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Technical note

Development and validation of a cell-based SEAP reporter assay for the detection of neutralizing antibodies against an anti-IL-13 therapeutic antibody

Karen Liao, Dan Sikkema, Catherine Wang, Keguan Chen, Stephen DeWall, Thomas N. Lee*

Clinical Immunology, GlaxoSmithKline, King Of Prussia, PA 19406, United States

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ABSTRACT

A cell-based bioassay capable of detecting neutralizing antibodies (NAb) specific to a therapeutic anti-IL-13 monoclonal antibody was developed, validated and used to analyze normal human and asthma serum samples. At the time of this study, a neutralizing assay was unavailable for anti-IL-13 antibody therapeutics with sufficient rigor for validation. Thus, we describe here a method and considerations for validation. The assay used IL-13 responsive HEK293 cells transfected with a secreted embryonic alkaline phosphatase (SEAP) reporter gene. Cells were plated at 5.4×10^4 per assay well due to 90% confluence on the subsequent day. Optimal IL-13 and anti-IL-13 concentrations were determined to be 600 pg/mL and 900 ng/mL respectively. We demonstrated the assay's cut point, sensitivity, specificity/cross reactivity, selectivity/matrix interference, and precision. Also, we demonstrated how the drug inhibitory concentration (IC_{50} , IC_{75} , and IC_{90}) can affect sensitivity and dynamic range/assay window. We characterized the differences in assay response between serum samples of normal population and asthma population. Asthma samples demonstrated an elevated OD ratio in average compared to normal samples. Thus, separate cut points were needed and calculated to be 1.78 and 2.43 for normal and asthma serum, respectively. The assay sensitivity was 670 ng/mL with the positive control (affinity purified rabbit anti-drug polyclonal antibodies). Potential false positives resulting from endogenous serum cytokines including IL-13, IL-4, and Interferon alpha ($INF-\alpha$) were evaluated and the results indicated that the interfering concentrations for these cytokines are much higher than the respective physiological concentrations. Based on these data, the risk of false positive by endogenous cytokines was considered to be low. In addition, irrelevant anti-drug positive control antibodies were evaluated for assay specificity and did not demonstrate neutralizing capability. Further, no matrix interference in the intended patient population was found when using a final assay serum concentration of 16.7%. The validated assay had acceptable intra- and inter- assay precision in that all %CVs were $\leq 25\%$. Overall, this assay successfully proceeded through validation and was used to determine NAb responses within serum samples.

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1. Introduction

Neutralizing assays can be ligand binding or cell-based in functionality. Currently, cell-based assays are the preferred

approach in that they are considered more biologically relevant. Yet, cell-based assays may yield increased variability, are susceptible to matrix interference and thus may be more developmentally laborious (McCutcheon et al., 2010). In addition, NAb may be difficult to measure in the presence of drug even though methods have been developed to overcome this issue (Hu et al., 2009). We describe here the development and validation of a cell-based colorimetric reporter assay used for the detection of Nab to anti-IL-13 therapeutic within human serum. The assay uses IL-13 responsive HEK293 cells stably transfected

* Corresponding author at: GlaxoSmithKline, 709 Swedeland Road, King Of Prussia, PA 19406, United States. Tel.: +1 610 270 4238; fax: +1 610 270 5155.

E-mail address: Thomas.n.lee@gsk.com (T.N. Lee).

with a secreted embryonic alkaline phosphatase (SEAP) reporter gene. This cell line responds to IL-13 through the STAT6 pathway inducing expression of SEAP which is collected in the supernatant. Subsequent detection is performed by addition of SEAP substrate to the supernatant which turns blue in the presence of SEAP. We first characterized the cellular response to IL-13 and established an optimal response of SEAP activity. Subsequently, we demonstrated a dose-dependent inhibitory effect on IL-13 induced SEAP activity by the anti-IL-13 mAb drug. We selected different drug levels within the linear response range to optimize the Nab assay sensitivity and assay window. This cell-based Nab assay was developed and optimized based on Guidance for Industry Assay Development for Immunogenicity testing of Therapeutic Proteins, FDA Draft Guidance 2009 (U.S. Department of Health and Human Services, 2009). Although neutralizing antibody detection methods have been published, we describe an inhibitory concentration (IC) optimization process to enhance sensitivity and present an IL-13 specific method which has the necessary sensitivity, precision and rigor to proceed into validation.

