



Research paper

Analytical validation of a highly sensitive microparticle-based immunoassay for the quantitation of IL-13 in human serum using the Erenna[®] immunoassay system

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ABSTRACT

IL-13 is a Th2 cytokine that has been shown to be an important mediator of airway inflammation contributing to asthma lesions. Given its proposed role in asthma, measurements of this cytokine in serum may provide insights into disease mechanisms, progression and pharmacodynamic effects of IL-13 targeted therapeutics. However, current commercially available ELISA immunoassays are frequently unable to detect baseline concentrations of IL-13 in serum from healthy individuals, which are below the limit of detection. Here we describe the use of the novel microparticle-based Erenna[®] IL-13 human immunoassay (Singulex, Inc.), which utilizes proprietary antibodies and single molecule counting technology, to quantify IL-13 from 100 μ L of serum from apparently healthy subjects and clinically defined symptomatic and asymptomatic asthma subjects. The lower limit of quantification of the Erenna assay was validated at 0.07 pg/mL and the assay detected baseline concentrations of IL-13 in 98% of serum samples tested. The calibration curve showed good precision over the entire linear range of 0.07–50 pg/mL, with inter-assay imprecision <10% CV except at the lowest concentration tested (<15%). The intra- and inter-assay imprecision of spiked serum samples containing three different IL-13 concentrations (2, 8, and 25 pg/mL) ranged from 2.2–2.4% and 6.1–6.8%, respectively. Using the Erenna IL-13 assay, we observe that serum IL-13 concentrations range from <0.07–1.02 pg/mL in apparently healthy subjects ($N=60$) with similar ranges in asymptomatic (0.07–0.66 pg/mL, $N=26$) and symptomatic (<0.07–1.26 pg/mL, $N=96$) asthma subjects. The Erenna immunoassay improved sensitivity by over two full logs compared to previous ELISA methods, while using smaller sample volumes. In addition, the Erenna assay reliably measured IL-13 in endogenous and spiked human serum samples that were not quantifiable using other methods. Taken together, these results show that this novel assay offers a significant improvement over previous methods for high-sensitive quantitative measurement of IL-13 in human serum samples obtained from both apparently healthy and asthmatic subjects, and can be used in future clinical studies to accurately measure concentrations of this cytokine prior to and following drug therapy in human serum.

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Abbreviations: IL-13, Interleukin-13; PD, pharmacodynamic; AHR, airway hyperresponsiveness; BAL, bronchial lavage fluid; LoD, limit of detection; LLoQ, lower limit of quantification; ULoQ, upper limit of quantification; CV, coefficient of variance; SD, standard deviation.

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1. Introduction

Interleukin 13 (IL-13) is a Th2 cytokine that has been shown to regulate inflammatory responses contributing to lesion development in allergy and asthma (Mentink-Kane and Wynn, 2004; Wills-Karp, 2004). Although IL-13 and IL-4 signal through shared receptors (LaPorte et al., 2008; Minton, 2008), it has been shown that IL-13 regulates asthma independently of IL-4 (Grünig et al., 1998). IL-13 has been directly implicated in several aspects of the pathophysiology of asthma (Foster et al., 2002), including airway hyperresponsiveness (AHR) and mucus production, as well as regulation of extra cellular matrix proteins which contribute to fibrosis and chronic airway remodeling (Kasaian and Miller, 2008; Morse et al., 2002). Thus, IL-13 has emerged as a therapeutic target for asthma, and pre-clinical results for use of an anti-IL-13 monoclonal antibody have been reported in mice (Yang et al., 2004, 2005), sheep (Kasaian et al., 2007) and non-human primate models (Bree et al., 2007; Kasaian et al., 2008) of respiratory disease.

IL-13 has been detected in bronchial tissue and induced sputum of asthmatic subjects. Increased concentrations of IL-13 mRNA (Prieto et al., 2000) and protein (Batra et al., 2004) have been quantified in bronchial lavage fluid (BAL) following allergen challenge. However, BAL samples are very difficult to obtain, and have hindered studies in sensitive groups such as pediatric subjects (Becker, 2002; Tang and Powell, 2001). IL-13 has been measured in nasal lavage fluid (Noah et al., 2006) and nasopharyngeal aspirates (Feleszko et al., 2006) of asthmatic and healthy children, but measurements of systemic concentrations in less invasive specimens such as serum have been difficult using ELISA-based assays (Karaman et al., 2007). While circulating serum concentrations of IL-13 may not represent the proposed site of action of therapeutic candidates against this pathway in asthma, accurate measurements of IL-13 in the circulation could enable an accessible demonstration of pharmacodynamic effects and/or target engagement of IL-13 targeting therapies.

Although increased concentrations of serum IL-13 have been described in chronic asthma, the limits of detection (LoD) for ELISA-based assays have led to conflicting measurements (Silvestri et al., 2006). A previous study using an ELISA IL-13 assay showed correlation between increased IL-13 concentrations and acute asthma (Lee et al., 2001), but there is little clarity concerning baseline concentrations of IL-13 in asymptomatic asthma patients compared to healthy controls. A small number of groups have reported mean IL-13 concentrations in healthy subjects of roughly 2–10 pg/mL using a bead-based multiplex cytokine assay (Stabler et al., 2004; Szodoray et al., 2007; Yasui et al., 2008). A recent study of atopic control subjects, who were otherwise healthy, showed a wider range of IL-13 concentration from less than the lower limit of quantification (LLOQ) to 1000 pg/mL, as might be expected for this more sensitive population (Hartl et al., 2006). Overall, these studies present a very broad range of IL-13 concentrations in human serum from populations that could be used as control cohorts in case control studies of asthma.

