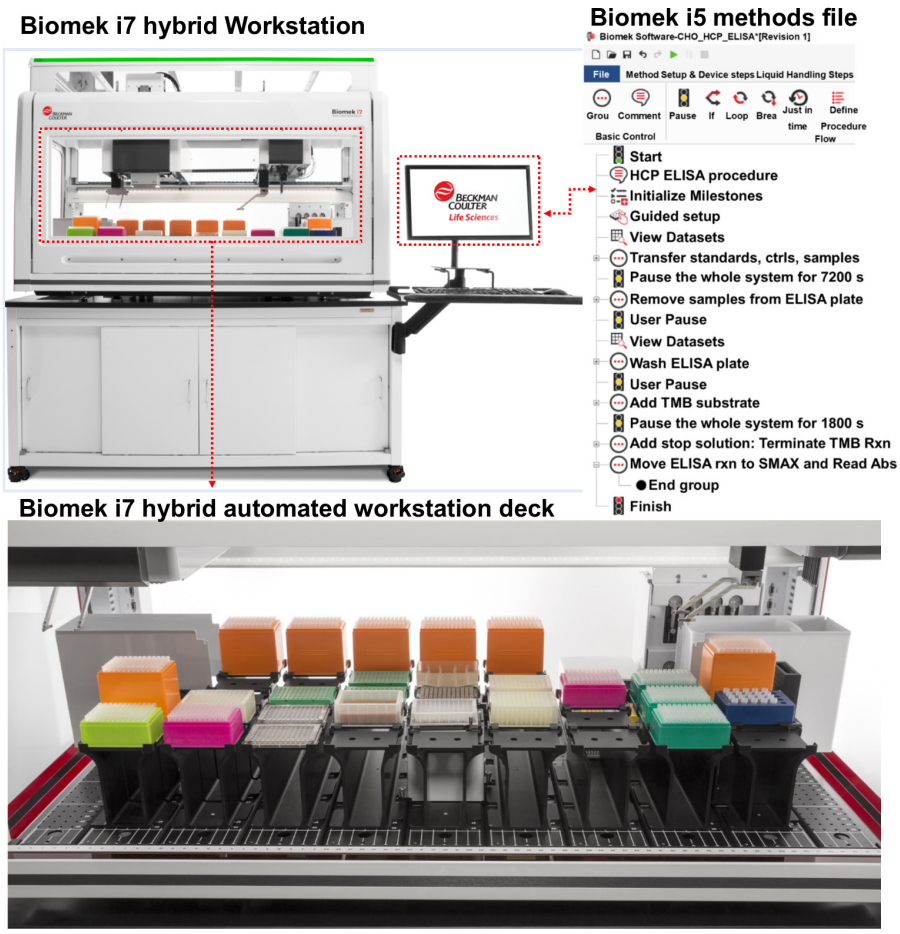

Figure 2. **Automation on Biomek i7 hybrid automated liquid handler.** Interaction between the workstation and the user is achieved using the Biomek methods written using Biomek i5 software.

Results

Standard curve analysis and kit validation

The kit was validated by two approaches using manual experimentation. First, we generated a standard curve using the standards provided in the kit (Figure 3A). We then generated secondary standards by spiking diH_2O with incremental concentrations of CHO HCP concentrate **(Figure 3B)**. The standard curves from both experiments had similar results that were consistent with manufacturer recommendations **(Figure 3A-B)**. The standards provided in the kit have concentrations of 0 ng/mL, 1 ng/mL, 3 ng/mL 12 ng/mL, 40 ng/mL and 100 ng/mL. We thus evaluated any detection threshold by designing new standards using spiked HCP concentrate protein that were 0 ng/mL, 0.1 ng/mL, 0.3 ng/mL, 1 ng/mL, 3 ng/mL 12 ng/mL, 40 ng/mL, 100 ng/mL and 1000 ng/mL in the spiked experiments. As expected, there was consistent incremental absorbance from the low to high concentration **(Figure 3C)** and no differences were observed when comparing the absorbance obtained from the kit standards and that of the spiked standards (Figure 3D). The total manual experimental time including the 2-hour 30 minutes incubation steps was 3 hours and 10 minutes for an 18-sample experiment.