2. Materials and methods

2.1. Reagents

HEK-Blue IL-4/IL13 cells, selection antibiotics (Blasticidin and Zeocin), recombinant IL-4, IL-13 neutralizing IgA, and SEAP substrate (Quanti-Blue) were purchased from Invivogen (San Diego, CA). Heat-inactivated fetal bovine serum (FBS) was purchased from SAFC Biosciences (Lenexa, KS). Dulbecco's Modified Eagle Medium (high glucose) (DMEM), PBS and Trypsin-EDTA and recombinant IFN- α were obtained from Invitrogen Corp. (Carlsbad, CA). Recombinant human IL-13 and humanized anti-IL-13 antibody (therapeutic mAb drug) were provided by GlaxoSmithKline BioPharm unit. Normal human sera were purchased from Bioreclamation, Inc (Hicksville, NY). Positive control antibodies directed against the drug were affinity purified from rabbit sera produced by Covance (Denver, PA).

2.2. Cell culture

The HEK-Blue IL-4/IL13 cells were cultured and maintained in DMEM containing 10% FBS, 10 μ g/ml blasticidin and 100 μ g/ml of Zeocin in a 75 cm² vented cap, canted neck cell culture flask (Corning, New York, NY). The flask is incubated in a 37 °C incubator with 5% CO₂ and 90% relative humidity. Cells were passed regularly with the confluence under 90%. On the assay day, cells were trypsinized and seeded in medium without selection antibiotics.

2.3. Assay procedure and controls

Patient sera or positive controls (PC) were centrifuged at 12,000 rpm for 10 min to generate clear serum. Centrifuged serum was first pre-incubated with 900 ng/mL anti-IL-13 drug for 15 min. Then, IL-13 at 600 pg/mL was added and incubated at room temperature for 1 h with shaking. After the neutralization incubation, 100 μ L was added to equal volume of freshly seeded cells at a density of 5.4×10^5 /mL in duplicate in a 96-

well plate. The final human serum concentration in the cell culture was 16.7%. The cells were incubated at 37 °C for 20 to 22 h. At the end of the incubation, supernatant was collected and mixed. Then, 40 μ L of the supernatant was transferred to a 96-well microtiter plate. 160 μ L of QUANTI-Blue™ solution was then added to each well. The microtiter plate was incubated at 37 °C for 30 min. SEAP level was determined by a spectrophotometer at 650 nm.

The Nab assay was monitored with five controls. The controls for cell response include a cellular background control (BC) and cellular maximum response control (MC). The assay performance was monitored by two positive controls (affinity purified rabbit anti-drug polyclonal antibodies, PC), a high positive control (2.5 μ g/mL) and a low positive control (1 μ g/mL). A negative control (NC) was also included. The BC was composed of cells without IL-13 or anti-IL-13 mAb but with only pooled normal human serum. The MC was composed of cells with IL-13, but without anti-IL-13 mAb. The positive controls were composed of cells with IL-13 and anti-IL-13 mAb and PC that was diluted in pooled human serum matrix. The NC was composed of cells with IL-13, anti-IL-13 mAb and pooled normal human serum. All controls contained a final human serum percentage of 16.7%. The average NC value on each plate was used to normalize other controls and test samples. The control and test sample OD ratios were then reported.

2.4. Assay validation and statistical analysis

2.4.1. Assay cut point

Forty normal human sera (NHS) and forty randomly selected pre-dose asthma patient sera were assayed in duplicate wells three times on three separate days. The response ratio for each sample was calculated by the formula of $\frac{\text{Mean OD}_{\text{Sample}}}{\text{Mean OD}_{\text{Negative Control}}}$. The variability of response over 3 days including the mean and variance were statistically analyzed (Statistica 8) to determine if a fixed or floating cut point would be most appropriate.

2.4.2. Assay sensitivity

Assay sensitivity was determined by the concentration in neat human serum at which the PC produced a response ratio equal to the established cut point. To determine this, the positive control was titrated in the pooled normal human serum. Positive control dose response curves and concentration at the cut point were analyzed and generated using a 4-parameter logistic fit by SoftMax Pro GxP v5.2 (Molecular Devices Corp, Sunnyvale, CA).