With current focus on IL-13 as a potential therapeutic target for asthma, the need for more sensitive and robust IL-13 immunoassays for investigating target engagement, pharma-

codynamics (PD) and pharmacokinetics (PK) becomes crucial. The development of immunoassays is especially important for modern biologics (Baumann, 2006) as candidate therapies progress towards clinical trials, where activity of a given therapy needs to be shown with respect to a baseline cohort of disease free, asymptomatic, or placebo control subjects. Current commercial immunoassays for IL-13 either cannot detect baseline concentrations of analyte due to high LLOQs, or they result in high numbers of non-quantifiable samples in healthy or asymptomatic subjects (Martin et al., 2006).

Recently, a novel immunoassay technology platform has been used for quantifying baseline concentrations of cytokines in serum from healthy individuals (Lu et al., 2008). This technology improves the sensitivity of conventional ELISA assays by up to three orders of magnitude (Todd et al., 2007) through a combination of two methods. First, the assay uses paramagnetic microparticles (MPs) as the solid phase support for immune-capture, which increases the surface area several-fold over plate-based ELISA methods (Lim and Zhang, 2007). Second, the instrumentation platform incorporates a digital counting system that detects single molecules as they pass through an interrogation space via capillary flow, resulting in a count of discrete detected events above background rather than a sum of total fluorescence including background. In addition, this technology has been used to improve the sensitivity of quantification for other critical analytes for which sensitivity has been a limiting factor, including VEGF (Puskas et al., 2008) and cardiac troponin-I in humans (Wu et al., 2006) and in rats (Schultze et al., 2008). In the present study, we demonstrate the application of this novel technology towards quantification of baseline concentrations of IL-13 in serum samples from healthy, asymptomatic, and symptomatic asthma subjects.

2. Materials and methods

2.1. Reagents

Recombinant human IL-13 (catalog # 213-IL/CF) was purchased from R&D Systems and used for preparation of spiked validation samples. The Erenna IL-13 human immunoassays were developed and manufactured by Singulex, Inc. (Alameda, CA) specifically for Wyeth. IL-13 calibrator (R&D Systems, Minneapolis, MN; catalog # 213-IL/CF) was prepared by Singulex, Inc. and supplied with the Erenna IL-13 human immunoassay at 1 µg/mL in calibrator matrix. This reference material was used to generate a calibration curve for each analytical run. Mouse anti-IL-13 antibodies Jin2 and Ab64 (both IgG₁, k; Wyeth Research) were used for capture and detection, respectively. These antibodies recognize distinct and non-overlapping epitopes of IL-13.

2.2. Collection, handling, and storage of serum from healthy and asthmatic subjects

Human serum samples from apparently healthy subjects ($N=60$) were obtained from Bioreclamation, Inc. (Hicksville, NY). A donor is determined to be “healthy” based on criteria set by either the FDA or the AABB (American Association of Blood Banks). Donors must have a temperature reading, blood pressure reading and a pulse reading within an acceptable

range. A minimum donor body weight is also required, and for repeat donors a valid explanation is necessary if the donor has exhibited a weight loss of more than 10 lb from donation to donation. A hematocrit reading within acceptable range is also necessary for a donor to be considered healthy. Information is recorded on each donor at the time of interview and kept on file as a part of the donor's permanent record.

Whole blood was drawn into serum separator tubes and incubated at RT for 2 h. Samples were centrifuged at 4 °C (1300 ×g; 20 min) within 30 min of collection. Aliquots of serum were immediately frozen at –70 °C or colder. Serum samples were shipped to the Wyeth Biomarker Laboratory on dry ice and stored at –70 °C or colder. Serum samples from 96 symptomatic and 26 asymptomatic asthmatic subjects were procured from 2 phase II clinical trials and all samples were obtained with the appropriate informed consents and institutional review board approval.

Critical inclusion criteria for enrollment in the symptomatic asthma trial were as follows: non-smoking men and women aged 18–80 years with diagnosis of asthma per the Global Initiative for Asthma Guidelines (GINA); stable on moderate- to high-inhaled corticosteroid (ICS) with or without long-acting β -agonist (LABA) and remain stable until day 112; a forced expiratory volume in 1 s (FEV1) of 55%–80% at screening and at baseline; demonstrated or historical improvement in FEV1 (reversibility) of $\geq 12\%$ at screening or at an unscheduled visit before baseline; and a mean score of ≥ 2 on the Asthma Control Questionnaire-5 (ACQ-5) by the baseline visit (Juniper et al., 1999). Symptomatic asthma subjects were excluded from the study if they had a respiratory disease other than asthma, an unstable comorbidity, an autoimmune disease, cancer or history of cancer within 5 years, or a significant respiratory or other infection or exacerbation of asthma within 4 weeks of the screening visit.

Critical inclusion criteria for enrollment in the asymptomatic asthma trial were as follows: non-smoking mild atopic asthmatic men and women aged 18–60 years whose only asthma medication was short-acting β -agonist (SABA) administered less than 2 times per week. Asymptomatic asthma subjects were excluded from the study if they had a significant respiratory or other infection or exacerbation of asthma within 4 weeks of the screening visit.

