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The Comparison of Chemiluminescent- and Colorimetric-detection Based ELISA for Chinese Hamster Ovary Host Cell Proteins Quantification in Biotherapeutics

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Abstract

Biologics manufacturing requires the clearance of Host Cell Proteins (HCPs) from recombinant therapeutic protein to acceptable low levels to ensure product purity and patient safety. To ensure adequate removal, a highly sensitive method, commonly in the form of Enzyme-Linked Immunosorbent Assay (ELISA), is necessary to quantify the HCPs amount in process intermediates and drug substance. We report the development of a chemiluminescent detection based ELISA (luminescent ELISA) in lieu of previously used colorimetric method (colorimetric ELISA) to improve assay sensitivity for the quantification of Chinese Hamster Ovary (CHO) HCPs in a monoclonal antibody product (mAb-A). For luminescent ELISA, Pierce Supersignal ELISA Femto was chosen as the substrate to replace colorimetric substrate TMB. The assay performance of luminescent and colorimetric ELISA was directly compared side-by-side. Our data show that luminescent ELISA has better signal/background ratio, broader linear range over logarithmic scales, and better linearity within the same linear range than colorimetric ELISA. Luminescent ELISA also demonstrates better low-end linearity, greater accuracy and precision. In addition, the Limit of Detection (LOD) and Limit of Quantification (LOQ) are significantly improved with luminescent ELISA as compared to colorimetric ELISA. In summary, luminescent ELISA is a more sensitive method and demonstrates superiority over colorimetric method for CHO HCP quantification.

Keywords: Host cell proteins; ELISA; Chemiluminescence; Chinese hamster ovary; Biotherapeutics; Monoclonal antibody

Introduction

Monoclonal antibodies (mAbs) have become a significant focus of the pharmaceutical industry due to their high specificity and their ability to engage a wide variety of targets [1,2]. While mAb therapeutics have been produced in a variety of genetically engineered host cell of non-human origin such as bacteria, yeast, plant, insect and mammalian cells, they are most commonly expressed in immortalized Chinese hamster ovary (CHO) cell lines [3-5]. CHO is a robust host that offers high productivity and glycosylation patterns similar to those found in endogenous human antibodies. Harvest of therapeutic antibodies of interest is relatively straight forward since the recombinant product is often secreted in the media. However, the harvest also contains significant amounts of proteins originated from the host, namely host cell proteins (HCPs), which are either secreted during fermentation or released into culture fluid as a result of cell lysis. Due to their non-human origin and thus potential immunogenic nature, HCPs can pose significant safety risk for patients and are part of process-related impurities that need to be controlled during bioprocess development [6-8]. Since after the purification steps, the residual HCPs amount in final drug substance is often very low in the parts per million (ppm) level, a highly specific, highly sensitive, and quantitative assay is desired to ensure their adequate removal and patient safety [9,10]. Due to its high specificity and sensivity, enzyme-linked immunosorbent assay (ELISA) is the most commonly accepted method by regulators for HCPs quantification [10]. Alternative immunospecific methods such as a quantitative slot blot assay [11] and solid-phase proximity ligation assay [12] as well as non-specific methods including mass spectrometry (MS) and 2D liquid chromatography (LC)-MS are also being developed or explored [8,10]. However, none of these methods are robust enough or can achieve the same level of sensitivity as ELISA, which remains the gold standard for HCP quantification. Commercially available HCP ELISA kits, commonly used as generic HCP assays in the early phase of development (Phase I/II) as well as previously reported late stage process-specific HCP ELISA often use colorimetric detection for signal generation [13,14]. Colorimetric detection limits the assay sensitivity for low levels of HCP especially in final drug product [13,15]. At Merck Research Laboratories, we have initially developed a process-specific colorimetric ELISA assay for one of our late stage CHO-produced monoclonal antibodies, mAb-A. Genetically engineered CHO cell line is used to manufacture mAb-A and thus a process-specific HCP ELISA using proprietary antibodies raised against the null CHO cells has been developed in-house for Phase III mAb-A to measure HCP components in the drug substance (DS).

The process-specific ELISA in its current format has a limit of quantification (LOQ) of 7.6 ng/ml in 5 mg/ml of drug substance (equivalent to 1.5 ppm). While this LOQ value is sufficient to demonstrate process clearance of HCPs, improvements can be made to increase the assay sensitivity to measure HCP concentration < 7.6 ng/ml. Since colorimetric detection limits the assay sensitivity for low levels of HCPs especially in the final drug susbtance, alternative method using chemiluminescent detection has been explored. Since its introduction in the late 1970s, chemiluminescence has been used in a variety of analytical and immunological tests such as high performance liquid chromatography [16], capillary electrophoresis

