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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

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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-α) 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 ≤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

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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 μg/ml blasticidin and 100 μg/ml of Zeocin in a 75 cm^2 vented cap, canted neck cell culture flask (Corning, New York, NY). The flask is incubated in a 37 °C incubator with 5% CO_2 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 μL 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 μL of the supernatant was transferred to a 96-well microtiter plate. 160 μL 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 μg/mL) and a low positive control (1 μg/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 $\frac{\text{Mean OD_Sample}}{\text{Mean OD_Negative Control}}$. 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.

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