2.3. Preparation of serum validation samples

For determination of intra- and inter-assay reproducibility, three sets of spiked serum validation samples corresponding to low, mid-range, and high expected serum concentrations of IL-13 were prepared in large batches, aliquoted, and frozen at –70 °C or colder. The low, mid and high spiked validation samples were each a pool of serum from 2–3 apparently healthy subjects spiked with recombinant IL-13 that resulted in measured concentrations on the low, middle and high-end of the calibration curve, respectively (see section 2.7). Samples were prepared in large batches and frozen at –70 °C or colder until used. For the spike recovery experiments, a different set of four serum samples were each spiked with three different IL-13 concentrations, to enable determination of spike recovery using samples from four different donors (see section 2.8). For both sets of spiked serum validation

samples, the samples were centrifuged (16,000 ×g) to remove particulates and separate the lipid layer prior to analysis in the immunoassay.

2.4. Assay development

Assays were developed at Singulex using antibodies Jin2 for capture and Ab64 for detection. Initially the capture antibody was either used to coat a MaxiSorp 384-well plate (CAT#464718, Nunc), or subsequently biotinylated and used to coat streptavidin-bound paramagnetic MPs (MyOne, CAT#650-01, Invitrogen) as a solid phase support. Detection antibody was fluorescently labeled with Alexa-647 (Invitrogen) as described previously (Wu et al., 2006). Optimal capture and detection antibody concentrations were determined with an antibody matrix. The analytical sensitivity, precision and range of the resulting plate-based and MP-based assays were compared to the original in-house ELISA assay.

2.5. Sample analysis

The IL-13 concentrations in human serum samples were measured at the Wyeth Biomarker Laboratory using the Erenna human IL-13 immunoassay system as previously described (Todd et al., 2007; Wu et al., 2006). Briefly, the IL-13 assay was performed as follows: 100 μ L sera or calibrator were added to 100 μ L assay buffer containing MPs coated with biotinylated capture antibody. The resulting mixture was incubated for 2 h in a 96-well plate. The MPs were then magnetically separated, washed 1 time and incubated with 20 μ L fluorescent dye labeled detection antibody for 1 h. After 5 washes via magnetic separation, 20 μ L of elution buffer was added, and eluted detection antibody was separated from MPs using a 384-well filter plate. The eluate was then read in the Erenna immunoassay system. All samples were tested in a manner blinded to reference test results.

2.6. Analytical validation of the calibration curve and range

An independent calibration curve was run on each 96-well assay plate using the IL-13 calibrator peptide solution provided with the assay kit. Calibration samples were prepared by serial dilution of the IL-13 calibrator peptide solution into calibrator diluent solution according to manufacturer specifications, resulting in a 7-point calibration curve with a range of 0.07–50 pg/mL. Assay linearity was determined from the linear portion of the standard curve response (measured vs. expected IL-13 concentration; back-interpolated standards) and the LoD ($2 \times$ standard deviation of 0 analyte signal/slope of respective standard curve) was calculated for the MP-based IL-13 immunoassay. The intra- and inter-assay imprecision parameters of the read-back values for each of the 7 calibrators were determined from 6 analytical runs. For each analytical run, 7 calibrator concentrations were analyzed in triplicate and a back-calculated concentration for each individual calibrator data point was obtained using a four-parameter best-fit curve.

2.7. Determination of intra- and inter-assay imprecision in spiked serum samples

To determine the intra- and inter-assay imprecision of the method, IL-13 concentration was measured in each of the 3 spiked serum validation samples (low, mid and high) in triplicate over 14 independent analytical runs. Serum samples were spiked with recombinant IL-13 protein at three different concentrations (final theoretical spiking concentrations of 2, 8, and 25 pg/mL). For intra-assay imprecision, the percent coefficient of variation (%CV) for each validation sample was determined in each analytical run and the mean %CV was calculated. The inter-assay imprecision was determined in 14 independent analytical runs and expressed as %CV.

2.8. Determination of spike recovery from spiked serum samples

The accuracy of the method was determined by the recovery of IL-13 from serum samples from four different individuals, spiked at three (3) different concentrations (final theoretical spiked concentrations of 2, 5, 30 pg/mL) at a ratio of 1:9 of spiking solution (vehicle): serum (v/v). The same concentrations (same v/v ratio) of IL-13 were also spiked into PBS/BSA, and recovery of IL-13 was assessed independently in the various spiked serum samples from the four individual subjects. In this study the measured concentration of the IL-13 spiked into PBS/BSA yielded the nominal concentration of IL-13 added to the serum samples. The accuracy of the method (% recovery) was therefore calculated by dividing the measured IL-13 concentration in spiked serum plasma samples by the expected concentration. The expected concentration equals the endogenous concentration plus the nominal spiked concentration.

2.9. Determination of short-term stability of IL-13 in serum samples

The short-term stability of IL-13 in serum from four (4) subjects was assessed in 1) serum immediately processed and frozen at -70°C ; 2) in serum incubated at RT for 2 or 24 h then frozen; and 3) in serum incubated at 4°C for 2 or 24 h then frozen. The IL-13 concentration in serum immediately processed and frozen yielded the baseline IL-13 concentration for comparison. The stability of IL-13 following one, two, and three freeze/thaw cycles (1 F/T, 2 F/T, and 3 F/T, respectively) was also determined for four (4) subjects. The concentration of IL-13 in serum samples following 1 F/T cycle yielded the baseline for comparison. The % change in the IL-13 concentration for each sample was calculated based on the measured baseline concentration.