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[17], immunoassays and DNA analyses [18,19]. Analytical methods using chemiluminescent detection are often characterized by their high sensitivity, broader dynamic range, and high signal-to-noise ratio [19]. In immunoassays, chemiluminescent horse radish peroxidases (HRP) substrates have shown improved sensitivity over colorimetric substrates [20]. Commercially available Thermo Scientific Super Signal ELISA Femto Maximum Sensitivity substrate uses an improved enhancer system with much greater sensitivity and has been successfully used in high throughput enzyme immunoassay [21], antibody microarrays [22], and blood-based diagnostic assays [23]. With its known advantage of fast light generation, high sensitivity (1.7 pg/ml), and improved low-end linearity, the ELISA Femto substrate was adopted for assay development and its assay performance was compared side-by-side with 1-step turbo TMB (sensitivity 70 pg/ml) based colorimetric detection method. The signal/noise ratio, linear range and linearity over logarithmic scales, precision and accuracy as well as the limit of detection (LOD) and limit of quantification (LOQ) of both methods were assessed and compared following ICH guidelines-Q2 (R1).

Materials and Methods

Commercial reagents and consumables

Hyclone phosphate buffer saline (PBS, 10×), carbonate-bicarbonate buffer packet PK40, blocker BSA in PBS (10×), neutrAvidin-horseradish peroxidase (HRP) conjugate, 1-step Turbo TMB ELISA substrate, and Supersignal ELISA Femto Maximum Sensitivity substrate were purchased from Thermo Fisher Scientific Inc.(Waltham, MA, USA); Tween-20 was purchased from Sigma Aldrich (St. Louis, MO, USA); Costar EIA/RIA ELISA clear bottom 96-well plate was purchased from Corning Inc (Corning, NY, USA); NUNC white opaque 96-well plate was purchased from Thermo Fisher Scientific Inc. (Waltham, MA, USA). ImmunoWare tubes and ImmunoWare reagent reservoirs were product of Thermo Scientific Pierce Inc (Waltham, MA, USA).

Merck proprietary reagents

Anti-HCP polyclonal antibodies were raised in goat by Pocono Farms& Laboratory, Inc. (Tobyhanna, PA, USA) against CHO null cell culture (mock) that doesn't have the gene encoding mAb-A and the anti-sera was affinity purified by a self-prepared mock HCP affinity column. Affinity-purified goat anti-HCP IgG was then aliquoted and partially labeled with biotin using EZ-link Sulfo-NHS-LC-Biotin kit from Thermo Scientific Inc (Waltham, MA, USA). Unlabeled anti-CHO HCP antibody (lot# 68780/140, 1.69 mg/mL) was used as coating antibody and biotinylated goat anti-HCP IgG (lot# 68780/147, 1.69 mg/mL) was used as detecting antibody in a sandwich ELISA format (Figure 1). CHO HCP stock generated from mock cell fermentation was used as standard (5.70 mg/mL, lot# 68383/106). The reagents are stored at -20°C, with one working aliquot stored at 2-8°C.

Instruments

ELISA plate wash was done using a BioTek Elx 405 Select semi-automatic plate washer (BioTek USA,Winooski, VT), and the absorbance/luminescence signal was read by a Molecular Devices SpectraMax M5 plate reader (Molecular Devices, Sunnyvale, CA).

ELISA working solutions

Coating buffer was prepared by dissolving one packet of the carbonate-bicarbonate buffer concentrate in 500 ml of deionized water; washing solution was prepared by adding Tween-20 to 1× PBS to a final concentration of 0.1%; blocking solution was prepared by adding Tween-20 and 10× Pierce Blocker to PBS to a final solution containing 1% BSA and 0.05% Tween-20; assay diluents was prepared by adding Tween-20 and 10× Pierce Blocker to PBS to a final solution containing 0.1% BSA and 0.05% Tween-20.

Performing CHO colorimetric ELISA and luminescent ELISA in Corning Costar clear EIA/RIA 96-well plate

The CHO HCP ELISA was performed using established protocols for colorimetric detection with the adaption on the substrate addition step for chemiluminescent detection (Figure 1). For chemiluminescent ELISA, SuperSignal ELISA Femto substrate, instead of 1-step Turbo TMB substrate, was added to the appropriate wells and light emission was measured at 425 nm. Briefly, the ELISA plate was coated by 100 µL/well of coating antibody solution (1 µg/ml) prepared in carbonate-bicarbonate buffer and incubated at room temperature with gentle shaking for 2 hrs. After 4 washes with 300 µL/well washing buffer (10 second incubation/wash) using BioTek Elx 405 Select plate washer, the plate was blot dried using tissue paper and incubated with 300 µL/well of blocking solution at room temperature for 1 hr. After wash, the ELISA reactions were performed at room temperature with the addition of CHO HCP standards or unspiked/spiked mAb-A samples (100 µL/well), followed by the subsequent incubation with biotinylated anti-CHO HCP antibody (1 µg/mL, 100 µL/well), NeutrAvidin-HRP conjugate (1:15,000 dilution in assay diluents, 100 µL/well), and 1-step TMB turbo ELISA substrate (100 µL/well) or Supersignal ELISA Femto Maximum Sensitivity substrate (100 µL/well). Plate was washed 4 times with 300 µL/well washing buffer between each incubation steps. For those wells with TMB as a substrate, plate was incubated in dark for 10 min and the reactions were terminated by the addition of 100 µL/well of 1 M sulfuric acid, and then the absorbance of those wells were read at 450 nm using a Molecular Devices plate reader; for wells with ELISA Femto as a substrate, the light emission was measured at 425 nm 10 min after the addition of substrate using the same plate reader.

