



Research Article

Development and Utility of an ELISA Method for Sensitive and Specific Detection of IgE Antidrug Antibodies

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Abstract. Biologics can potentially induce unwanted immune responses, leading to formation of antidrug antibodies (ADA) of various affinity, isotypes, and subclasses. Among them, antigen and drug-specific immunoglobulin E (IgE) antibodies have been reported to have potential correlation with hypersensitivity and anaphylaxis in particular. Recent regulatory guidance on immunogenicity testing has recommended the measurement of antigen-specific IgE antibodies for biologics with a reported high risk of anaphylaxis using assays with sensitivities in the high pg/mL to low ng/mL range. Nevertheless, IgE ADA remains challenging to detect due to their being the least abundant isotype in blood serum samples and the potential for interference in the bioanalytical methods due to high levels of endogenous immunoglobulin G (IgG) and immunoglobulin M (IgM) ADA, not to mention the nonspecific total serum IgE antibodies. Another challenge in developing IgE ADA assays is the need to create a surrogate drug-specific IgE antibody positive control to monitor the performance of the assay for the intended use. In this case study, utilizing a human IgE antidrug antibody positive control and a human IgE receptor as capture, an enzyme-linked immunosorbent assay (ELISA) method was developed for the measurement of IgE ADA, meeting the regulatory expectations, with excellent assay sensitivity, selectivity, specificity, and tolerance towards potential interference in serum samples. This assay format could be readily adapted and implemented to assess drug-specific IgE antibodies in the event of drug-related anaphylaxis in clinical and in nonclinical development programs.

KEY WORDS: antidrug antibody; ELISA; hypersensitivity; IgE; immunogenicity.

INTRODUCTION

Biologics have the propensity to elicit unwanted immune responses, leading to the formation of antidrug antibodies (ADA), which can potentially alter pharmacokinetic profiles, pharmacodynamic outcomes, and the efficacious effects of the therapeutics (1–5). The immunogenicity of biologics can also induce various adverse events. One potential effect of this immunogenicity is induction of hypersensitivity reactions, frequently in the form of either immediate (Type I) or

immune complex-mediated (Type III) reactions (6). Assessment of immunogenicity follows a multi-tiered approach starting with the screening and confirmation of the presence of ADA in patients, followed by measurement of ADA titers. Using a risk-based approach, additional efforts are often needed to evaluate the neutralizing activity of the ADA. Further characterization may also be necessary to understand the affinity, binding epitope, and the distribution of the ADA isotypes and subclasses. Among the immunoglobulin isotypes, immunoglobulin E (IgE) responses have been considered to be a risk factor in the development of hypersensitivity or allergic reactions and anaphylaxis in particular (7). While considerable efforts have been given to detection of immunoglobulin G (IgG) and immunoglobulin M (IgM) ADA, measurement of IgE ADA remains a challenging task (8).

There have been reports of anaphylactic reactions being attributed to administration of a few monoclonal antibody (mAb) biologics, resulting in serious and potentially life-threatening, adverse events (6,7,9). Anaphylaxis is a Type I hypersensitivity reaction mediated by the cross-linking of IgE on the surface of mast cells or basophils with the allergenic epitopes. Regulatory guidance recommends that antigen-

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specific IgE assays should be implemented where a biologic has a high risk of initiating anaphylaxis or when an anaphylactic event has been observed as a clinical program progresses through IND stages into the licensure and post-approval stages (2). Although the measurement of drug-specific IgE antibodies is not a routine ADA assessment, development of an IgE ADA assay is warranted in the event of anaphylaxis. Efforts have been made to develop sensitive, highly specific IgE ADA assays across the industry, with the goal of better understanding the potential correlation between anaphylaxis and the presence of IgE ADA and thus the management of anaphylaxis (10–12). The commonly used bridging assay detects all ADA isotypes in a sample. Therefore, this assay format would not be able to differentiate whether the detected ADA response is that of an IgE isotype. It is therefore necessary to develop an alternative method for assessing the potential presence of IgE ADA when anaphylaxis has been identified. There are unique challenges in developing IgE ADA assays because of the inherently low levels of IgE ADA in circulation, the potential interference of high levels of IgG ADA, and other endogenous interfering substances (e.g., total serum IgE antibodies and drug target) and the administered drug itself.

It is critical to develop an appropriate positive control to ensure that an ADA assay is suitable for its intended use. Conventional methods of generating ADA assay polyclonal positive controls would not provide a specific IgE ADA positive control, as the resulting purified IgG positive control would be the most dominant isotype produced. Alternatively, chimeric mAb positive controls have been developed and utilized to monitor system suitability of ADA assays and, in particular, positive controls used in drug-specific IgE assays (10,11). In this manuscript, we report the development and validation of an enzyme-linked immunosorbent assay (ELISA) method with superior sensitivity, selectivity, and specificity for the detection of drug-specific IgE antibodies. This methodology could be readily adapted and implemented to assess IgE drug-specific antibodies in clinical and nonclinical development of therapeutic biologics, where anaphylactic events are observed and deemed to be related to the drug administered.

MATERIALS AND METHODS

Equipment and Materials

The following instruments and reagents were used: ELX-405 Plate Washers (BioTek Instruments, Winooski, VT); Octet RED96 system (Molecular Device); SpectraMaxPlus 384 Plate Reader and SoftMax GXP PRO version 5.4.4 (Molecular Devices, Sunnyvale, CA); Expi293 expression system, GeneOptimizer®, Human IgE Isotype Control (Catalog #DIA HE1-01), goat Antihuman IgE (Catalog #A18795), EZ-Link Sulfo-NHS-LC-Biotin, Streptavidin-Poly HRP, and Nunc™ 96-Well Clear Polystyrene Microplate (Thermo Fisher Scientific, Rockford, IL); Plasmid Gigaprep Kit (Sigma); LamdaFab select affinity chromatography and Superdex200 gel filtration chromatography (GE Healthcare Life Sciences); Donkey Anti-Goat IRDye680 (Catalog #926068074) and Odyssey Imager (LI-COR Biosciences, NE); pooled normal human serum (PNHS) and individual

human donor serum samples (BioIVT, Westbury, NY); and nonspecific Human IgG₄ control (Clone 3A5.040) and IgG anti-MAB072 idiotype mAb positive control (Clone H05-IgG) (Teva Pharmaceuticals).

Generation and Characterization of IgE Anti-MAB072 Antibody Positive Controls

Generation of the IgE Anti-MAB072 Antibody Positive Controls

To generate a suitable positive control for the IgE anti-MAB072 antibody assay, antigen-binding fragments (Fabs) specific to complementary determining regions (CDR) of monoclonal antibody drug MAB072 (produced by Teva Pharmaceuticals) were isolated from a phage display library in a similar manner to those described for the generation of anti-idiotypic antibodies (13). The amino acid sequences of the Fabs were formatted onto a human IgE heavy chain backbone and human lambda light chain in silico, followed by back-translation into DNA sequences. The resulting sequences were synthesized de novo by assembly of synthetic oligonucleotides. The resulting genes were subcloned into a mammalian expression vector and verified by DNA sequencing.

The expression of IgE anti-MAB072 idiotype antibody positive controls was performed using the Expi293 expression system. Briefly, large-scale plasmid preparations were made using an endotoxin-free Plasmid Gigaprep Kit. The heavy and light chain-expressing plasmids were co-transfected into Expi293 cells according to the manufacturer's protocol, followed by incubation at 37 °C and 8% CO₂, for 5–6 days with constant shaking. The expression was determined by western blot analysis of the extracellular medium. For purification, cells were harvested, and conditioning media was collected by filtration using a 0.2 µm filter. The recombinant antibodies were purified from the extracellular medium using LamdaFab select affinity chromatography, followed by Superdex200 gel filtration chromatography.