2.10. Statistical analysis

All data are shown as mean $\pm$ SD. All results and calculations were performed using Microsoft Excel, GraphPad Prism v5, and SMD Curve Fit. The mean, SD, %CV, and %Bias of the back-calculated concentrations were calculated for each analytical run (to assess intra-assay imprecision) and for all analytical runs (to assess inter-assay imprecision). While there is currently no formal guidance for selecting the LLoQ of immunoassays, the LLoQ of the present assay was pre-specified as the lowest concentration associated with less

than 25% CV and 25% bias. The upper limit of quantification (ULoQ) is the highest calibrator concentration that can be measured with a %CV and %Bias $\leq 25\%$. Biological and analytical variation was expressed as %CV.

3. Results

3.1. IL-13 assay development

The resulting assay performance of the Erenna IL-13 plate-based and MP-based assays are compared with the performance of the original ELISA-based assay in Table 1. The Erenna MP-based assay demonstrated the lowest LLoQ while the original ELISA assay showed the highest LLoQ. Overall, the Erenna plate- and MP-based IL-13 assays provide an 8-fold and 140-fold improvement over the original ELISA assay, respectively.

3.2. Erenna IL-13 immunoassay characterization

The full range and low-end standard calibration curves for the Erenna MP-based assay are shown in Fig. 1. A high degree of correlation was observed over both the full dynamic range of the assay (Fig. 1A; $y = 1.0520x - 0.2109$, $R^2 = 0.9996$) and the low-end portion of the curve (Fig. 1B; $y = 1.0153x - 0.0155$, $R^2 = 0.9996$). Analytical precision was determined over 6 consecutive assay runs, and the mean intra- and inter-assay imprecision (%CV) and accuracy (%Bias) are shown in Table 2. Inter-assay imprecision was less than 15%, with a range of 2.9–14.3% and average percent bias range of -4.8 to 1.9%. Intra-assay imprecision was less than 15%, with a range of 2.8–12.8% and average bias range of -2.8 to 6.3%. The LLoQ of the assay was 0.07 pg/mL, a concentration at which the inter-assay %CV and %Bias were both validated at 14.3% and 0% respectively. The ULoQ of the assay is 50 pg/mL, a concentration at which the %CV and %Bias were 2.9% and 1.9%, respectively. Since serum samples require no pre-dilution, the reportable range of the assay for IL-13 concentrations in human serum samples is 0.07–50 pg/mL. Taken together these data demonstrate the ability for the Erenna immunoassay to provide sensitive measurement of IL-13 concentrations across a broad dynamic range.

3.3. IL-13 assay precision in human serum

The performance of the semi-automated assay was verified by determining the intra- and inter-assay imprecision of the spiked serum validation samples (Table 3). Serum samples were spiked with recombinant IL-13 protein at 3 different concentrations (final theoretical spiking concentrations of 2, 8, and 25 pg/mL). Interpolated mean spiked IL-13 concentrations

Table 1

Sensitivity improvement of the Erenna IL-13 MP-based immunoassay over two other IL-13 immunoassay methods using identical antibody pairs.

Immunoassay	Format	Calibrator	LLoQ (pg/mL)	Improvement
Original IL-13 ELISA	ELISA	R&D Systems	9.8	–
Erenna plate assay	Plate	Singulex	1.2	8×
Erenna MP assay	MP	Singulex	0.07	140×