Performing CHO HCP colorimetric and luminescent ELISA in Nunc opaque 96-well plate

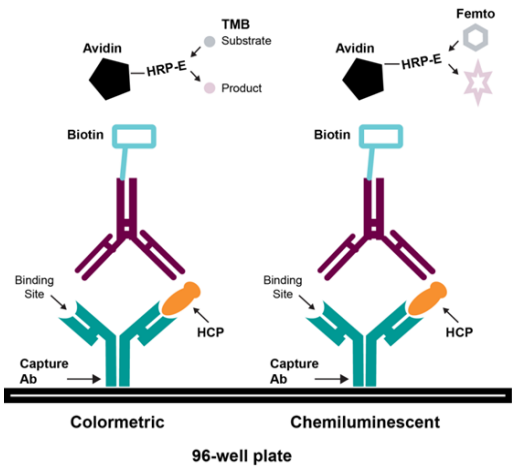

Figure 1: Schematic illustration of colorimetric and luminescent ELISA performed. Both ELISA methods are performed on the same 96-well plate and following the same procedure of HCPs capturing by coated anti-HCP antibodies, primary detection by biotinylated anti-HCP antibodies, and secondary detection by NeutrAvidin-HRP conjugates. The signal detection is either obtained by measuring absorbance at 450 nm generated from catalyzing TMB substrate (left) or by measuring the luminescence at 425 nm generated from oxidizing of ELISA Femto substrate (right).

To avoid luminescence signal cross-interference from adjacent wells, NUNC white opaque 96-well plate that provides maximum reflection and low cross-talk was chosen for luminescent ELISA development and optimization. The ELISA procedure follows the same steps of coating, washing, blocking, standards and samples incubation, primary detection (biotin-antibody conjugate), secondary detection (NeutrAvidin-HRP conjugate), and substrate incubation steps as described previously. The ELISA Femto substrate solution was prepared fresh on day of use by mixing equal volume of the signal enhancer with the Femto substrate. The steps and reagent volumes used in each step are summarized in Supplemental Table 1. For colorimetric assay, the reaction was stopped with the addition of 1 M sulfuric acid after 10 min incubation in TMB substrate, and then the reaction mixtures were transferred to a Costar clear 96-well plate using multi-channel pipette or and the absorbance of each well was measured at 450 nm using Molecular Devices SpectraMax microplate reader.

Standards and samples preparation for evaluating colorimetric and luminescent ELISA assay performance in Corning Costar 96-well clear plate

The ELISA assay performances including signal/noise ratio, dynamic range, low-end linearity, accuracy and precision were compared side-by-side using CHO HCP standard in triplicate prepared in a series of 3-fold dilution over the range from 2000 ng/ml to 0.034 ng/ml. Additional experiments were performed using standards in a series of 2-fold dilution ranging from 200 to 0.195 ng/ml or 50 to 0.049 ng/ml. Different amount of CHO HCPs (25 ng/ml, 5 ng/ml, 2 ng/ml and 1 ng/ml) were also spiked into mAb-A drug substance (final concentration 5 mg/ml) to assess the accuracy and precision of each assay.

Standards and samples preparation for evaluating colorimetric ELISA and luminescent ELISA performance in NUNC white opaque 96-well plate

The ELISA assay performances including signal/noise ratio, the assay linearity, accuracy, precision, LLOD and LLOQ were compared over the standards range from 1.56 ng/ml to 100 ng/ml using the same amount of reagents (Supplemental Table 1) optimized for luminescent ELISA in NUNC white opaque plate.

Data analysis

All experiments were done in triplicate. The comparison of colorimetric and luminescent ELISA was performed side-by-side in three repeated experiments with slightly variation on standard range. The data analysis was performed using Molecular Devices Soft max Pro v 5.3 software and Microsoft Excel. The chemiluminescence light emission at 425 nm (E425) or absorbance values at 450 nm (A450) are plotted against standard concentrations using the software's built in 4-parameter fit, linear fit, or log-log curve fitting. The signal/noise ratio of each standard data point is determined using the signal generated by a standard at a given concentration to that of concentration 0 (blank). The linear range over logarithmic scales is determined as where the correlation coefficient (R2) has a value >0.99. The concentrations of standards are then back-calculated from the standard curve fit equation to assess the precision (CV%) and accuracy (recovery%) of the assay. In all conditions, the same curve-fitting method is applied for both colorimetric and luminescent ELISA for direct comparison. The recovery% of standards is calculated as the ratio of back-calculated concentration to the expected concentration × 100%.