2.4.3. Intra- and inter-assay precision

Assay precision was assessed by looking at the performance (%CV) of all quality control (QC) samples, including background control (BC), high positive control (HPC), low positive control (LPC), maximum signal control (MC), and negative control (NC). To assess intra-assay precision, the above controls were run in four sets of duplicate wells on each of three plates on one day. For inter-assay precision, the above control samples were assayed by two analysts in four days.

2.4.4. Specificity and cross-reactivity

2.4.4.1. Anti-IL-13 drug cross-reactivity/specificity. To evaluate specificity of the anti-IL-13 drug, a non-specific humanized antibody of the same isotype (IgG1) and a commercial anti-IL-13 antibody (Invivogen, San Diego, CA) were analyzed and compared to the drug. All three antibodies were titrated in parallel and assessed within the same assay plate. The response ratios for the three curves were analyzed and compared.

2.4.4.2. Positive control cross-reactivity/specificity. To evaluate specificity for the PC, an anti-framework antibody (goat anti human IgG-Fc, Jackson ImmunoResearch Laboratories, West Grove, PA) and two irrelevant anti-drug positive controls raised from the same species (Covance, Denver, PA) were tested in parallel with the specific PC in the Nab assay. Considering the pooled human serum would interfere with the binding of anti-human IgG-Fc (anti-framework PC) to the anti-IL-13 mAb drug, the PCs were titrated in cell culture medium instead of pooled normal human serum in regular Nab assay and the rest of the assay followed the procedure described in Section 2.3 assay procedure and controls.

2.4.5. Matrix interference

HEK-Blue IL-4/IL-13 cells can respond to IL-13, IL-4, and to a lesser degree INF- α . Thus, elevated serum level of the three cytokines could potentially result in a false positive outcome. To evaluate the concentrations which could potentially induce a false positive result, the cells were assayed for their responsiveness to these cytokines. Each cytokine was 1:2 serial diluted to yield a 12-point curve with a starting concentration of 200 ng/mL. These diluted samples were then diluted 1:3 with culture medium, to mimic the dilution scheme of neutralization procedure, and 100 μ L of the diluted cytokines were transferred to a cell plate with 100 μ L of 5.4×10^5 /mL cells per well. The cells were incubated at 37 °C for 20 to 22 h. SEAP activity was determined subsequently.

To assess matrix interference, the PC was spiked into five individual normal human sera and five individual asthma sera at concentrations of 5 μ g/mL and 1 μ g/mL. The spiked serum for each sample was assayed alongside the unspiked sample on one plate. The response ratios of both unspiked and the PC spiked samples were normalized to the mean OD of the four NC sets on the plate. Results were determined by applying the cut point previously determined within the corresponding population.

3. Results and discussion

3.1. IL-13 dose response

At the plating concentration of 5.4×10^5 cells per mL, approximately 90% confluence could be achieved at 24 h post-seeding. This enabled a dose-dependent increase in SEAP activity upon treatment with increasing concentrations of IL-13 (Fig. 1A). All data were normalized to a control without IL-13 (BC) and thus presented as OD ratios. The linear range of cellular response was with IL-13 concentrations at 390–6250 pg/mL. The maximum cellular response ($E_{\max}$) was achieved with approximately 12.5 ng/mL of IL-13. The final assay concentration of IL-13 was