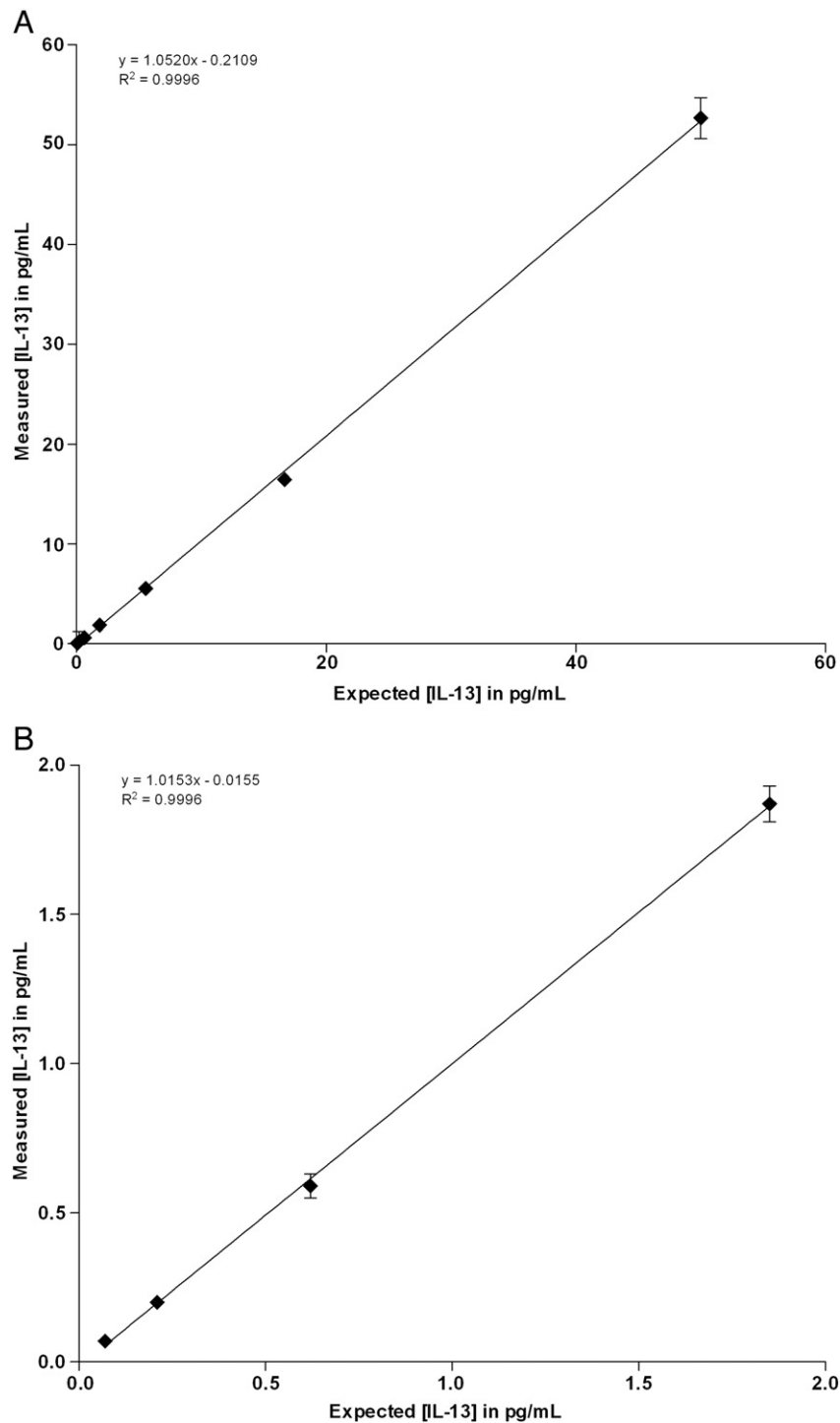


Fig. 1. Analytical performance of the Singulex IL-13 assay. Back-interpolated 7-point calibrations were generated using serial dilutions of recombinant IL-13 as the standard analyte. The full range (A) and the low-end (B) standard curve with linear correlations are shown. Each standard curve was generated using the average of three replicate determinations.

over all runs were 3.00, 10.08 and 23.38 pg/mL for each of the low, mid and high validation samples, respectively. The measured IL-13 concentration in these samples yielded the nominal concentration of the IL-13 added to the serum samples. Inter-assay imprecision (%CV) for the spiked validation samples

was less than 10%, and the low, mid, and high spiked validation samples had 6.6%, 6.1% and 6.8% CV's, respectively. Mean intra-assay imprecision (%CV) was less than 5%, and the low, mid, and high spiked validation samples had %CV's of 2.4%, 2.4% and 2.2%, respectively (Table 3).

Table 2

Analytical intra- and inter-assay precision of the Erenna IL-13 MP assay validated over 6 independent analytical runs.

	Calibration standards, [IL-13] in pg/mL						
	50	16.67	5.56	1.85	0.62	0.21	0.07
<i>Intra-assay</i>							
Mean % CV	10.1	2.8	4.0	4.7	6.1	10.4	12.8
Mean % Bias	−2.8	6.3	1.0	−1.4	−1.6	−2.4	4.8
Replicates (N)	6	6	6	6	6	6	6
<i>Inter-assay</i>							
Mean (pg/mL)	48.62	17.38	5.61	1.82	0.61	0.21	0.07
SD	1.34	0.75	0.13	0.03	0.03	0.01	0.01
% CV	2.8	4.3	2.3	1.7	4.9	4.8	14.3
% Bias	−2.8	4.3	0.9	−1.6	−1.6	<0.1	<0.1
Samples (N)	6	6	6	6	6	6	6

3.4. IL-13 recovery and short-term stability in human serum

Spiked serum samples (final theoretical spiking concentrations of 2, 5, and 30 pg/mL) had overall recoveries varying from 79.8 to 104.5%, with a mean recovery of 97.1% (Table 4). The overall mean %Bias for the three spiked concentrations across the four serum samples was −3.9% (Table 4), with a range from −20.2% to 4.5%. Analyte stability at room temperature and 4 °C is also shown in Table 4. Endogenous IL-13 concentrations in serum were stable at RT and 4 °C for up to 24 h with changes <15% (range of −2.8–6.2%), and measured IL-13 concentrations in serum were stable between 1 and 3 freeze thaw cycles with mean change from baseline <7%.

3.5. Distribution of IL-13 in healthy, asymptomatic and symptomatic asthma subjects

The validated Erenna IL-13 immunoassay was used to determine concentrations of IL-13 in serum samples from 96 symptomatic, 26 asymptomatic asthma and 60 apparently healthy subjects. Significant overlap was observed in the ranges of distribution of these three subject groups (Fig. 2). The 99th% cutoff value was 0.69 pg/mL for symptomatic and 0.56 pg/mL for asymptomatic subjects, compared to a 99th% cutoff value of 0.90 pg/mL for apparently healthy subjects.

Baseline values for each reference sample population and corresponding combined values are shown in Table 5. The average and range of baseline IL-13 is consistent among symptomatic subjects (mean ± SD: 0.23 ± 0.16 pg/mL; Range: <LLOQ–1.26 pg/mL), asymptomatic asthma subjects

Table 3

Precision of the Erenna IL-13 assay in spiked human serum.