Results and Discussion

The assay performance of luminescent ELISA and colorimetric ELISA was directly compared by a variety of assay parameters such as signal/noise ratio, linear range and linearity over logarithmic scales, accuracy and precision, lower limit of detection (LLOD) and quantification (LLOQ). To reduce the variations caused by assay plate type, the comparison of assay performance was carried out in both 96-well clear plate and opaque plate. Several standard curve-fitting methods built in the Softmax Pro v5.4.1 were applied to determine the optimum method for HCP quantification.

Assay performance comparison for colorimetric ELISA and luminescent ELISA in Corning Costar clear 96-well plate using conditions optimized for colorimetric ELISA

ELISA assays using 1-step Turbo TMB (colorimetric) or ELISA Femto (luminescent) as a substrate for CHO HCP testing were performed side-by-side on the same 96-well Costar clear EIA/RIA plate following the procedure described in materials and methods. The standard curves were first fit using a 4-parameter non-linear regression. As seen in Figure 2A, colorimetric ELISA standard curve (bottom) displayed a sigmoid shape with the absorbance value at 2000 ng/ml reaching a plateau, in contrast, luminescent ELISA standard curve at 2000 ng/ml remains in the rising phase (top). The C values

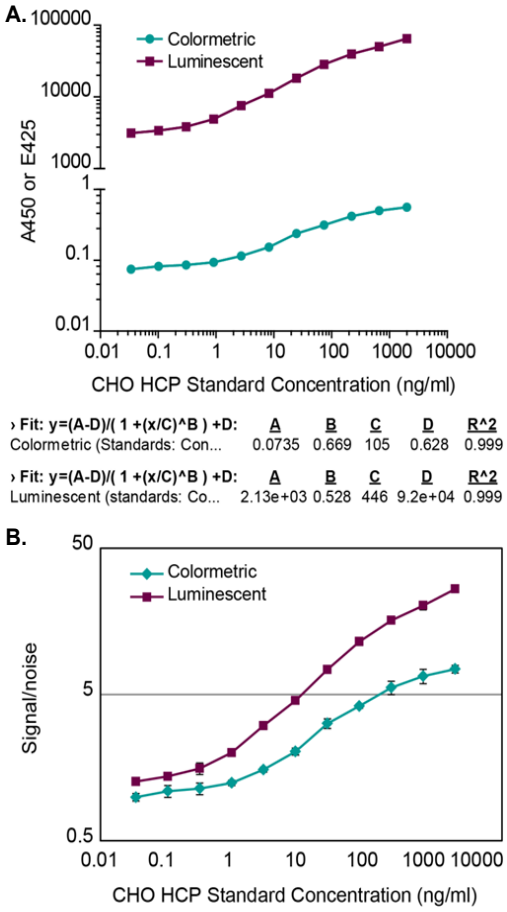

Figure 2: Standard curves from colorimetric and luminescent ELISA performed in clear 96-well plate. A. Absorbance values (A450) or luminescence emissions at 425 nm (E425) were plotted against standard concentrations on log-log scales and the curve equations were fit using 4-parameter non-linear regression. B. Signal/noise ratio values plotted against CHO HCP standard concentration over logarithmic scales.

from the 4-parameter fit standard curve equation of colorimetric and luminescent ELISA, which represent the standard concentrations where the signal response is ~½ of the maximum signal response, are 105 and 446 respectively, indicating that luminescent detection has a much broader range. To assist the direct comparison between the two detection methods, relative signal response or signal/noise ratio of each standard was also plotted against its concentration on logarithmic scales (Figure 2B). Luminescent detection showed significant higher signal/noise ratio at all standard concentrations and its standard curve has steeper slope than colorimetric method. As shown in Table 1, the absorbance values at 450 nm (A450) generated by colorimetric method ranged from 0.075 to 0.561 at standard concentrations from 0 to 2000 ng/ml; in contrast, light emissions (425 nm) from luminescent method have a reading from 2461 to 64780 at the same standard range. At 2000 ng/ml standard concentration, luminescent ELISA has a signal/noise ratio of 26.32, which is equivalent to 3.5 fold of the S/N for colorimetric ELISA (7.467, Table 1). The mean S/N for luminescent detection method (8.677 ± 8.736) is also significantly higher than the mean S/N for colorimetric method (3.191 ± 2.348) as analyzed by paired t-test (p=0.017, n=11, Table 1).