Characterization of the IgE Anti-MAB072 Antibody Positive Controls

The specificity of the IgE anti-MAB072 antibody positive controls was determined using western blot and the Octet RED96 system. For western blot analysis, 2 µg of MAB072 and a human IgG₄ isotype control were loaded on a nonreducing SDS-PAGE, transferred to nitrocellulose membranes, and blocked. The membranes were then incubated with the IgE anti-MAB072 antibody positive controls at a concentration of 1 µg/mL for 60 min to detect the loaded drug and IgG₄ control. Following incubation, membranes were washed three times with 0.1% TBS-T, incubated with goat antihuman IgE (diluted 1:1000) for 60 min, washed again three times with 0.1% TBS-T, and incubated with rabbit anti-goat IRDye680 diluted 1:5000 for 30 min. The membranes were washed, and specific binding was detected using the Odyssey Imager.

Additional evaluation of the binding specificity of the IgE anti-MAB072 idiotype positive controls was conducted on the Octet RED96 system in a 96-well format at 30 °C with

1000 rpm agitation, following the instrument manufacturer's instructions. Briefly, streptavidin (SA) sensor tips were first pre-wet for 10 min in Octet reaction buffer (PBS with 0.05% bovine serum albumin), and then 25 nM biotinylated MAB072 or the biotinylated IgG₄ isotype control was captured on the SA sensor for 7.5 min, followed by a 1-min wash in reaction buffer to establish the baseline signal. The SA sensor tips were then immersed into wells containing IgE anti-MAB072 antibodies at a concentration of 12.5 nM for 15 min to initiate specific association. The data were then imported into ForteBio Octet Data Analysis 8.2 and parallel referencing by subtracting the IgE antibody binding in the absence of ligand and then fit the data using a 1:1 L model where $R_{\max}$ was set to global.

The apparent binding affinity of the IgE anti-MAB072 antibody positive controls with MAB072 was determined using the Octet RED96 system. As described above, 25 nM biotinylated MAB072 was captured on pre-wet SA sensor tips, followed by a baseline wash. The sensor tips were then incubated with IgE anti-MAB072 antibody positive controls at concentrations of 12.5, 6.25, 3.2, 1.6, and 0.08 nM. Association (or binding) was allowed to occur for 20 min, followed by dissociation for 120 min in Octet reaction buffer. The data was then fitted by ForteBio Octet Data analysis 8.2, parallel referencing the IgG₄ isotype control using a 1:1 fit model for calculating the association rate (K_a , on-rate), dissociation rate (K_{off} , off-rate), and dissociation constant (KD) of the IgE anti-MAB072 antibody positive controls. The experiment was performed minimally in triplicate, and mean values were reported.

Generation of IgE Receptor FcεRIα

Human IgE receptor FcεRIα with histidine tag was synthesized and then subcloned into a mammalian expression vector and verified by DNA sequencing. The recombinant FcεRIα protein was expressed using Expi293 expression system (as described in [Generation of IgE Receptor FcεRIα](#)). The expression mixture was harvested via 2500 xg spin and filtration with a 0.2-μm Opticap filter. The harvested material was loaded onto HisPrep Ni-NTA affinity column on purifier system at 2.0 mL/min overnight. The column was washed, and the protein was eluted. Using an Amicon 3 k MWCO filter, the protein was concentrated to about 2.5 mL, followed by gel filtration on SuperDex 200 using Dulbecco's phosphate buffered saline. Fractions collected from the gel filtration were analyzed on size exclusion HPLC. Selected fractions were pooled and filtered through a 0.2-μm syringe filter.

IgE Anti-MAB072 Antibody Assay Procedure

An ELISA method was developed to detect IgE anti-MAB072 antibodies, which consists of a screening assay and a confirmatory assay. The screening assay was designed to detect IgE antibodies capable of binding to MAB072. Samples that were reactive in the screening step were further tested to confirm their binding specificity, by adding MAB072 in the confirmatory assay to inhibit the assay response of the reactive samples. In this method, a high affinity IgE receptor FcεRIα was utilized as a capture reagent. Biotinylated MAB072 followed by streptavidin-poly HRP (SA-HRP) was used as the detection reagents (Fig. 1). Additionally, an IgE anti-MAB072 antibody mAb was utilized as the assay positive

control, to facilitate detection of drug-specific IgE antibodies and to monitor the system suitability during assay development, validation, and sample analysis. Details of this procedure are described as follows.

In the screening assay, Nunc® Immobilizer™ Amino plates were coated with 1 μg/mL of the IgE receptor FcεRIα overnight at 2–8 °C, followed by blocking the plate with 0.1 M HEPES containing 1% casein and 0.5% Tween-20 for 1 h at room temperature. Serum samples and controls pre-diluted at the minimum required dilution (MRD, 1:50) were added to the coated plates and incubated for 2 h at room temperature to capture IgE antibodies in the samples and controls. After washing the plate, IgE anti-MAB072 antibodies that were captured on the plate were then allowed to bind to biotinylated MAB072 at 1 μg/mL during an overnight incubation at room temperature, followed by the addition of SA-HRP to detect the captured immune complex. The activity of the bound HRP was quantitated by color development after the addition of 3,3',5,5'-tetramethylbenzidine. The absorbance of the reaction mixture was measured at 450 nm (as the measurement wavelength) and 650 nm (as the reference wavelength), upon adding diluted sulfuric acid to stop color development. The absorbance measurement was proportional to the level of MAB072-specific IgE antibodies in the samples. A sample would be scored as screen-positive if the absorbance was equal to or greater than the plate-specific cut point (PSCP). The PSCP was calculated by multiplying the mean optical density (OD) value of the negative control (NC) by the screening assay cut point factor (CPF), which was predetermined using drug-naïve donor serum samples or baseline samples collected during clinical trials.

Samples tested positive in the screening assay were evaluated for the binding specificity using the confirmatory assay. In the confirmatory assay, the samples and controls were tested following the screening procedure described above, except that the biotinylated MAB072 buffer was supplemented with 50 μg/mL of unlabeled MAB072 to inhibit the binding of biotinylated MAB072 to the captured IgE drug-specific antibodies at the detection step. A sample would be confirmed positive if the percent reduction of OD, in the presence vs. absence of MAB072, was equal to or greater than the confirmatory cut point (CP).

This drug-specific IgE method was fully validated to have the requisite sensitivity, precision, selectivity, specificity, and robustness for its intended use. More importantly, this bioanalytical method was validated for its tolerance to endogenous, potentially interfering substances, including the drug MAB072, the drug target, IgG ADA, and nonspecific IgE antibodies, as described in detail in [Sections Screening Assay CPF and Confirmatory, Assay Sensitivity and Hook Effect, Establishment of Positive Control Concentrations, Target Interference, Interference of Serum Total IgE Antibodies, Interference of IgG ADA, Assay Selectivity and Matrix Effect, Assay Precision, Method Robustness and Positive Control Stability](#).

Screening Assay CPF and Confirmatory CP

The screening assay CPF was established by testing serum samples from a panel of 80 drug-naïve donors by two analysts, three times each over a minimum of 3 days. In each screening assay plate, individual samples were analyzed in duplicate along with 8 replicates of negative control samples.