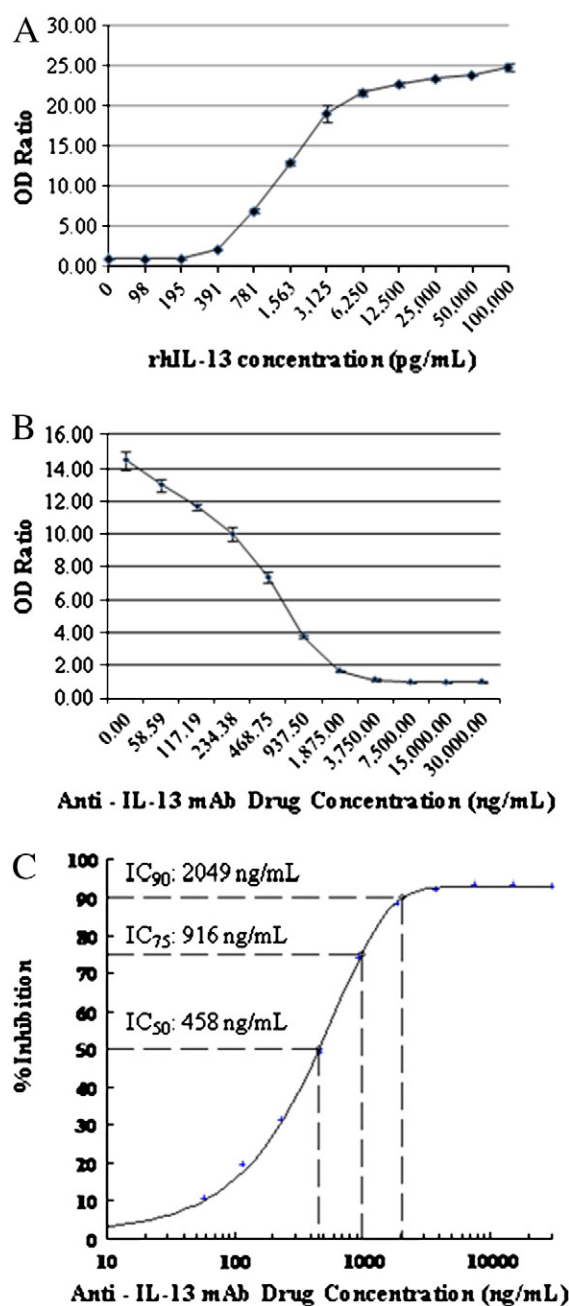


Fig. 1. Cell responsiveness to IL-13 and anti-IL-13 mAb drug. (A) Response of HEK-Blue cells to rhIL-13 in 16.7% human serum. rhIL-13 was serially diluted with pooled human serum. The titration curve was 1:3 diluted with cell culture medium and then 100 μ L of the diluted samples were added to 100 μ L of cells in duplicate wells of a 96-well plate. Cell culture supernatant was collected for SEAP activity after a 20-hour incubation. OD ratio = OD sample / OD BC. (B) Anti-IL-13 mAb drug inhibition curve in 16.7% human serum graphed by OD ratio (OD ratio = OD sample / OD BC) and (C) graphed by % inhibition (% inhibition = $100 - 100 \times (\text{OD with drug} / \text{OD without drug})$). Anti-IL-13 mAb drug was serially diluted in cell culture medium; the diluted drug was mixed with equal volume of 600 pg/mL rhIL-13 prepared in culture medium and equal volume of pooled normal human serum. 100 μ L of the mixture was added to 100 μ L cells in duplicate wells of 96-well plate. SEAP activity was detected after a 20-hour incubation.

Table 1

Comparison of assay sensitivity and assay window at various concentrations of anti-IL-13 mAb drug.

Anti-IL-13 Drug (ng/mL)	Assay Window	Sensitivity ($\mu\text{g/mL}$)
2000 (IC ₉₀)	14.6	2.17
900 (IC ₇₅)	7.2	0.65
450 (IC ₅₀)	2.7	0.46

Assay window: $\text{OD}_{\text{max}}/\text{OD}_{\text{NC}}$; Sensitivity: PC concentration intersecting the cut point.

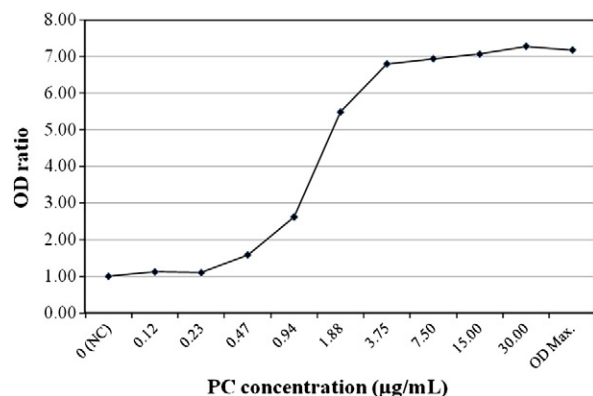


Fig. 2. PC response curve with a fixed concentration of anti-IL-13 drug and final human serum percentage of 16.7%. The PC was diluted in pooled human serum. Then the diluted PC was mixed with equal volume of 900 ng/mL (IC₇₅) anti-IL-13 mAb drug and 600 pg/mL IL-13. The mixed solution was incubated at RT for 60 min, and then 100 μL of the mixture was added to 100 μL cells in duplicate wells of 96 well plate. SEAP activity was detected after a 20 hour incubation. $\text{OD ratio} = \text{OD}_{\text{sample}}/\text{OD}_{\text{NC}}$.

selected to be 600 pg/mL. Cells treated with 600 pg/mL of IL-13 elicited approximately a six-fold induction of SEAP activity over background. This concentration was within the linear range of response; thus, allowing small changes in NAb assay response to be attributable to the presence of drug or NAb activity.