Validation standard	Low	Mid	High
<i>Inter-assay imprecision</i>			
Mean [IL-13] pg/mL	3.00	10.08	23.38
SD	0.2	0.6	1.6
%CV	6.6	6.1	6.8
Samples (N)	14	14	14
<i>Intra-assay imprecision</i>			
Mean %CV	2.4	2.4	2.2
Range %CV	0.2–6.6	0.3–8.2	0.2–5.2

Table 4

Spike recovery and analyte stability using the Erenna IL-13 assay.

<i>Spike recovery</i>				
Mean % Change	97.1%			
Mean % Bias	−3.9%			
Replicates (N)	4			
	Room temp (RT)		Cold storage (4 °C)	
<i>Short-term stability</i>				
Time (hours)	2	24	2	24
Mean % Change	6.2%	−2.8%	1.3%	13.4%
Replicates (N)	4	4	4	4
<i>Long-term stability</i>				
Freeze–thaw cycles	2		3	
Mean % Change	−1.5%		6.8%	
Range of % Change	−15.1 to 9.1%		−18.2 to 50%	
Replicates (N)	4		4	

(mean ± SD: 0.20 ± 0.13 pg/mL; Range: 0.07–0.60 pg/mL) and healthy subjects (mean ± SD: 0.25 ± 0.19 pg/mL; Range: <LLOQ–1.02 pg/mL). Consistent with this observation, reference ranges calculated by the mean ± 2SD method and 99th% cutoff values were comparable between the two groups. All but one sample was quantifiable at baseline in both healthy subjects (98.33% quantifiable) and asymptomatic asthma subjects (98.96% quantifiable) using the Erenna IL-13 assay.

3.6. Biological variability of IL-13 in asymptomatic asthma subjects

Biological variability was measured in a reference sample population of 26 asymptomatic asthma subjects over 15 days. Measurements taken at baseline (Day 1) and on two subsequent days (Days 13 and 15) are shown in Table 6. Mean IL-13 serum concentration and range for each individual is shown in Fig. 3.

4. Discussion

Many biomarkers are present in circulation at very low concentrations often close to the LLOQ of existing immunoassays. In the case of asthma, there is a specific need for improved sensitivity in order to measure the very low circulating concentrations of IL-13 to study the role of IL-13 as well as study pharmacodynamic effects of drugs. While IL-13 immunoassays and reagents are commercially available, they typically lack sensitivity needed to measure IL-13 in baseline healthy subjects, and result in a large number of non-quantifiable samples in asymptomatic asthma subjects.

Here we introduce a new method for quantifying IL-13 in human serum using the novel Erenna immunoassay system. The improved sensitivity of this method (LLOQ of 0.07 pg/mL) enables measurements of IL-13 in most subjects. When compared to previous methods which use the identical antibody pair (Table 1), the Erenna MP-based assay increased sensitivity by up to two full logs. When compared to the upper 99th% percentile limit determined in this study, the Erenna MP-based IL-13 assay was the only assay tested which could quantify baseline concentrations of IL-13 in serum from symptomatic, asymptomatic asthmatic and healthy subjects