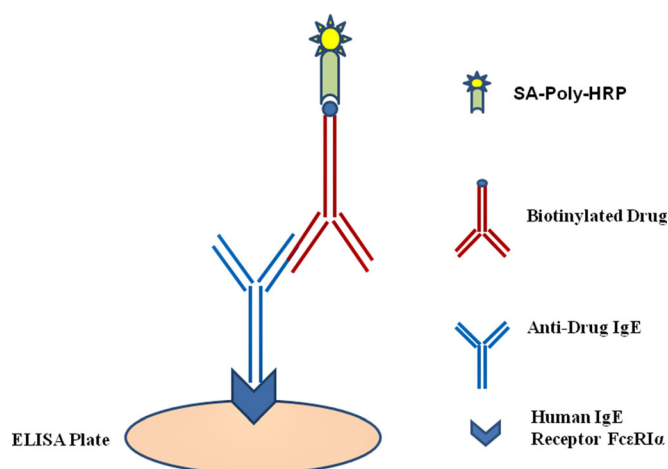


Fig. 1. Detection of IgE antidrug antibodies utilizing an ELISA

Normalized signal-to-noise (S/N) ratios were calculated by dividing the individual mean sample OD values by the mean OD value of the NC samples for each assay plate. The boxplot method was utilized to identify statistical outliers. The remaining S/N ratios were then compiled to calculate the screening CPF with a 5% false-positive rate, following the robust parametric approach.

The confirmatory assay CP, which was used to confirm a sample as positive, was defined as the percent (%) inhibition of the assay signal in the presence of the drug. The confirmatory CP was determined by testing serum samples from a panel of 100 drug-naïve donors by two analysts, three times each over a minimum of 3 days. Individual samples were analyzed in duplicate in the presence or absence of MAB072. The individual sample mean OD values in the presence of MAB072 were divided by the respective sample mean OD values in the absence of MAB072. The % inhibition was calculated using the following equation:

$$\%OD\ inhibition = \left(1 - \frac{Mean\ OD\ with\ drug}{Mean\ OD\ without\ drug}\right) * 100$$

where OD is the absorbance measurement.

The confirmatory CP was calculated with a 1% false-positive rate, following the robust parametric approach.

Assay Sensitivity and Hook Effect

The screening assay sensitivity was defined as the concentration of the positive control antibody, which consistently produced an assay signal at the PSCP. To determine the assay sensitivity of the screening assay, the IgE anti-MAB 072 antibody positive control was spiked into pooled normal human serum (PNHS) at 5 ng/mL and diluted 1:2 with PNHS, yielding samples at concentrations of 2.5, 1.25, 0.625, 0.313, and 0.2 ng/mL. Individual samples were tested in duplicate in screening assays. The mean assay responses (OD) were plotted and interpolated against the positive control concentrations on a Log₁₀ scale using 5-parameter curve-fitting model in SoftMax Pro. Eighteen curves were generated by two analysts, each analyst performing three assay runs per day over three different days. The final assay sensitivity was

calculated as the mean of these 18 measurements, upon back-transformation to a linear scale.

Additionally, prozone effect (hook effect) was evaluated by spiking 100 µg/mL of the IgE anti-MAB072 antibody positive control into PNHS, followed by a 1:5 serial dilution in PNHS. These samples were analyzed in the screening assay, and the assay responses (OD) were plotted against the positive control concentrations.

The confirmatory assay sensitivity was defined as the positive control concentration at the confirmatory CP. Separate confirmatory assays were set up to determine the confirmatory CP. The IgE anti-MAB072 antibody positive control was spiked into PNHS at 5 ng/mL and serially diluted 1:2, yielding samples at concentrations of 2.5, 1.25, 0.625, and 0.313 ng/mL. Individual samples were tested in duplicate in confirmatory assays. The % inhibition values were plotted and interpolated against the positive control concentrations on a Log₁₀ scale using SoftMax Pro. The final assay sensitivity was calculated as the mean of the individual concentrations at the confirmatory CP, upon back-transformation to a linear scale.

Establishment of Positive Control Concentrations

A low positive control (LPC), a high positive control (HPC), and the NC were established as the assay system suitability controls for the screening and confirmatory assays. The LPC was prepared by spiking the IgE anti-MAB072 antibody positive control into PNHS at a concentration that was statistically determined to give a 1% assay rejection rate, based on the sensitivity curves described in Section [Assay Sensitivity and Hook Effect](#), according to the following formula:

$$LPC\ Concentration = Mean\ Assay\ Sensitivity + t_{0.01df} * SD$$

where *df* is the estimated total degree of freedom, *SD* is the standard deviation of the sensitivity measurements, and *t* is the distribution.

The HPC was prepared by spiking the positive control antibody into the PNHS at 8.7 ng/mL of the positive control.

Drug Tolerance

The assay's tolerance to the interference of MAB072 was assessed by spiking PNHS with the IgE anti-MAB072 antibody positive control at the LPC concentration (1.7 ng/mL) and with MAB072 at 300, 200, 100, 50, 25, 10, 5, or 0 µg/mL. The samples were allowed to stand at room temperature for at least 30 min to allow for the formation of MAB072-IgE anti-MAB072 antibody positive control complex prior to testing the samples in the screening assay.

Target Interference

Potential interference of the target of MAB072 was evaluated by spiking PNHS with the IgE anti-MAB072 antibody positive control at the LPC concentration and the drug target at 100, 50, 20, 10, 5, 2, 1, or 0 µg/mL. The range of the target concentration was selected to cover the potential concentration of the target in subjects treated with MAB072. The samples were analyzed in the screening assay.

Interference of Serum Total IgE Antibodies

Potential interference of human total serum IgE was assessed by spiking PNHS with the IgE anti-MAB072 antibody positive control at the LPC concentration and nonspecific human total IgE at 20, 15, 10, 5, 2, 1, 0.5, and 0 µg/mL. The samples were analyzed in the screening assay.

Interference of IgG ADA

Potential interference of IgG anti-MAB072 antibody was assessed by spiking PNHS with the IgE anti-MAB072 antibody positive control at the LPC concentration and IgG anti-MAB072 idiotypic mAb at 100, 50, 20, 10, 4, 2, 1, and 0 µg/mL. These samples were tested in the screening assay.

Assay Selectivity and Matrix Effect

The assay selectivity was evaluated by testing serum samples from 10 drug-naïve donors, which were spiked with the IgE anti-MAB072 antibody positive control at the LPC level, in the screening assay. The respective unspiked individual serum samples were also analyzed to monitor baseline assay response.

Potential interference of hemolytic and lipemic serum matrix was also tested in the screening assay. Specifically, hemolyzed blood was spiked into NC, LPC, and HPC samples, such that the proportion of hemolyzed blood was 2% and 10% in neat serum. Triglycerides were spiked at 150 and 500 mg/dL into NC, LPC, and HPC samples, to evaluate the lipemic matrix effect.

Assay Precision

The intra- and inter-assay precisions were evaluated using NC, LPC, and HPC data that were generated in 12 assay plates, tested by 2 analysts over 3 days, with each analyst testing 2 assay plates per day in both the screening and confirmation assays. Three sets of controls (NC, LPC, and HPC) were tested in duplicate wells on each plate.

Method Robustness and Positive Control Stability

The robustness of the method was tested by assessing the impact of minor variations to the procedure. A set of 14 NC replicates, 5 LPCs in duplicate, and 5 HPCs in duplicate were analyzed under two extreme conditions – one with minimum and the other with maximal incubation times at each of the procedural steps, such as plate coating time, blocking time, sample incubation time, detection antibody incubation time, as well as with SA-HRP incubation time and substrate incubation time.

The IgE anti-MAB072 antibody at the HPC concentrations was evaluated for its stability after six freeze/thaw cycles, upon storage at 2–8 °C for 1 to 7 days, or at room temperature for 4 to 24 h, prior to testing these control samples in the screening assay.