3.2. Dose response of anti-IL-13 drug

A dose dependent inhibition of IL-13 induced SEAP activity was observed upon addition of the humanized anti-IL-13 antibody (Fig. 1B). Three concentrations of anti-IL-13 drug within the linear range (IC₉₀, IC₇₅, and IC₅₀) were determined to be 2049, 916, and 458 ng/mL, respectively (Fig. 1C). These inhibitory concentrations were evaluated for assay inhibition and the resulting assay window available for signal re-establishment

Table 2

Cut points with 99% confidence limit.

	Mean	Variance				SD	Z	Cut point (Mean + $Z \times \text{SD}$)
		Sample	Day	Error	Total			
Normal Human Serum	0.94	0.102	0.007	0.024	0.133	0.36	2.33	1.78
Pre-dose Serum of Asthma Patient	1.87	0.035	0.007	0.014	0.056	0.24	2.33	2.43

SD (standard deviation): square root of total variance.

Z: standard normal deviate.

Table 3

Assay sensitivity.

Run ID	PC concentration ($\mu\text{g/mL}$) at cut point of 1.78	Mean (Assay Sensitivity) ($\mu\text{g/mL}$)	SD	%CV
Day 1-1	0.48	0.67	0.17	24.67
Day 1-2	0.56			
Day 1-3	0.55			
Day 2-1	0.91			
Day 3-1	0.67			
Day 3-2	0.88			
Day 4-1	0.65			

once NAb activity was present. It was demonstrated that the IC₇₅ provided the best profile of assay window and sensitivity. Drug at IC₉₀ resulted in largest assay window; however the assay sensitivity was reduced by more than 3- fold compared to that at IC₇₅. On the other hand, drug at IC₅₀ showed a slight improvement in assay sensitivity, but the assay window became marginal (2.7) (Table 1). Therefore, anti-IL-13 drug at IC₇₅ (900 ng/mL) was selected as the neutralizing assay drug concentration. This approach ensured that the assay had the best combination of sensitivity and assay window.

3.3. Positive control neutralizing activity dose response

With the IL-13 inhibited by anti-IL-13 drug, the suppressed SEAP activity was re-established upon PC addition; when positive control antibody bound and neutralized anti-IL-13 mAb, the released IL-13 restored the cellular response. A dose-dependent curve was generated with PC concentrations between 117 ng/mL – 30 $\mu\text{g/mL}$. Overall, increasing concentrations of PC increased SEAP activity and PC near 3.75 $\mu\text{g/mL}$ restored the signal to maximum control (MC) level (Fig. 2). Based on the PC response curve, a high positive control (2.5 $\mu\text{g/mL}$) and low positive control (1 $\mu\text{g/mL}$) were selected for assay validation along with three other assay controls (BC, NC, and MC).

3.4. Assay validation

3.4.1. Cut point determination

Forty normal human sera (NHS) and forty randomly selected pre-dose asthma patient sera were analyzed at 16.7% final serum concentration on three different days. Data was first analyzed by box plot analysis to identify outliers. No outliers were found in all runs. The combined data was found to be within a normal distribution. Variance component analysis was used to assess variability from samples, assay days or experimental error

Table 4

Assay precision.

Control	Intra-Assay Precision			Inter-Assay Precision		
	Mean (n = 12)	SD	%CV	Mean (n = 30)	SD	%CV
HPC (OD ratio)	5.64	0.19	3.41	5.71	0.57	9.95
LPC (OD ratio)	2.64	0.15	5.59	2.44	0.33	13.62
MC (OD ratio)	5.88	0.30	5.14	6.24	0.75	12.01
BC (OD ratio)	0.51	0.01	2.64	0.58	0.05	9.40
NC (% Inhibition)	83.11	0.58	0.70	82.93	3.02	3.64

OD ratio = OD_{sample}/OD_{NC}.

% inhibition = 100 – 100 (OD_{NC}/OD_{MC}).