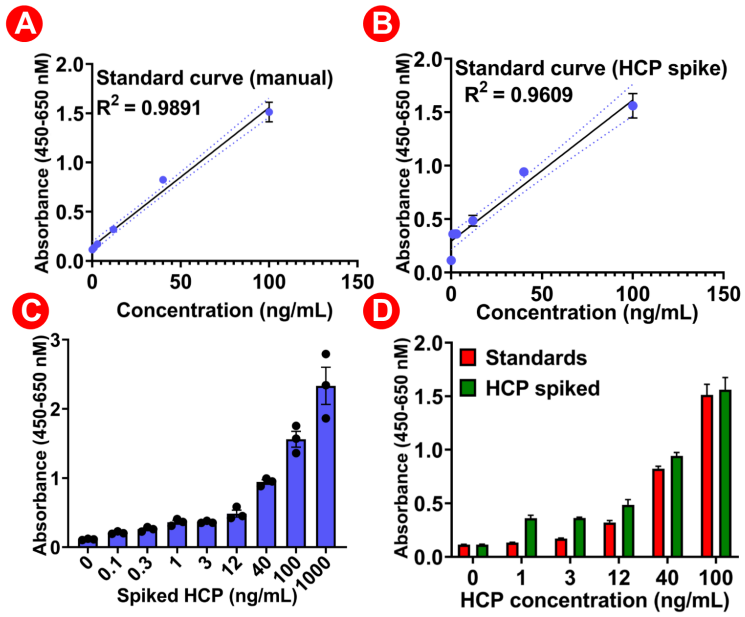

Figure 3. Manual standard curve analysis and kit validation A) Standard curve from kit standards **B)** Standard curve using spiked samples **C)** Absorbance versus spiked HCP concentrations (ng/mL). **D)** Comparison of absorbance from kit standards versus spiked standards. All experiments performed in triplicate and manually.

To automate the kit, we first performed the standard curve experiment on the Biomek i7 to determine consistency of the automated liquid transfer steps using the Span-8. There were consistent similarities between the manual standard curve (R^2, 0.9891), and the standard curve from data generated using Biomek (R^2, 0.9932) **(Figure 3A, 5A)**. The total estimated time for an 18-sample automated experimental was 2 hours 47 minutes including the 2-hour 30 minutes incubation steps. Compared to the manual run there was an estimated reduction in the automated experimental time by 23 minutes.

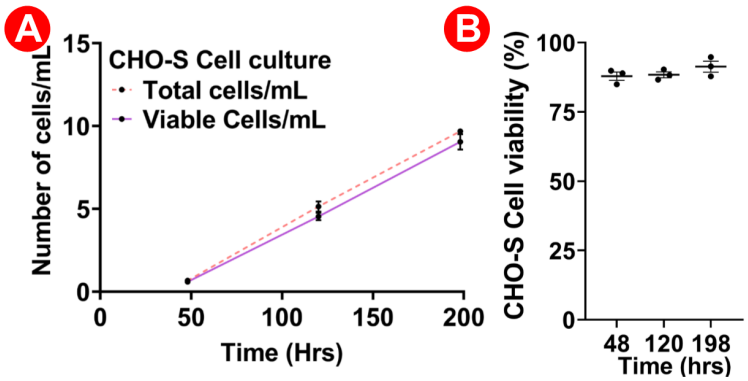

Figure 4. **CHO-S Cell culture and viability. A)** CHO-S cell proliferation over time. The number of cells (viable versus dead) at specific sample harvesting time points **B)** Percent viable cells in samples collected for time points analysis.

We thus designed a low-to medium-throughput experiment to measure the concentration of HCP in HCCF of cultured CHO-S cells and in protein lysates of CHO-S at baseline. Here CHO-S cells were cultured for 198 hours, and HCCF and cell pellets were collected at 48, 120, and 198-hour time points in triplicates. We determined that cell proliferation was consistent with previous reports [1] **(Figure 4A)** and that cells were healthy and viable (>85% cell viability) at each sample collection point **(Figure 4B)**. We collected HCCF and cell pellets using centrifugation and the sorted cell pellets were used to isolate CHO-S protein. Automated experiments were then performed on the Biomek i7 using the kit standards and unknown samples of HCCF and CHO-S protein lysate at 48, 120, 198-hours.