RESULTS

Characterization of the IgE Anti-MAB072 Antibody Positive Control

Three IgE anti-MAB072 antibody positive control clones were confirmed to be of IgE isotype and to bind specifically to MAB072 by western blot using a goat antihuman IgE detector antibody, where the gel was overloaded with the drug and the IgG4 isotype control to confirm that the IgE anti-MAB072 antibodies are specific to the drug CDRs but not the IgG₄ backbone (Fig. 2). The apparent affinity of these antibody clones was evaluated using an Octet instrument (Fig. 3). All of these IgE anti-MAB072 antibody clones were demonstrated to have apparent high affinity towards the drug, with equilibrium dissociation constant (K_D) in the range of 20–120 pM range, and could be utilized as positive controls for monitoring the performance of the IgE anti-MAB072 assay for its intended use (Table 1). The IgE anti-MAB072 mAb clone H05 with the highest apparent affinity was selected as the positive control to provide suitable assay sensitivity. The IgE anti-MAB072 antibody positive control was also shown to be stable in serum in the drug-specific IgE antibody assay, for 6 freeze/thaw cycles, or in storage at 2–8 °C for over 7 days or at room temperature for over 24 h.

Establishment of Assay Cut Points

The screening cut points are defined as the level of the assay response, at or above which a sample was scored screen-positive, and below which it was scored negative. For this screening assay, a floating PSCP was calculated by multiplying the mean NC OD value of each assay plate by a CPF and applied to the samples in the plate. The CPF was assessed by analyzing a total of 480 data points generated from a panel of 80 individual donor serum samples. An aberrant assay run containing 30 data points was excluded from the statistical analysis. Individual data points having a CV greater than 20% were also excluded. Additionally, 9 statistical outliers were identified and excluded from statistical calculation of the CPF. Thus, a total of 431 data points were used to determine the CPF, which was calculated to be 1.11 (Fig. 4).

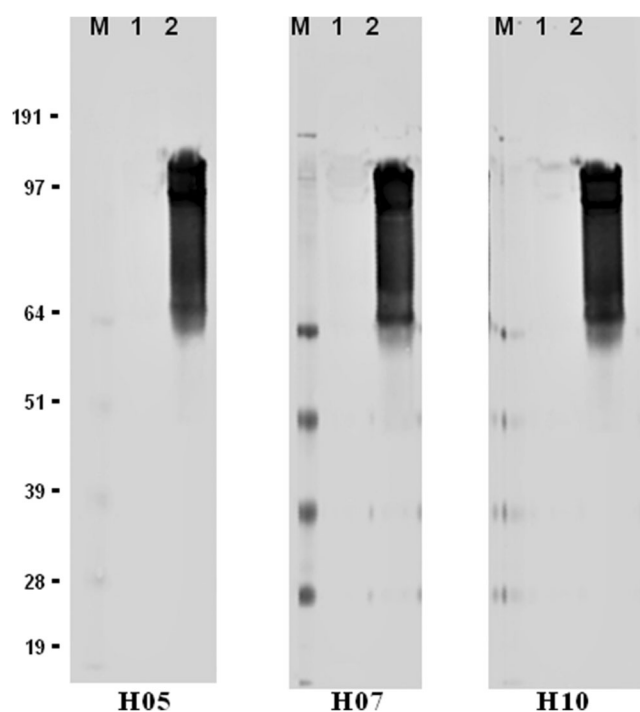


Fig. 2. Specificity evaluation of the IgE anti-MAB072 antibody positive controls by western blot. Monoclonal antibody drug MAB072 and human IgG₄ isotype control were resolved by SDS-PAGE, transferred on nitrocellulose, and then detected with H05/H07/H10 as primary antibody (indicated below each western blot) followed by goat antihuman IgE and donkey anti-goat IRDye680. Lane M, Pre-stained marker; lane 1, Isotype control IgG₄ mAb; lane 2, MAB072

The confirmatory CP was defined as the % inhibition at or above which a sample was scored confirmed positive and below which the sample was confirmed negative. In this experiment, a total of 600 data points were collected. Among them, 27 data points with CV > 20% for the OD values of duplicate wells were excluded from the statistical analysis. The remaining 573 data points were used in statistical analysis to determine the confirmatory CP, which was calculated to be 26.1% (Fig. 5).

The screening assay CPF, the confirmatory CP, and other assay performance parameters are summarized in Table II.

Assay Sensitivity and Selection of LPC Concentration

For the screening assay, each assay curve was interpolated to calculate the IgE anti-MAB072 antibody positive control concentration at the PSCP. The individual concentrations obtained from 18 dose-response curves were utilized to calculate the mean positive control concentration at PSCP, which was determined to be 0.37 ng/mL. The assay sensitivity was calculated to be 1.05 ng/mL with 95% confidence. Following the recommendations outlined in the regulatory guidance (2) and the industry white paper (14), the LPC concentration was estimated to be 1.70 ng/mL, to achieve 1% assay rejection rate.

To assess the confirmatory assay sensitivity, each confirmatory assay curve was interpolated to calculate the IgE anti-MAB072 antibody positive control concentration at the confirmatory CP. The individual concentrations obtained

from 35 confirmatory assay curves were used to calculate the mean positive control concentration at confirmatory CP, which was determined to be 0.65 ng/mL. The confirmatory assay sensitivity was determined to be 2.26 ng/mL with 95% confidence.

Tolerance to Potential Interferences

This IgE drug-specific ELISA method was evaluated for its tolerance to the potential interference of MAB072, the drug target, IgG ADA, and nonspecific total human serum IgE (Fig. 6). All samples spiked with the surrogate IgE anti-MAB072 PC at the LPC level and the MAB072 at 300 µg/mL concentration remained positive. Nonetheless, the assay signals for samples containing the drug at 25 µg/mL and below appeared to decrease substantially (Fig. 6a). This observation could be attributed to unidentified assay artifacts, as the assay responses in samples spiked with greater levels of the drug were restored to that of the sample without the drug. Therefore, the assay was concluded to be able to detect the IgE anti-MAB072 antibody positive control at the LPC concentration of 1.7 ng/mL in the presence of MAB072 tested at up to the concentration of 300 µg/mL, which substantially exceeded the projected maximal concentration of 100 µg/mL of the drug anticipated in the clinical samples.

The level of drug target can accumulate in the circulation due to drug-target complex formation in patients treated with a biologic, which causes either false-positive detection of ADA or theoretically false-negative results, depending on the assay formats and the biochemical properties of the target. The target of MAB072 was not anticipated to interfere with the detection of IgE anti-MAB071 antibodies in this ELISA format, regardless of the levels of the target. Nonetheless, high levels of the drug target were spiked into the NC and LPC samples to assess potential interference of the drug target. The spiked negative control samples remained negative in this test, whereas the LPC samples were still positive, indicating no interference.

IgE antidrug antibodies are present at an extremely low level and are prone to interference by other specific and nonspecific endogenous immunoglobulins. To evaluate the potential interference of nonspecific total serum IgE and IgG antidrug antibodies, the NC and LPC samples were spiked with up to 10 µg/mL of a nonspecific human IgE antibody or 500 µg/mL of an IgG monoclonal anti-MAB072 antibody. While all spiked LPC samples remained positive, the spiked NC samples were still all negative in the assay. The apparent 29% reduction of the assay signal in the presence of 31.5 µg/mL of IgG anti-MAB072 antibody PC is likely due to unidentified assay artifacts, as the assay signals of other spiked samples containing higher levels of the IgG antibody were restored (Fig. 6d). Overall, the data suggested that the assay will be able to tolerate potential interference of IgG antidrug antibodies.