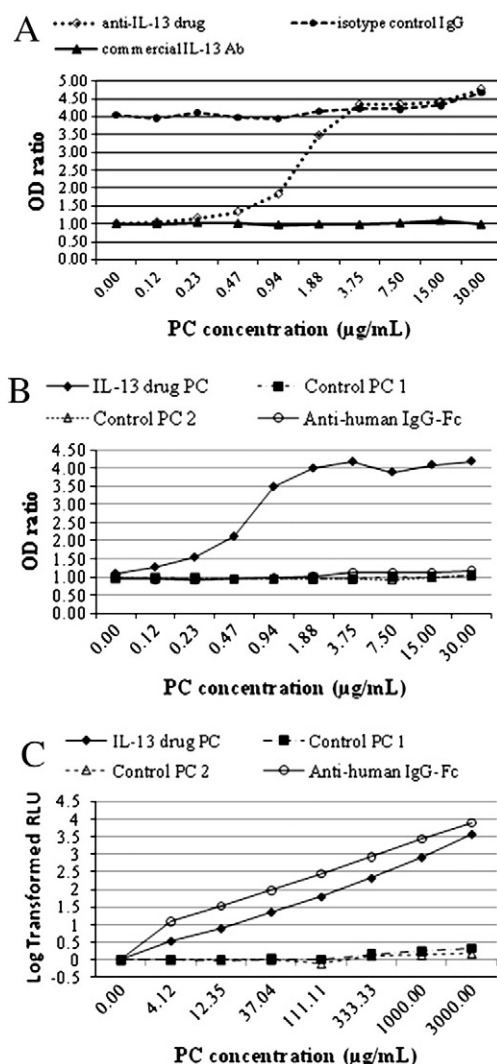


Fig. 3. Assay specificity. (A) Cross reactivity of anti-IL-13 mAb drug. (B) PC cross reactivity: neutralizing activity for the specific and control PCs. The NAb assay was conducted in cell culture medium without human serum. (C) PC cross reactivity: drug binding activity for the specific and control PCs in a bridging ADA assay. The assay was conducted in assay diluents (1% BSA/PBS) without human serum.

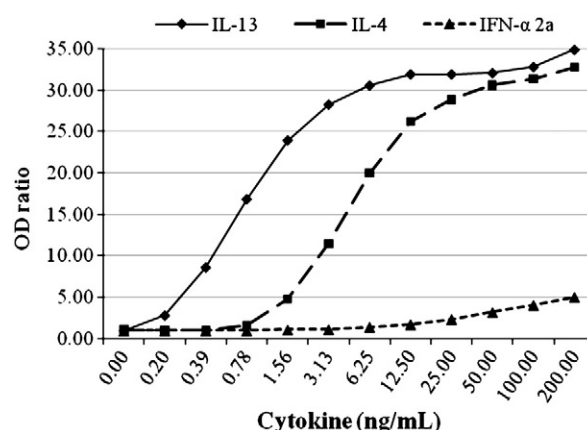


Fig. 4. Matrix interference: response of HEK-Blue IL-4/IL13 cells to IL-4, IL-13 and IFN-α2a. OD ratio = OD_{sample}/OD_{BC}.

(representing unidentified source of variance). Means and variances were found to be similar across runs in both NHS and asthma serum. Therefore a fixed cut point in OD ratios was applied to both populations (Shankar et al., 2008). Cut points with a 99% confidence limit were calculated to be 1.78 and 2.43 for normal and asthma serum, respectively, by the formula of mean + 2.33 × SD (Table 2). The cut point in normal human serum was applied to evaluate assay controls, while the cut point in asthma serum was applied to clinical sample evaluation.

3.4.2. Assay sensitivity

The assay sensitivity was determined by the concentration at which the PC produces a response ratio (OD ratio) equal to the cut point established in normal human serum (1.78). Seven PC dose response curves were generated across 4 days in this validation. OD ratios from each PC titration were analyzed by a four-parameter logistic function (SoftMax Pro 5.2, Molecular Device); the concentration of PC response curve intersecting with the cut point (1.78) was obtained for each curve. The average of the seven values was reported as the assay sensitivity and estimated to be 0.67 μg/mL (Table 3). It was noted that for restoration of assay response, a near identical molar amount of positive control NAb was effective at neutralizing the therapeutic antibody (670 ng/mL PC vs 900 ng/mL drug antibody).