To accurately quantify the HCPs in drug substance, the ELISA standard curves need to be fit using the appropriate mathematical models. Several models are available in SoftMax software that include linear, semi-log, log-log, 4-parameter and 5-parameter fit. The method used to form the calibration curve dictates the working range and overall accuracy of the assay [24]. For broad range CHO HCP standard curves as shown in Figure 2, 4-parameter non-linear regression fit gave the best correlation coefficient of R^2=0.999. Using the 4-parameter fit equation, the concentration of each CHO HCP standard was back-calculated according to its corresponding A450 or E425 value. The precision and accuracy of the back-calculated concentration was demonstrated by its relative standard deviation calculated from triplicate (CV%) and the recovery% (the ratio of back-calculated concentration to the expected concentration) (Table 2). Using CV% ≤ 30 and 70 ≤ Recovery% ≤ 130 as acceptance criteria for accuracy and precision, luminescent ELISA has a working/dynamic range from 0.914 ng/ml to 666.7 ng/ml, which is 27 fold of that for colorimetric ELISA (2.743 ng/ml to 74.07 ng/ml, Table 2). In addition, we assessed the linearity of both colorimetric and luminescent ELISA at various standard concentration ranges using several mathematic models and determined their linear range with a log-log fit curve. For luminescent detection, the linear range over logarithmic scales is 0.31-2000 ng/ml (R^2=0.991), 27 fold of that for colorimetric method (0.91-222.22 ng/ml, R^2=0.992). Moreover, at the 0.91-222.22 ng/ml standard range, the R^2 of luminescent detection for log-log linear fit is 0.998, showing better linearity than colorimetric method at the same range (R^2=0.992, Figure 3). The better linearity within the same concentration range was also observed in experiment covering the standard range of 0.781-200 ng/ml, where luminescent

CHO HCP Standard (ng/ml)	Colorimetric ELISA A450	Luminescent ELISA E425	Colorimetric ELISA S/N	Luminescent ELISA S/N
0	0.075 ± 0.003	2461 ± 265.3	1	1
0.034	0.075 ± 0.004	3124 ± 140.6	0.993	1.269
0.102	0.082 ± 0.008	3389 ± 151.6	1.091	1.377
0.305	0.086 ± 0.008	3842 ± 351.9	1.139	1.561
0.914	0.093 ± 0.004	4924 ± 216.0	1.244	2.001
2.743	0.115 ± 0.004	7537 ± 432.9	1.533	3.063
8.23	0.153 ± 0.008	11179 ± 536.5	2.039	4.543
24.69	0.238 ± 0.018	18228 ± 335.9	3.17	7.407
74.07	0.313 ± 0.011	28313 ± 1656	4.17	11.51
222.2	0.419 ± 0.044	39576 ± 1913	5.577	16.08
666.7	0.502 ± 0.056	49976 ± 3293	6.683	20.31
2000	0.561 ± 0.034	64780 ± 3319	7.467	26.32
Mean			3.191	8.677
Stdev			2.418	8.736
P value (paired t-test on mean S/N, n=11)				0.017

Table 1: Comparison of the signal/noise (S/N) ratios of CHO HCP standards from colorimetric ELISA and luminescent ELISA performed in Corning Costar clear 96-well plate (n=3 for each standard concentration).

CHO HCP standard (ng/ml)	Colorimetric ELISA		Luminescent ELISA	
	%CV	%Rec	%CV	%Rec
2000	59.97	137.66	33.69	113.15[b]
666.7[l]	105.06	160.25	26.93[a]	87.78[b]
222.2[l]	51.35	111.11[b]	16.72[a]	106.95[b]
74.07[l, c]	11.90[a]	94.67[b]	16.75[a]	112.54[b]
24.69[l, c]	21.87[a]	118.46[b]	4.86[a]	100.98[b]
8.230[l, c]	17.90[a]	88.77[b]	12.54[a]	85.78[b]
2.743[l, c]	18.16[a]	89.84[b]	15.76[a]	89.39[b]
0.914[l]	31.8	84.94[b]	15.29[a]	72.43[b]
0.305	93.76	129.78[b]	37.1	85.82[b]
0.102	125.17	247.84	22.16[a]	140.18
0.034	57.38	171.49	27.08[a]	267.85
0	75.77		107.53	

Table 2: Comparison of the precision (%CV) and accuracy (%Rec) of back-calculated concentration using 4-parameter fit for colorimetric and luminescent ELISA performed in Corning Costar clear 96-well plate.