Assay Precision, Selectivity, and Robustness

This drug-specific IgE assay has also been demonstrated to have good precision and selectivity and is able to measure IgE anti-MAB072 antibodies in hemolyzed and lipemic serum samples (Table II). The intra- and inter-assay precisions are

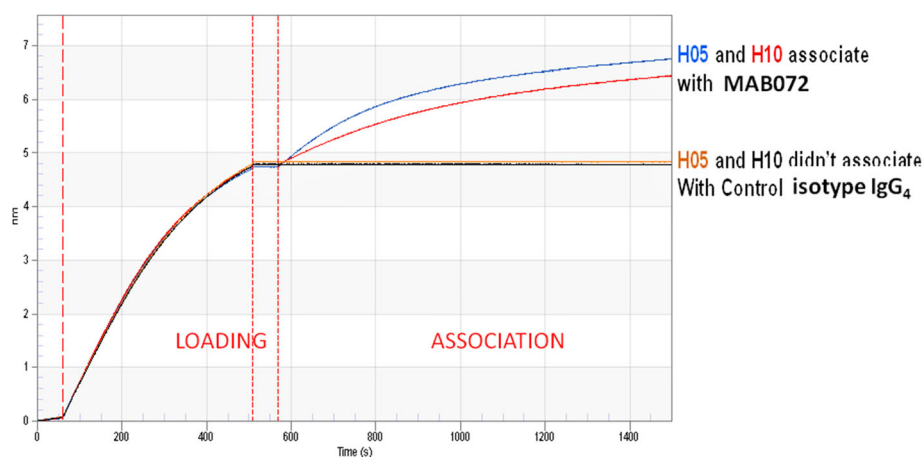


Fig. 3. Specificity evaluation and apparent affinity measurement of the IgE anti-MAB072 antibody positive controls by Octet. MAB072 and biotinylated IgG₄ isotype control IgG₄ mAb were loaded on SA sensors. The specificity for the IgE anti-MAB072 antibody positive control was determined. The affinity was also measured

summarized in Table III. Additionally, the robustness of the method was evaluated by subjecting the assay to the extreme conditions defined in the method. This method was demonstrated to perform within the acceptance criteria, with both the minimum and maximal allowable time for plate coating, plate blocking, sample incubation, detector antibody incubation, as well as the SA-HRP and substrate incubation times. The stability results for IgE anti-MAB072 antibody PC are summarized in Table II (data available through [Supplementary Materials](#)).

DISCUSSIONS

Administration of biologics can lead to hypersensitivity reactions in subjects being treated with these drugs. Type I hypersensitivity reactions (anaphylaxis), which are potentially life-threatening adverse events, result from the drug cross-linking and activating mast cells or basophils by antigen-specific IgE molecules bound to the cell surface. Anaphylaxis has been reported among patients receiving some of the biologics targeting a variety of biological pathways, including cetuximab, gemtuzumab, infliximab, omalizumab, and rituximab (6,10,15,16). While IgE anti-infliximab antibodies were detected among patients with severe Type I hypersensitivity reactions, IgE antibodies against galactose- α -1,3-galactose (α -gal) have been reported in patients treated with mAb therapeutic that was expressed in the mouse myeloma cell line SP2/0.

Recent regulatory guidance recommends that IgE antigen-specific antibodies to biologics should be evaluated

where there is a high risk for anaphylaxis or where anaphylaxis has been observed (2). While much effort has been given to the detection and characterization of total ADA, the measurement of IgE antidrug antibodies remains a challenging task. First, antigen-specific IgE is present in circulation at an extremely low concentration. As a result, the measurement of such specific IgE antibodies may be prone to the interference from ADA of other isotypes (e.g., IgG and IgM) and nonspecific total IgE antibodies, as well as the drug target and the drug itself. Secondly, utilization of a surrogate IgE positive control is critical in ensuring that the drug-specific assay is suitable for the reliable detection of IgE drug-specific antibodies as exemplified by the assays used for the testing and characterization of ADA. Nevertheless, the typical approaches used to produce either a polyclonal or monoclonal ADA positive control are not suitable for generating a drug-specific IgE positive control. A chimeric IgE antidrug antibody positive control, generated by fusing the variable regions of a drug-specific antidrug idiotype antibody with the human IgE constant regions, has been utilized in electrochemiluminescent antidrug IgE antibody assays (11). MAB072 is a human IgG₄ monoclonal antibody therapeutic targeting a soluble cytokine in circulation. The drug has the potential to induce anaphylaxis in patients treated with the drug. It is therefore important to develop an assay for measuring IgE antidrug antibodies immediately after reporting anaphylaxis in patients who were treated with the drug during clinical trials. To facilitate development of the IgE anti-MAB072 antibody assays, we have generated several IgE anti-MAB072 idiotype antibodies with high apparent

Table I. Apparent Affinity of IgE Anti-MAB072 Antibody Positive Controls

IgE anti-MAB072 antibody positive clones	KD (M) ^a	k _a (1/Ms) ^b	k _{off} (1/s) ^c
H05	2.01×10^{-11}	1.01×10^6	2.05×10^{-5}
H07	2.44×10^{-11}	9.37×10^5	2.28×10^{-5}
H10	1.19×10^{-10}	4.11×10^5	2.92×10^{-5}

^a KD, dissociation constant; k_a, association rate (on-rate); k_{off}, dissociation rate (off-rate). IgE = Immunoglobulin E

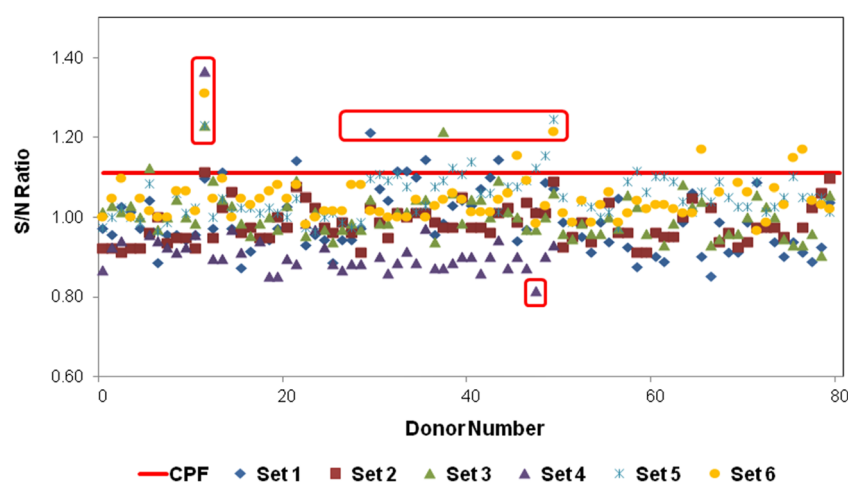


Fig. 4. Establishment of the screening assay cut point factor. Cut point factor (CPF) = 1.11. Data points within red boxes: outliers. Number of donors $N = 80$, tested by 2 analysts, 3 times each over a minimum of 3 days, generating 6 sets of data points. A total of 9 statistical outliers removed, in addition to samples with duplicate CV > 20%

affinity for the drug, in the low pM range. Anaphylaxis is induced by antigen cross-linking IgE molecules that bind to the mast cells or basophils. In animal models, high affinity antigen-specific IgE antibodies have been demonstrated to have a pathogenic role in anaphylaxis and were pathogenesis (17). These high affinity IgE anti-MAB072 antibody positive controls may be used as a tool to better monitor assay performance measuring high affinity IgE anti-MAB072 antibodies.