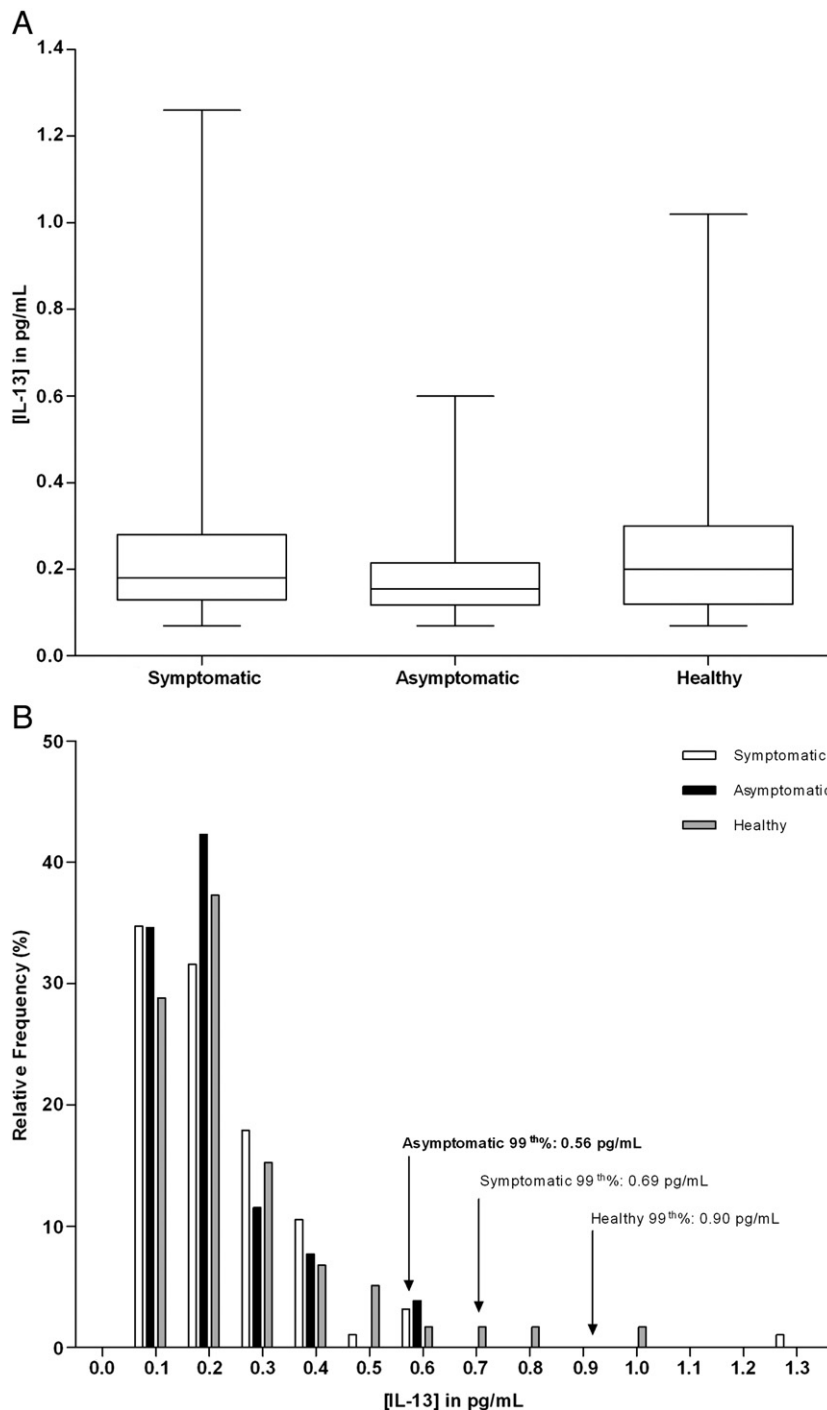


Fig. 2. Distributions of IL-13 in serum from asymptomatic, symptomatic and healthy subjects represented in a box-and-whiskers plot (A). Box boundary illustrates the interquartile range with line across denoting the median and whiskers showing the range of quantifiable values. All quantifications are the results of three determinations. Relative frequency distributions of the 3 subject groups are plotted in (B) with their respective 99th% cutoff value.

below the 99th% of the reported reference range. Together these results suggest that the present MP-based assay method is a significant improvement over previous methods.

We show that this novel assay offers a significant improvement over previous methods for ultra-sensitive quan-

titative measurement of IL-13 in human serum samples obtained from both apparently healthy and asthmatic subjects, and could be considered for use in clinical studies of asthma. We have used this assay to determine a preliminary assessment of the range of circulating concentrations of IL-13

Table 5

Baseline IL-13 measurements in healthy and asymptomatic asthmatic subjects (Day 1).

Performance parameter	Symptomatic asthmatic subjects	Asymptomatic asthmatic subjects	Healthy subjects
Total (N)	96	26	60
Mean [IL-13] (pg/mL)	0.23	0.20	0.25
Median [IL-13] (pg/mL)	0.18	0.16	0.20
± SD	0.16	0.13	0.19
Measured range [IL-13] (pg/mL)	<LLOQ–1.26	0.07–0.60	<LLOQ–1.02
99th% Cutoff value (pg/mL)	0.69	0.56	0.90
Non-quantifiable (N < LLOQ)	1	0	1
Quantifiable (% > LLOQ)	98.96%	100%	98.33%

in both healthy ($N=60$), asymptomatic asthmatic ($N=26$) and symptomatic asthmatic ($N=96$) subjects. We show that the average and range of baseline IL-13 is consistent among symptomatic asthma subjects (mean $\pm$ SD: 0.23 ± 0.16 pg/mL; Range: <LLOQ–1.26 pg/mL), asymptomatic asthma subjects (mean $\pm$ SD: 0.20 ± 0.13 pg/mL; Range: 0.07–0.60 pg/mL) and healthy subjects (mean $\pm$ SD: $0.25 \pm$

0.19 pg/mL; Range: <LLOQ–1.02 pg/mL). We also demonstrate that although intra-individual biological variation of baseline IL-13 in serum from asymptomatic asthma subjects is high, this biological variation occurs within the limits of the observed healthy reference range.

The significance of these results is two-fold: 1) there is substantial overlap in peripheral IL-13 concentrations in healthy, symptomatic and asymptomatic samples and 2) there is approximate 5–10 fold variability in IL-13 concentrations across each of the subgroups. Given the well characterized performance of the assay presented in this paper, the variability observed here is likely to reflect true biological variability of this cytokine between subjects, emphasizing the need for an accurate and precise assay for tracking peripheral IL-13 concentrations within any patient/subject over time. The impact on the use of IL-13 as a biomarker for clinical studies of asthma, given the overlap in peripheral concentrations of IL-13 between healthy subjects and asymptomatic/symptomatic asthma patients, is that the clinical utility of measuring peripheral IL-13 concentrations as a disease marker may be limited. However, the utility of measuring peripheral IL-13 concentrations as a pharmacodynamic (PD) marker is clear for therapeutic agents that target IL-13 directly. This method has been developed thus far for use in human serum, but future development could extend the utility of this assay for use in different clinical

Table 6

Baseline intra-subject variability for 3 different non-episodic time-points in 26 asthmatic patients over 15 days.