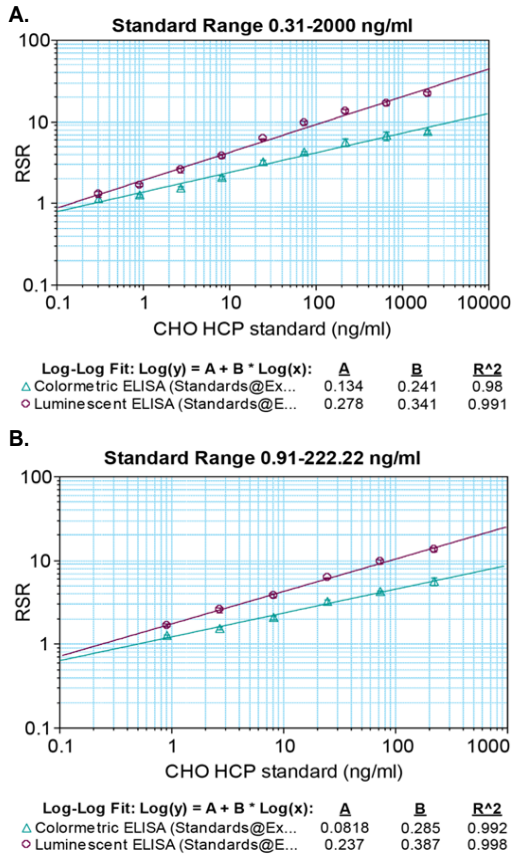

Figure 3: The linear range and linearity comparison over logarithmic scales between colorimetric ELISA and luminescent ELISA. A. Log-log linearity comparison over the range of 0.31-2000 ng/ml. B. Log-log linearity comparison over the range of 0.91-222.22 ng/ml.

method has a correlation coefficient of R=0.999 for log-log linear fit curve, as compared to R^2=0.994 for colorimetric method. In addition to the broader log-log fit linear range and better linearity within the same log-log fit range, luminescent detection also showed better low-end linearity at standard concentrations from 0.034 to 2.743 ng/ml, where the linear correlation has a R^2=0.992 in comparison to R^2=0.961 for the colorimetric ELISA (Figure 4). Using the low-end linear curve, the concentration of CHO HCPs at 0.914 ng/ml can be more precisely determined by luminescent ELISA, but not by colorimetric method (Supplemental Table 2).

Within the log-log linear range of 0.91-222.22 ng/ml, the accuracy and precision of both ELISAs were compared. Our results show that within this range luminescent method is significantly more precise (CV%, p=0.03, single tail t-test) than colorimetric method (Table 3). Using the log-log fit curves within this range to back-calculate standard concentrations from 0.03 to 2000 ng/ml, all standard concentrations exhibited comparable or better CV and recovery values than colorimetric method, which also holds true when back-calculating HCP concentration using 4-parameter fit (data not shown). Despite the standard curve fit methods, luminescent ELISA always has smaller CV values (~1/2) than colorimetric method. However, when using log-log fit curve to determine the concentration within this range, luminescent method also exhibit better accuracy (100.65 ± 9.12% recovery) than colorimetric method (102.66 ± 18.77% recovery), although not statistically different (p=0.41, single tail t-test).

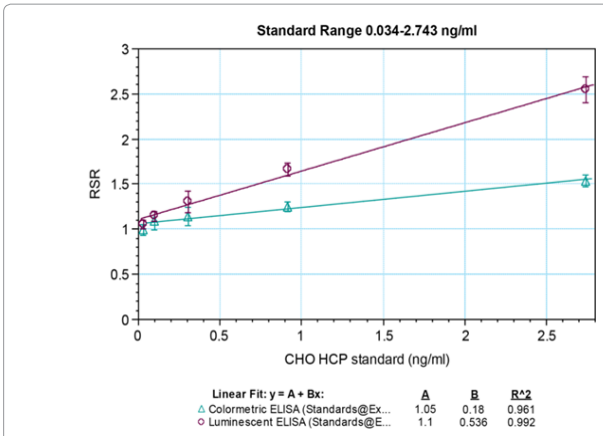

Figure 4: Low-end linear curves of colorimetric and luminescent ELISA at concentrations ranging from 0.034 to 2.743 ng/ml. The mean signal value ± standard deviation from triplicate wells was plotted against standard concentration by a linear fit method. Larger variations are observed in colorimetric method at this concentration range. The standard curve is also less linear than luminescent ELISA (R2=0.961 vs. 0.992).