FcεRI is the high affinity receptor for the Fc region of IgE. It is a tetrameric immune recognition receptor consisting of one α subunit that binds (FcεRI α) to the Fc domain, one β sub-unit (FcεRI β), and two γ subunits (FcεRI γ) that regulate important accessory signaling. IgE interacts monovalently with FcεRI α . To this end, we generated the extracellular domain of high affinity FcεRI α as the capture reagent to develop this sensitive and specific IgE anti-MAB072 antibody ELISA method. In this method, unlabeled FcεRI α was directly coated on the plate as the capture reagent during an

assay run, and the only conjugated reagent was the biotinylated drug used as the detection reagent at the detection step.

This method has since been validated to have superior sensitivity, precision, selectivity, and specificity. Utilizing the screening and confirmatory cut point CPs that were statistically determined, the concentrations of the IgE anti-MAB072 positive control at the cut points were found to be 0.37 ng/mL and 0.65 ng/mL, respectively. Similar assay observations have been reported in IgE ADA assays, which utilized the biotinylated drug as the capture reagent (10,11). Nonetheless, it is important to estimate the assay sensitivity with greater consistency. When a 95% confidence was applied to calculate the assay sensitivities, the assay sensitivity was found to be 1.04 ng/mL and 2.26 ng/mL for the screening and confirmatory assays, respectively, which are well within the regulatory recommendations for antigen-specific IgE assays. By comparison, the traditional bridging assay used for measuring total anti-MAB072 antibodies has a sensitivity of 22 ng/mL (data not shown). The LPC concentration was statistically

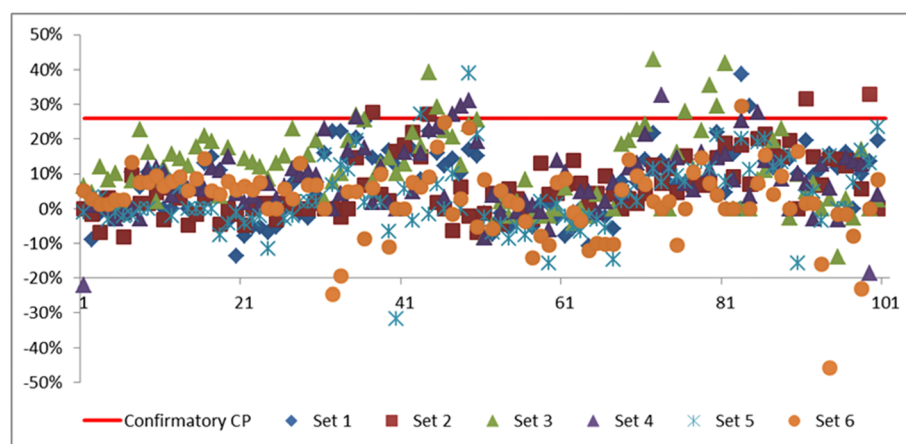


Fig. 5. Establishment of the assay confirmatory cut point. Assay confirmatory CP = 26.1%. Number of donors $N = 100$, tested by 2 analysts, 3 times each over a minimum of 3 days, generating 6 sets of data points

Table II. Summary of IgE Antidrug Antibody ELISA Method

Validation parameters	Result
MRD	1:50
Screening assay CPF	1.11
Screening assay relative sensitivity	1.05 ng/mL (95% confidence)
Screening assay hook effect	No hook effect was observed with up to 100 µg/mL
PC concentration	LPC: 1.7 ng/mL HPC: 8.7 ng/mL
Drug tolerance limit	Tolerate > 300 µg/mL of drug tested at the 1.7 ng/mL LPC level
Human total IgE tolerance	Tolerate > 10 µg/mL human total IgE tested at the 1.7 ng/mL LPC level
Drug-specific IgG interference	No interference observed with 500 µg/mL of IgG antidrug antibody tested
Matrix interference	Hemolysis: no interference observed at up to 10% hemolysis tested Lipemia: no interference observed at up to 500 mg/dL triglycerides tested
Selectivity	100% of unspiked individual samples tested negative 100% of PC-spiked individual samples at LPC level tested positive
Confirmatory CP	26.1% signal reduction for asthma populations
Precision of screening assay and confirmatory assay	See Table III
Stability of assay controls	Stable up to 6 freeze/thaw cycles
Freeze/thaw	Stable up to 24 h
Bench top (room temperature)	Stable up to 7 days
Short term storage at 2 to 8 °C	

CP = cut point; CPF = cut point factor; IgE = Immunoglobulin E; IgG = Immunoglobulin G; HPC = high positive control; LPC = low positive control; MRD = minimum required dilution; PC = positive control

estimated to be 1.7 ng/mL for the screening assay, assuming a 1% assay rejection rate.

Interference of the drug and the drug target is a critical issue in the assessment of immunogenicity (18). Such interference can adversely affect the reliable detection of ADA. Additionally, the interference can confound the interpretation and assessment of the ADA's potential impact on the PK/PD profile and correlation with safety adverse

events in patients. This IgE anti-MAB072 antibody ELISA method exhibited exceptional tolerance towards high levels of MAB072 in samples, capable of readily detecting 1.7 ng/mL of the IgE anti-MAB072 antibody positive control in the presence of 300 µg/mL of the drug tested, whereas the traditional bridging ADA method can measure 500 ng/mL of the ADA positive control in the presence of no more than 144 µg/mL of MAB072 (data not shown). The observation

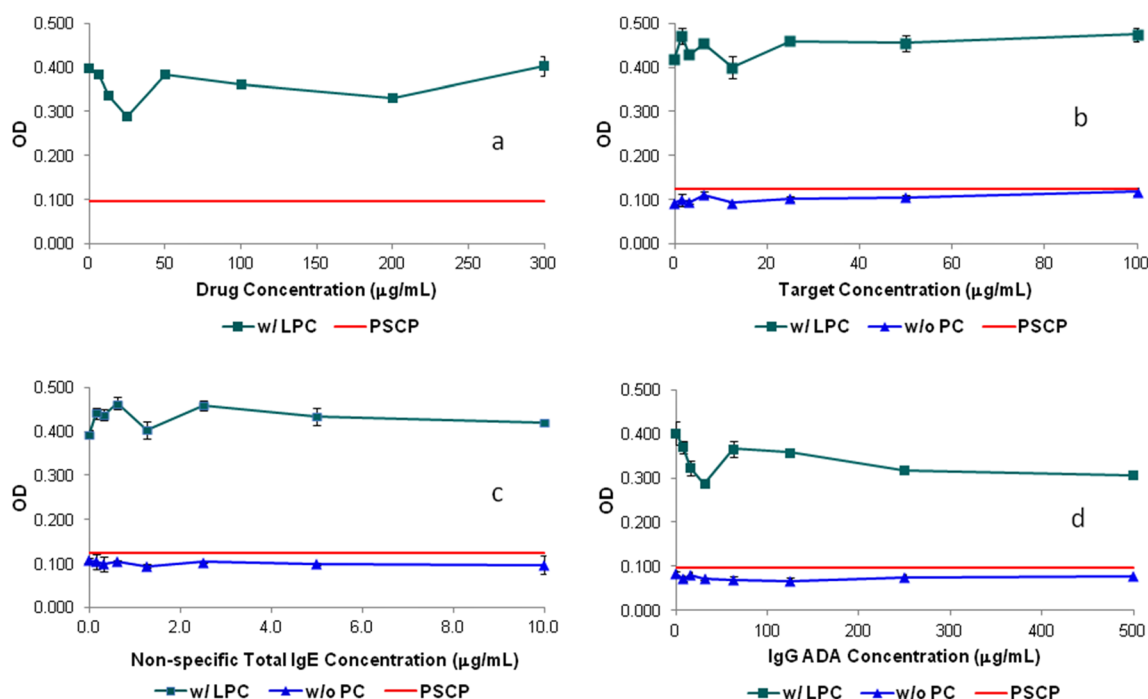


Fig. 6. Evaluation of potential interferences. Interferences of **a** MAB072; **b** drug target; **c** nonspecific total IgE; **d** IgG ADA. PC = positive control; LPC = low positive control; PSCP = plate-specific cut point