3.4.3. Assay precision

To assess intra-assay precision, the QC samples (HPC, LPC, MC, NC and BC) were run in four sets of duplicate on three plates on the same day. As shown in Table 4, the intra-assay precision was much lower than the target value (25%), with the highest %CV of 5.59% in LPC. Inter-assay precision was assessed by the QC performance across four days by two analysts; with the highest %CV of 13.62% in LPC for the inter-assay precision.

3.4.4. Specificity

To assess the anti-IL-13 antibody drug cross reactivity, two control antibodies including an irrelevant humanized IgG isotype control and a commercial anti-IL-13 antibody were tested in parallel with the specific antibody drug, as shown in Fig. 3A. The irrelevant control IgG did not affect/suppress IL-13 activity

Table 5

Matrix interference: Detection of spiked PC in normal and disease serum.

Samples	NHS					Asthma sera				
	NHS 1	NHS 2	NHS 3	NHS 4	NHS 5	AS1	AS2	AS3	AS4	AS5
Unspiked	1.22	0.76	0.78	0.67	0.92	1.97	1.75	1.82	1.81	1.90
Spiked (1 µg/mL)	2.61	1.67	1.93	1.41	2.51	3.86	3.56	3.62	3.48	4.02
Spiked (5 µg/mL)	4.78	3.81	4.52	3.09	4.82	5.93	5.90	5.88	5.82	6.22
Cut point	1.78					2.43				

Values are OD ratios (OD_{sample}/OD_{NC}).

while the commercial anti-IL-13 did suppress IL-13 activity. However, the PC did not restore the commercial anti-IL-13 suppressed IL-13 activity. We also assessed the PC cross reactivity in the Nab assay. The specific PC, two irrelevant rabbit anti-human IgG1 antibody PCs, and the goat anti-human IgG-Fc were evaluated. Only the drug-specific PC showed neutralizing activity (Fig. 3B). To demonstrate PC-drug (anti-IL-13 mAb) binding, all four PCs including the goat anti-human IgG-Fc were also evaluated in a previously validated ADA bridging assay. Binding occurred with the specific PC and the anti-human IgG-Fc, but was not demonstrated with the irrelevant antibodies (Fig. 3C).

3.4.5. Matrix interference

The HEK-Blue IL-4/IL-13 cells are reported to respond to IL-4, IL-13 and to INF- α . To evaluate potential false positives, cells were tested for the responses to the three cytokines. As shown in Fig. 4, IL-13 caused a false positive result at or above 100 pg/mL; IL-4 caused a false positive result at 640 pg/mL; while INF- α caused a false positive response at 1325 pg/mL. Based on published information (St et al., 2009; (Lewkowich et al., 2001) and our in-house data, which suggest that drug unbound (free) IL-4 and IL-13 are undetectable (<4 pg/mL) in normal and asthma patient serum, we concluded that the possibility of a false positive result caused by free IL-13 and IL-4 was negligible. For INF- α , the cells were not sensitive enough for endogenous levels to cause false positive responses (Dabitao et al., 2011).

Matrix interference was also assessed by testing PC spiked individual serum. As shown in Table 5, all five HPC (5 µg/mL) spiked normal human sera tested positive. Three out of five LPC (1 µg/mL) spiked normal human sera tested positive while two were below the cut point. However, both HPC and LPC spiked asthma samples tested above the cut point (positive). Since this assay was intended for use in asthma patient sample testing, we decided that the matrix interference was minimal and acceptable for assay purposes. It was noticed that the basal SEAP activity was higher in asthma samples compared to normal human serum. The increased background of the asthma samples lead to an increased assay cut point for the asthma samples. This phenomenon may be attributable to multiple factors including clinical, genetic, or environmental background of the diseased population.

For a further matrix consideration, contaminating alkaline phosphatase activity could have been present in some cell culture mediums or samples. To address this, heat-inactivated serum was used for cell culture and individual samples analyzed within the assay were normalized to the NC.

4. Conclusion

In summary, we have demonstrated an optimized assay for the detection of neutralizing antibodies directed against an anti-IL-13 therapeutic monoclonal antibody. The assay was suitable for validation, and provided a valuable format for assessing neutralizing activity. The assay had sufficient sensitivity combined with acceptable precision. It was specific in that it did not demonstrate a neutralizing response to the anti framework antibody or other anti-drug antibodies not directed to anti-IL-13. We also concluded that optimizing the drug inhibition with the IC values yielded maximum assay sensitivity and dynamic range.

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