The limit of detection, normally refers to the lower limit of detection (LLOD) is the lowest quantity of a substance that can be distinguished from the absence of that substance (i.e., a blank value) within a stated confidence limit. The lower limit of quantification (LLOQ) is the lowest quantity of a substance that can be accurately quantified. There are a variety of ways to determine the LLOD and LLOQ of an analytical method, here we calculated the theoretical LLOD and LLOQ of ELISA according to the guidelines of ICH-Q2 (R1) using the formula: LLOD=3.3 × θ/S, and LLOQ=10 × θ/S, where θ is the standard deviation of blank concentration back-calculated from the calibrate curve, S is the slope of the standard curve. We also determined the experimental LOQ as the lowest and highest standard concentration which can be accurately determined (CV<20% and recovery within the range of 100 ± 20%). Using the log-log fit curve within linear range of 0.31-2000 ng/ml (R^2=0.991), the calculated LLOD and LLOQ for luminescent ELISA is 0.245 and 0.816 ng/ml, respectively; in contrast, within its log-log linear range (R^2=0.992), colorimetric method has a LLOD and LLOQ of 0.819 and 2.483 ng/ml, respectively, which is ~2 times higher than that for luminescent method. When calculated using log-log linear curve fitting in the same range of 0.91-222.22 ng/ml (R^2=0.998), luminescent ELISA has a LLOD=0.365 ng/ml and LLOQ=1.216 ng/ml, remaining much lower than that of colorimetric method (Table 4). In addition, lower LLOD and LLOQ is consistently observed in luminescent ELISA from replicated experiments (data not shown) in spite of the methodology used for calculating LLOD and LLOQ, indicating that luminescent detection is indeed more sensitive than colorimetric ELISA for CHO HCP detection and quantification.

One common method to validate the precision and accuracy of ELISA is using the standard curve to measure mAb-A DS with known amount of spike. In our experiments, different amount of CHO HCP was spiked into 5 mg/ml drug substance and spike recovery was calculated to indicate the accuracy of quantification. Using clear 96-well plate, we observed low recovery on the back-calculated concentration in the spiked samples when the unspiked sample wells are adjacent to the high concentration standards, which lead us to change the assay plate for luminescent ELISA from Costar clear plate to NUNC white opaque plate for assay optimization to avoid cross-interference of luminescence signals.

		CV%		Recovery%	
		Colorimetric	**Luminescent**	**Colorimetric**	**Luminescent**
Log-log fit	Mean ± SD	20.29 ± 9.12	11.79 ± 3.67	102.66 ± 18.77	100.65 ± 9.12
	P value (one tail)	0.03		0.41	
4-parameter fit	Mean ± SD	25.50 ± 14.27	13.65 ± 4.58	97.97 ± 13.59	94.68 ± 14.91
	P value (one tail)	0.04		0.35	

Table 3: Comparison of mean CV% and recovery% of colorimetric method to that of luminescent method using different fitting method (n=6, for standards ranging from 0.91 to 222.22 ng/ml).

Detection method	Log-log fit curve range (R^2)	LLOD (ng/ml)	LLOQ (ng/ml)
Colorimetric	0.91-222.22 ng/ml (0.992)	0.819	2.483
Luminescent	0.91-222.22 ng/ml (0.998)	0.365	1.216
Luminescent	0.31-2000 ng/ml (0.991)	0.245	0.816

Table 4: The LLOD and LLOQ for luminescent and colorimetric ELISA.

The comparison of colorimetric method to luminescent method using conditions optimized for luminescent ELISA in NUNC white opaque 96-well plate

To avoid cross-talk among adjacent wells, chemiluminescence detection based assays are often carried out in white or black opaque plate. In this study, we also compared the assay performance between luminescent ELISA and colorimetric ELISA using conditions optimized for luminescent ELISA in Nunc white opaque 96-well plate, which provides maximum reflection and low cross-talk. Performing luminescent ELISA in white opaque plate using conditions previously described in clear plate dramatically raised the signal response or relative luminescence unit by 6 to 15 fold. Thus, the reagents concentration used in ELISA assays performed in opaque 96-well plate were re-optimized and finalized as shown in Supplemental Table 1. The assay performance of luminescent and colorimetric ELISA was also compared side-by-side using the same amount of assay reagents. The signal/noise ratio, linearity over logarithmic scales, accuracy and precision, as well as the LLOD and LLOQ of both assays were re-assessed.