Table III. Inter- and Intra-assay Precision

Parameters	Screening assay			Confirmatory assay	
	NC	LPC	HPC	LPC	HPC
Grand mean	0.075	0.138	0.406	49.3	80.2
variance for analyst	2.82×10^{-12}	1.33×10^{-12}	1.25×10^{-11}	3.12	9.89×10^{-8}
variance for day	9.47×10^{-5}	7.63×10^{-5}	3.17×10^{-3}	15.7	4.20×10^{-10}
variance for plate	2.90×10^{-5}	1.22×10^{-4}	3.36×10^{-3}	29.8	8.54
variance for replicate	2.82×10^{-5}	1.45×10^{-4}	4.31×10^{-3}	55.9	8.96
Intra-assay %CV ^a	10.1	11.8	21.6	18.8	5.20
Inter-assay %CV ^b	13.0	6.30	13.9	8.80	0.0

^a Intra-assay precision (%CV) = $100 \times \text{SQRT}(\text{variance for plate} + \text{variance for replicate}) / \text{grand mean}$, where CV is coefficient of variation; SQRT is square root

^b Inter-assay precision (%CV) = $100 \times \text{SQRT}(\text{variance for analyst} + \text{variance for day}) / \text{grand mean}$, where CV is coefficient of variation; SQRT is square root

HPC = high positive control; LPC = low positive control; NC = negative control

that a non-bridging IgE method possesses greater drug tolerance limit than a bridging total ADA assay has also been previously reported (11). This is likely because such a non-bridging assay format does not require bridge formation of the ADA with two labeled drug conjugates in order to detect the antibodies as with the commonly used bridging assay, and thus more resistance to the interference of the drug (19). Furthermore, utilization of a high affinity FcεRIα as the capture reagent would allow for effective capture of IgE antibodies and removal of potential interfering substances in a sample during the washing steps, prior to the addition of biotinylated MAB072 for the detection of IgE anti-MAB072 antibodies, achieving greater assay sensitivity and specificity. Additionally, in this ELISA method, the unlabeled drug in a sample would not compete against the immobilized FcεRIα for binding with IgE ADAs, potentially yielding further improvement towards assay's tolerance towards drug interference. By comparison, in a conventional assay, where a conjugated drug is used as the capture reagent, the unlabeled drug in a sample would compete against the capture reagent for binding with IgE ADAs (10,11).

While high levels of the drug can prevent the detection of ADA, leading to false ADA negative results, the drug target can cause either false-positive or false-negative measurement depending upon the nature of the target and the assay design (20). When a labeled drug is used as the capture reagent, the drug target may compete with ADA for binding to the labeled drug, leading to target interference (10,11). Since the drug target is not expected to bind to the FcεRIα capture reagent, there would be no target interference anticipated with this ELISA IgE ADA method. Indeed, there was no interference observed in the NC and LPC samples that were supplemented with a high level of the drug target.

Nonspecific serum IgE antibodies can compete with the IgE anti-MAB072 antibodies for binding with FcεRIα during the capture step, leading to negative results. Because of the extremely low levels of IgE ADA, it is imperative to evaluate the potential effect of nonspecific serum IgE antibodies. This is particularly critical when an IgE ADA assay is used in evaluating patients who are known to have a higher serum concentration of nonspecific total serum IgE antibodies than

the normal population. To this end, this IgE anti-MAB072 antibody assay was shown to be able to measure the IgE antibody positive control against MAB072 in the presence of nonspecific IgE antibodies at a level exceeding what is expected in clinical samples.

Along with the total ADA assays, this IgE anti-MAB072 antibody ELISA method has been implemented to assist with the evaluation of anaphylactic events associated with MAB072 during the clinical trials. Further, the methodology can be readily adapted for assessing IgE antidrug antibodies in clinical and nonclinical development programs, where drug-related anaphylactic events are reported.

CONCLUSIONS

In conclusion, we have developed an ELISA method for measuring IgE drug-specific antibodies, which can be readily adapted and implemented to assess subjects exhibiting anaphylaxis during clinical and nonclinical studies. This assay design has been demonstrated to possess excellent sensitivity, tolerance to potential interference of drug and drug targets, resistance to interference of specific non-IgE ADA and nonspecific serum IgE antibodies, and meeting the requirements presented in the latest regulatory guidance. When evaluating the potential correlation between the onset of hypersensitivity and the measurement of drug-specific IgE antibodies, cautions need to be exercised because of the inherent instability of serum IgE antibodies (17).

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COMPLIANCE WITH ETHICAL STANDARDS

Conflict of Interest All authors except Dr. Linglong Zou are employees of Teva Pharmaceuticals Inc.

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Supplementary Materials

Table 1. Screening Assay Relative Sensitivity Data and Estimation of LPC Concentration

Run #	Mean OD for PC						Mean OD of NC	PSCP	Relative Sensitivity (ng/mL)
	5 ng/mL	2.5 ng/mL	1.3 ng/mL	0.6 ng/mL	0.3 ng/mL	0.2 ng/mL			
1	0.194	0.114	0.104	0.084	0.076	0.074	0.071	0.078	0.337
2	0.159	0.098	0.084	0.077	0.072	0.068	0.069	0.077	0.891
3	0.214	0.126	0.112	0.089	0.088	0.083	0.081	0.089	0.472
4	0.156	0.122	0.103	0.096	0.089	0.085	0.079	0.088	0.250
5	0.169	0.124	0.104	0.094	0.088	0.083	0.079	0.088	0.351
6	0.203	0.142	0.109	0.094	0.086	0.086	0.081	0.090	0.475
7	0.188	0.132	0.099	NR ^a	0.069	0.063	0.065	0.072	0.405
8	0.228	0.156	0.111	0.085	0.074	0.066	0.065	0.072	0.290
9	0.158	0.121	0.095	0.082	0.076	0.069	0.073	0.081	0.569
10	0.162	0.132	NRErrore. L'origine riferimento non è stata trovata.	0.091	0.079	0.071	0.068	0.075	0.228
11	0.278	0.197	0.127	0.094	0.081	0.076	0.071	0.079	0.261
12	0.285	0.199	0.132	0.097	0.081	0.078	0.083	0.092	0.550
13	0.151	0.122	0.097	0.093	0.082	0.080	0.082	0.091	0.766
14	0.185	0.133	0.105	0.084	0.072	0.065	0.065	0.072	0.309
15	0.148	0.115	0.100	0.088	0.079	0.076	0.077	0.085	0.535
18	0.145	0.128	0.109	0.102	0.097	0.094	0.093	0.103	0.808
19	0.254	0.170	0.116	0.089	0.076	0.069	0.061	0.067	0.096
20	0.191	0.150	0.113	0.094	0.080	0.073	0.065	0.072	0.128

Errore. L'origine riferimento non è stata trovata. Non-reportable due to duplicate wells with CV > 20%.

mL=milliliter; ng=nanogram; NC=negative control; NR=non-reportable; OD=optical density; **Errore. L'origine riferimento non è stata trovata.**PC= positive control; PSCP=plate-specific cut point.