The total number of samples analyzed including standards, negative control (diH_2O), positive control (200ng CHO HCP concentrate), and HCCF and CHO-S protein lysates at specified time points was 42 samples, or approximately half of a 96 well plate assay. We observed high linearity in the standard curve (R^2, 0.9932, **Figure 5A**). Using extrapolation and slope intercept equation, the HCP concentration in the unknown samples was calculated **(Figure 5B)**. As expected, the negative control contained low to undetectable levels of CHO HCP **(Figure 5B)**. While 200 ng of CHO HCP concentrate was spiked into diH_2O and used as positive control, the calculated values of HCP measured approximately 200 ng in the positive control samples **(Figure 5B)**.

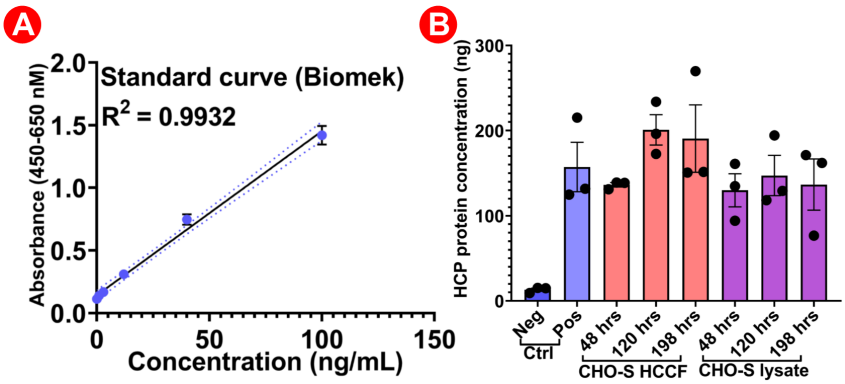

Figure 5. **Automated CHO HCP ELISA to measure unknown CHO protein in CHO-S samples**. **A)** CHO HCP standard curve results generated on Biomek i7 work station **B)** Quantification of CHO protein in CHO-S HCCF and protein from CHO-S cell lysate generated on the Biomek i7 workstation.

Our analysis suggested detectable levels of CHO HCP protein in both the CHO HCCF fluid and CHO protein lysates at all time points analyzed **(Figure 5B)**. However, we noted minimal variability in the amount of HCP protein at different time points. We speculate that this could be due to the use of healthy cells with low death rate and once that were at an early passage (P1-P3) without any transfected recombinant protein **(Figure 4A)**. Moreover, the kit lower limit detection (LOD) defined as the concentration corresponding to a signal two standard deviations above zero standard is 0.3 ng/mL. The lower limit of quantitation (LOQ), defined as the first dosed standard is 1 ng/mL. While there is no detection limit established as acceptable for HCP levels, <100 ng is considered for setting HCP specifications[7,8]. The estimated time to completion of the experiment was 2-hours, 59 minutes, 39 seconds for a 42-sample assay plate including the 2.5-hour incubation times needed in the protocol. Importantly, we evaluated similarities in the data acquired manually or though the Biomek automation protocol by comparing the absorbance from manual versus automated results. The datapoints were strikingly similar at all concentrations **(Figure 6)**, suggesting that automation of the protocol did not interfere with the experimental procedure, reagent stability and data acquisition.

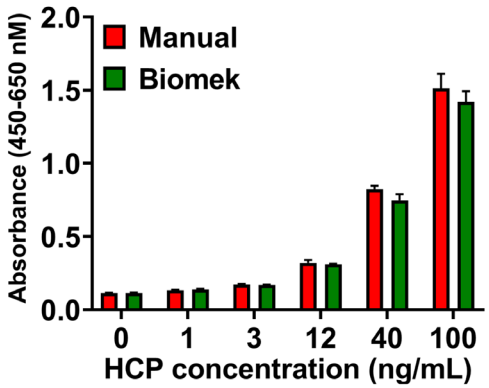

Figure 6. **Manual versus Automated standard curve absorbance data.** The standard curve data was strikingly similar when data was generated manually (red) or by automation (green)