Under the assay conditions shown in Supplemental Table 1, colorimetric ELISA has a signal response range from 0.056 to 0.121, the log-log fit linear standard curve has a correlation coefficient R^2=0.95, considerably lower than that of luminescent ELISA, which has a signal response range from 4036 to 47731 and a linearity of R^2=0.998 (Figure 5).The accuracy and precision of both colorimetric and luminescent ELISA assays were evaluated using the back-calculated standard concentration and the measured spiked DS concentration. For standards, luminescent has significantly lower CV values (9.76 ± 8.87 vs. 28.17 ± 15.96, p=0.006, n=8) than colorimetric method, indicating improved precision. For accuracy, the recovery of standards at all concentrations in luminescent ELISA ranges from 94.43% to 109.73% (100 ± 6.15%); in contrast standard recovery in colorimetric ELISA ranges from 68.30 to 162.62%, showing much higher variation than luminescent method (Supplemental Table 3). For spiked DS recovery, luminescent ELISA was capable of accurately measure 25 ng/ml and 5 ng/ml HCP spike in 5 mg/ml mAb-A drug substance, with much lower CV values and closer to 100% recovery than colorimetric method; for 1 ng/ml HCP spiked in mAb-A DS (0.2 ppm) recovery, luminescent method was relatively accurate with a CV=30.5% and a recovery=77.35%, while colorimetric method only showed 39.39% recovery (Table 5).The LLOD and LLOQ of luminescent ELISA and colorimetric ELISA were calculated according to the standard deviation of blank (θ) and the slope of calibration curve (S) following the formula LLOD=3.3 × θ/S and LLOQ=10 × θ/S (Table 6). As shown in Table 6, luminescent ELISA is ~9 times more sensitivity than colorimetric ELISA when using 0.25 µg/ml biotin-anti-HCP, 1:120,000 dilution of NeutrAvidin-HRP and 50 µL of substrate. Additionally, colorimetric ELISA was also repeated in Costar clear 96-well plate using concentrations of reagents described above, however, the signal response only ranged from 0.056 to 0.074 at the standards concentrations from 1.563 to 100 ng/ml. The signal response was not sensitive enough to differentiate concentration changes, confirming the much lower sensitivity of colorimetric substrate than luminescent substrate.

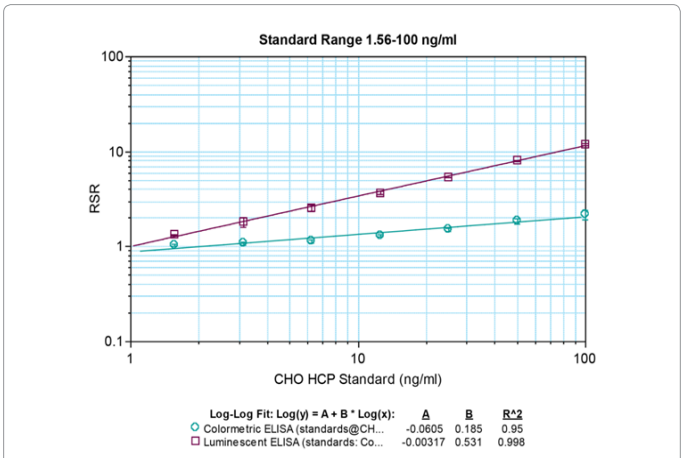

Figure 5: The log-log fit standard curves of colorimetric and luminescent ELISA using conditions optimized for luminescent ELISA and performed in NUNC opaque 96-well plate.

Conclusion

The sensitivity of luminescent detection was compared side-by-side with colorimetric detection in a sandwich ELISA format under conditions optimized for either colorimetric ELISA or luminescent ELISA. The performance of both assays including parameters such as signal/noise ratio, linear range and linearity over logarithmic scales, precision and accuracy, as well as LLOD and LLOQ were fully evaluated and compared. Our results show that luminescent detection has enhanced signal/noise ratio, broader linear range on logarithmic scales and better linearity within the same range. In addition, luminescent detection also shows better lower end linearity of its standard curve, which allows the accurate quantification of HCP concentration at as low as 1 ng/ml. Under the same experimental conditions, luminescent ELISA is able to detect and accurately quantify lower amount of HCP than the colorimetric method. Moreover, when calculating HCP concentration from its standard curve, better recovery (closer to

	Colorimetric method		Luminescent method	
	CV%	Recovery%	CV%	Recovery%
Unspiked DS	-		20.9	
25 ng/ml spiked DS	9.8	109	5.5	98.75
5 ng/ml spiked DS	14.4	121.04	6.3	94.52
1 ng/ml spiked DS	-	39.39	30.5	77.35

Table 5: The CV and recovery of spiked DS as back-calculated from the log-log fit calibration curve of luminescent or colorimetric ELISA.

	Colormetric method	Luminescent method
LLOD (ng/ml)	12.47	1.34
LLOQ (ng/ml)	37.78	4.06

Table 6: The LLOD and LLOQ of colormetric and luminescent ELISA performed in Nunc white opaque plate using conditions optimized for luminescent ELISA.

100%) and lower CV values were observed with luminescent ELISA. Lastly, chemiluminescent ELISA using the Femto substrate doesn't require a stopping step. Chemiluminescent signals can be measured immediately after the addition of substrate and for 10 min after, since the signal will remain at a plateau for that amount of time. In summary, chemiluminescent ELISA proves to be more sensitive than its colorimetric counterpart for mAb-A HCP quantification. Although the experiments were performed using polyclonal antibodies raised against mAb-A null culture HCP, the same principal should apply to the quantitation of HCP in other drug substance samples with minimal assay development work needed. In conclusion, improvement of the HCP ELISA detection limit without compromising assay robustness offers the opportunity to better control HCP clearance during each of the purification steps and reduces the risk of HCP associated immunogenic response in patients.

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