Table 2. Evaluation of Assay Hook Effect

PC (ng/mL)	Sample Mean OD					
	Run #1	Run #2	Run #3	Run #4	Run #5	Run #6
100000.0	3.958	3.959	3.959	3.960	3.958	3.960
20000.0	3.956	3.957	3.957	3.959	3.958	3.952
4000.0	3.926	3.950	3.954	3.950	3.959	3.901
800.0	3.948	3.959	3.960	3.893	3.960	3.947
160.0	2.806	2.766	3.267	3.959	3.045	3.345
32.0	0.890	NR ^a	1.000	1.487	0.886	1.075
6.4	0.310	0.279	0.250	0.389	0.228	NRErrone. L'origine riferimento non è stata trovata.
1.3	0.094	0.105	0.099	0.138	0.100	0.122
0.3	0.077	0.082	0.072	0.078	0.074	0.080
0.1	0.079	0.082	0.072	0.073	0.069	0.084
PSCP	0.077	0.088	0.072	0.079	0.072	0.072
Hook effect?	No	No	No	No	No	No

Errore. L'origine riferimento non è stata trovata. Non-reportable due to duplicate wells CV >20%.

CV=coefficient of variation; mL=milliliter; ng=nanogram; NR=Non-reportable; OD=optical intensity;

Errore. L'origine riferimento non è stata trovata.PC=positive control; PSCP=plate-specific cut point.

Table 3. Evaluation of Drug Tolerance Limit

MAB072 Concentration (µg/mL)	Sample Mean OD with LPC at 1.7 ng/mL	%CV	PSCP
300	0.404	5.3%	0.097
200	0.331	0.6%	0.097
100	0.362	1.0%	0.097
50	0.385	1.2%	0.097
25	0.289	0.0%	0.097
12.5	0.337	3.0%	0.097
6.25	0.384	2.5%	0.097
0	0.399	0.9%	0.097

CV=coefficient of variation; LPC=low positive control; µg=microgram; mL=milliliter; ng=nanogram;
OD=optical density; PSCP=plate-specific cut point.

Table 4. Interference of the Drug Target

Concentration of the Drug Target (µg/mL)	Sample Mean OD	%CV	Sample Mean OD	%CV	PSCP
	with LPC at 1.7 ng/mL		NC		
100	0.475	3.1%	0.118	6.5%	0.124
50	0.456	4.0%	0.106	3.3%	0.124
25	0.459	0.2%	0.102	4.9%	0.124
12.5	0.400	6.4%	0.093	0.5%	0.124
6.25	0.454	1.6%	0.111	6.1%	0.124
3	0.429	1.1%	0.094	4.8%	0.124
2	0.472	3.8%	0.099	12.9%	0.124
0	0.419	2.0%	0.092	4.1%	0.124

CV=coefficient of variation; LPC=low positive control; µg=microgram; mL=milliliter; NC=negative control; ng=nanogram; OD=optical density, PSCP=plate-specific cut point.

Table 5. Interference of Human Total IgE Antibodies

Human Total IgE (µg/mL)	Sample Mean OD	%CV	Sample Mean OD	%CV	PSCP
	with LPC at 1.7 ng/mL		NC		
10	0.420	0.5%	0.097	22%	0.124
5	0.434	4.3%	0.099	4%	0.124
2.5	0.460	2.3%	0.104	1%	0.124
1.25	0.404	4.8%	0.094	5%	0.124
0.625	0.464	3.1%	0.105	2%	0.124
0.313	0.437	2.9%	0.100	17%	0.124
0.156	0.442	2.8%	0.105	16%	0.124
0	0.393	2.2%	0.107	4%	0.124

CV=coefficient of variation; IgE=immunoglobulin E; LPC=low positive control; µg=microgram; mL=milliliter; NC=negative control; ng=nanogram; OD=optical density, PSCP=plate-specific cut point.

Table 6. Interference of IgG Anti-MAB072 Antibody

IgG Anti-MAB072 Antibody Concentration (µg/mL)	Sample Mean OD	%CV	Sample Mean OD	%CV	PSCP
	with LPC at 1.7 ng/mL		NC		
500	0.306	1.0%	0.078	0.018	0.097
250	0.317	2.3%	0.075	0.011	0.097
125	0.358	0.0%	0.066	0.113	0.097
62.5	0.366	5.2%	0.070	0.123	0.097
31.3	0.288	1.6%	0.072	0.036	0.097
16	0.323	5.2%	0.080	0.010	0.097
8	0.371	3.9%	0.071	0.080	0.097
0	0.403	6.6%	0.084	0.038	0.097

CV=coefficient of variation; IgG=immunoglobulin G; LPC=low positive control; µg=microgram; mL=milliliter; NC=negative control; ng=nanogram; OD=optical density; PSCP=plate-specific cut point.

Table 7. Evaluation of Assay Selectivity

Donor ID	Individuals Unspiked			Individuals Spiked with 1.7 ng/mL PC		
	Mean OD	%CV	PSCP 0.069	Mean OD	%CV	PSCP 0.069
D01	0.061	0	Negative	0.107	4	Positive
D02	0.060	6	Negative	0.104	12	Positive
D03	0.060	4	Negative	0.089	4	Positive
D04	0.063	4	Negative	0.119	4	Positive
D05	0.061	1	Negative	0.115	2	Positive
D06	0.061	3	Negative	0.124	7	Positive
D07	0.061	3	Negative	0.115	4	Positive
D08	0.062	4	Negative	0.102	1	Positive
D09	0.063	3	Negative	0.106	2	Positive
D10	0.062	0	Negative	0.093	4	Positive

CV=coefficient of variation; ID=Identification; OD=optical density; PC= positive control; PSCP=plate-specific cut point.

Table 8. Interference of Hemolysis and Lipemia

Matrix Type	Level	NC		LPC (1.7 ng/mL)		HPC (8.7 ng/mL)	
		Mean OD	PSCP 0.069	Mean OD	PSCP 0.069	Mean OD	PSCP 0.069
Hemolyzed Blood	2%	0.062	Negative	0.113	Positive	0.376	Positive
	10%	0.061	Negative	0.117	Positive	0.381	Positive
Triglyceride	150 mg/dL	0.059	Negative	0.127	Positive	0.373	Positive
	500 mg/dL	0.060	Negative	0.120	Positive	0.392	Positive

dL=deciliter; HPC=high positive control; LPC=low positive control; mg=milligram; mL=milliliter; ng=nanogram; NC=negative control; OD=optical density; PSCP=plate-specific cut point.

Table 9. IgE Anti-MAB072 Antibody Positive Control Stability

Testing Condition	NC		HPC (8.7 ng/mL)	
	Mean OD	Results	Mean OD	Results
1 F/T cycle	0.077	Negative	0.385	Positive
2 F/T cycles	0.071	Negative	0.468	Positive
3 F/T cycles	0.072	Negative	0.380	Positive
4 F/T cycles	0.072	Negative	0.338	Positive
5 F/T cycles	0.070	Negative	0.408	Positive
6 F/T cycles	0.073	Negative	0.362	Positive
24±2 hrs at 2-8°C	0.074	Negative	0.285	Positive
7±1 days at 2-8°C	0.073	Negative	0.402	Positive
4±1 hrs at RT	0.072	Negative	0.418	Positive
24±2 hrs at RT	0.073	Negative	0.300	Positive
PSCP	0.080			

F/T=Freeze/Thaw; HPC=high positive control; hrs=hours; NC=negative control; OD=optical density; RT=room temperature; PSCP=plate-specific cut point.