Subject ID (N = 26)	IL-13 Concentration (pg/mL)			Average	SD	%CV
	Day 1	Day 13	Day 15			
1	0.15	0.13	0.16	0.15	0.01	8.5
2	0.09	0.10	0.08	0.09	0.01	12.7
3	0.15	0.12	0.14	0.14	0.02	11.5
4	0.15	0.20	0.15	0.17	0.03	17.5
5	0.14	0.18	0.17	0.17	0.02	12.4
6	0.15	0.17	0.15	0.15	0.01	7.9
7	0.26	0.29	0.23	0.26	0.03	10.0
8	0.11	0.11	0.10	0.11	0.00	3.2
9	0.20	0.19	0.15	0.18	0.03	15.4
10	0.09	0.44	*	0.27	0.25	95.2
11	0.12	0.23	0.12	0.15	0.06	40.9
12	0.42	**	0.12	0.27	0.21	77.3
13	0.17	0.17	0.19	0.18	0.01	7.8
14	0.60	0.29	0.41	0.44	0.15	35.5
15	0.08	0.10	0.09	0.09	0.01	8.2
16	0.12	0.18	0.13	0.14	0.03	23.0
17	0.07	0.08	0.09	0.08	0.01	12.0
18	0.18	0.40	0.16	0.25	0.14	55.3
19	0.20	0.23	0.19	0.21	0.02	10.2
20	0.16	0.22	0.18	0.19	0.03	14.3
21	0.20	0.37	0.24	0.27	0.09	32.9
22	0.33	0.46	0.39	0.39	0.07	16.5
23	0.33	0.38	0.33	0.35	0.03	8.3
24	0.43	0.45	0.46	0.45	0.02	3.4
25	0.11	0.15	0.14	0.13	0.02	15.6
26	0.17	0.25	0.22	0.21	0.04	18.9
Mean	0.20	0.23	0.19	0.21	0.05	16.7 ⁺
± SD	0.07	0.08	0.08	0.08	0.00	3.16 ⁺
Range	0.07–0.60	0.08–0.46	0.08–0.46	0.08–0.45	0–0.25	3.2–55.3 ⁺

*No data available on this day for this subject.

**Measurement was determined to be an outlier (1.80 pg/mL), and excluded.

⁺Excludes data with only 2 baseline samples.

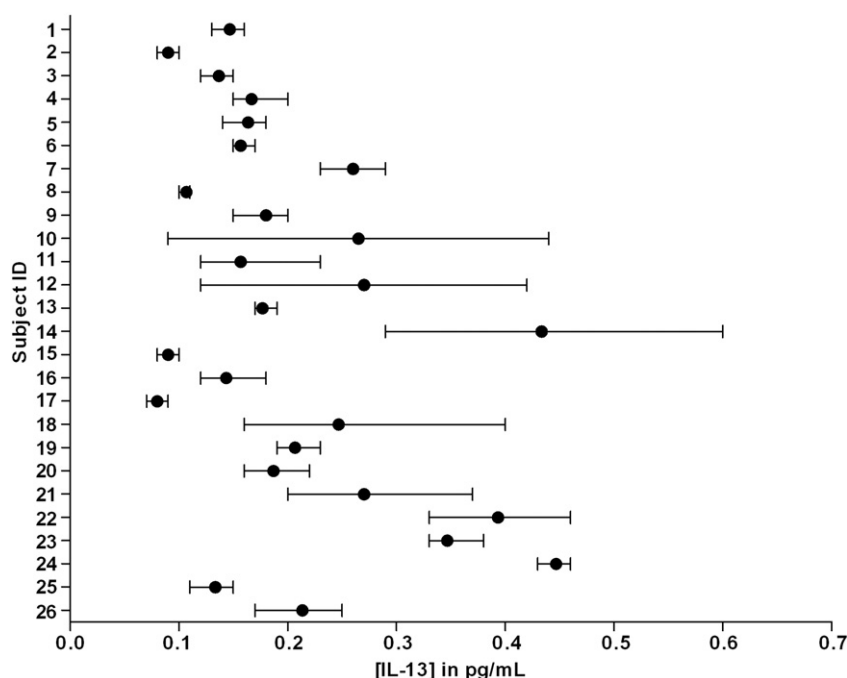


Fig. 3. Long-term biological variability of IL-13 in serum from asymptomatic asthma subjects. Subjects ($N=26$) were sampled over fifteen days, and each quantification was the result of three determinations. Symbols represent the mean, and bars represent the absolute range of quantified values.

specimens (i.e. plasma, sputum, and BLF) or for use in pre-clinical PD studies in animals (i.e. rats, mice, dogs, and monkeys). Taken together, the sensitivity of the present IL-13 assay will enable PD measurements in both healthy and asthmatic human populations for the development of IL-13 targeted therapies in asthma.

More work is necessary to evaluate how individual changes in circulating IL-13 concentrations correlate with early and late stages of acute respiratory inflammation and development of chronic disease. Results of such studies will form the basis upon which circulating concentrations of IL-13 can be used as an effective biomarker during clinical trials involving PK/PD and mechanism of action (MOA) studies (Kasaian et al., 2007). With an LLoQ of 0.07 pg/mL, the Erenna IL-13 assay described here far exceeds the sensitivity of IL-13 detection by bioassay, which is typically in the ng/mL range (Bree et al 2007; Miller et al., 2008). Thus, it offers the unique ability to detect IL-13 at concentrations expressed in biological samples, and is a powerful tool for investigating the IL-4/IL-13 pathway *in vivo*. Such new tools are necessary to further our understanding of the molecular basis of asthma pathology, and to effectively evaluate promising new therapies targeting the IL-4/IL-13 pathway.

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