Summary

The current automated method successfully demonstrated the automation of the mammalian CHO HCP ELISA assay using the Beckman Coulter Biomek i7 liquid handler. We demonstrated the 42-assay plate sample analysis containing triplicate standard curve samples, triplicate negative control, triplicate positive control, and 6 unknowns analyzed in triplicate for a total of 18 unknown samples. Total estimated time on the Biomek was approximately 3 hours with two incubation steps totaling 2 hours and 30 minutes. In a comparative 18 sample run between manual experiments and automated experiments, there was a decrease in assay time by 23 minutes. This suggests that automation could maintain not just the accuracy and reproducibility of the assay but can also lower time to data acquisition and analysis. The current method was performed using Span-8, however in high-throughput assays, the Biomek methods can be revised to use the multichannel head capable of transferring up to 1mL of liquid in a single transfer. This can be useful in the wash step that requires a bulk transfer of >300 µL volume per lane to accelerate the 4x wash steps by using the multi-dispense function. Labware integrations (e.g., plate shakers, plate washers, and plate readers) can contribute to throughput efficiency by decreasing interactions with the system to eradicate human error rate and increase walk-away time for the analyst. Moreover, the Biomek data acquisition and reporting tool (DART) is a software package designed to gather data and synthesize runtime information from Biomek log files capturing each manipulation of the sample during the Biomek method development. This eliminates time-consuming and error-prone "cut-and-paste" style management of sample data. DART seamlessly organizes data from Biomek liquid handlers, integrated lab equipment and benchtop devices into a single data source. Importantly, this automated method demonstrated that HCP ELISA automation cannot only produce highly reliable results from throughput analysis but can also decrease the protocol run time and increase analyst walk-away time.

References

1. Lesley M Chiverton et al, "Quantitative definition and monitoring of the host cell protein proteome using iTRAQ - a study of an industrial mAb producing CHO-S cell line," Biotechnology Journal, vol. 11, no. 8, pp. 1014-1024, 2016.
2. K. N. Valente, N. E. Levy, K. H. Lee, K. H. Lee and A. M. Lenhoff, "Applications of proteomic methods for CHO host cell protein characterization in biopharmaceutical manufacturing," Current Opinion in Biotechnology, vol. 53, no. , pp. 144-150, 2018.
3. H. S. Marzieh, T. . Bahman, H. . Shahin, Y. . Fereshteh, K. . Razieh, S. . Mohammad, N. . Mahdi and V. . Javad, "Increased the specificity and sensitivity of monospecific antibody against host cell protein (HCP) in quality control of hepatitis B recombinant vaccine," Journal of paramedical sciences, vol. 2, no. 3, pp. 24-29, 2011.
4. G. . Walsh, "Biopharmaceutical benchmarks 2014," Nature Biotechnology, vol. 32, no. 10, pp. 992-1000, 2014.
5. T. L. Martin, E. J. Mufson and M.-M. . Mesulam, "The light side of horseradish peroxidase histochemistry.," Journal of Histochemistry and Cytochemistry, vol. 32, no. 7, pp. 793-793, 1984.
6. G. . Rey and M. W. Wendeler, "Full automation and validation of a flexible ELISA platform for host cell protein and protein A impurity detection in biopharmaceuticals.," Journal of Pharmaceutical and Biomedical Analysis, vol. 70, no. , pp. 580-586, 2012.
7. W. R. M. H. S. Z. M. B. a. J. R. W. Haiyan Liu, "HCP Antigens and Antibodies from Different CHO Cell Lines," Bioprocess international, 2017.
8. R. F. C. M. S. Daniel G. Bracewell, "The future of host cell protein (HCP) identification during process development and manufacturing linked to a risk-based management for their control," Biotechnology and Bioengineering, vol. 112, p. 1727–1737, 2015.

Materials

Equipment	Manufacturer
Biomek i7 hybrid automated liquid handler	Beckman coulter Life Sciences
Spectramax i3x plate reader	Molecular Devices, INC
Orbital shaker	Benchmark Scientific, INC
Orbi-shaker	Benchmark Scientific, INC
37ºC cell culture incubator 7	VWR Scientific Products
Allegra X-15R centrifuge	Beckman Coulter Life Sciences

Reagents	Manufacturer	Part Number
CHO HCP ELISA 3G kit	Cygnus Technologies, INC	F550-1
Mammalian CHO HCP protein concentrate		F553H
CHO STM cells (cGMP banked)	Fisher Scientific, INC	A11557-01
CD CHOO Medium		10743-029
mammalian M-PER protein extraction reagent		78501

Reagents	Manufacturer	Part Number
BC90 Pipette Tips	Beckman Coulter Life Sciences	B85884
BC230 Pipette Tips		B85906
BC1070 Pipette Tips		B85945

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