DEVELOPMENT AND ANALYTICAL VALIDATION OF AN ENZYME–LINKED IMMUNOSORBENT ASSAY FOR THE MEASUREMENT OF SERUM S100A12 AND SERUM CALPROTECTIN IN INFLAMMATORY BOWEL DISEASE

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Abstract

Background: Inflammatory bowel disease (IBD) is characterized by prolonged symptomatic episodes of risk of relapse and remission. Current diagnosis of IBD rely heavily on use of faecal biomarkers such as faecal calprotectin (fCAL) which has been noted to have certain limitations. Quantitative determination of levels of fCAL through the application of enzyme–linked immunosorbent assay (ELISA) technique is well–established**.**

Aim: the primary goal of this study was to develop and validate S100A12 ELISA (Immunodiagnostik™ AG, Stubenwald–Allee 8a, D–64625 Bensheim, Germany) for the determination of S100A12 in serum (sA12), and to validate MRP8/14 Calprotectin S100A8/A9 ELISA (Bühlmann Laboratories AG, Baselstrasse 55, CH–4124 Schönenbuch, Switzerland) and IDK® Calprotectin ELISA (Immunodiagnostik™ AG, Stubenwald–Allee 8a, D–64625 Bensheim, Germany) for the determination of calprotectin in serum (sCAL).

Method: The assay was validated by determining sensitivity, linearity, recovery, imprecision, carry over, analytical interference and stability. A two–site sandwich enzyme–linked immunosorbent assay (ELISA) was developed and analytically validated using faecal and serum samples from healthy controls and patients presenting with inflammatory bowel disease directed against commercially available ELISA kits manufactured by Bühlmann Laboratories AG, Schönenbuch, Switzerland and Immunodiagnostik™ AG, Bensheim, Germany. To accomplish this goal, a two–site sandwich ELISA for serum S100A12 and faecal calprotectin was set up and validated by evaluating faecal S100A12 ELISA assay for

use with serum S100A12 samples, and faecal calprotectin ELISA assay for use with serum calprotectin samples.
Results: Linearity versus recovery data for BMN®-Cp (100.8 vs. 82.1%), IDK®-Cp (98.4 vs. 89.5%) and IDK®-A12 (103.7% vs. 107.8%) are within the target of between 80–120% acceptance criteria for immunoassays. %CV for intra–assay versus inter–assay variability for BMN®-Cp (3.1 vs. 3.2), IDK®-Cp (2.9 vs. 4.7) and IDK®-A12 (7.0 vs. 3.8) are <20% acceptable criteria for imprecision study.ULMR for BMN®-Cp, IDK®-Cp and IDK®-A12 were $2.4x10^6$, $2.5x10^4$ and $5.4x10^2$ ng/mL respectively. LoB versus LLoD were 577 vs. 597, 0.673 vs. 1.119 and 1.145 vs. 1.633 ng/mL for BMN®-Cp, IDK®-Cp and IDK®-A12 respectively. LoQ was 3615, 2880 and 522 ng/mL for BMN®-Cp, IDK®-Cp and IDK®-A12 respectively. No significant assay drift, carry over or instability was observed for the assays.
Conclusion: The assays described are sufficiently sensitive, linear, accurate, precise and reproducible for routine clinical laboratory application. Further studies to evaluate the clinical utility of the assays in assessing IBD are needed.
Key words: *ELISA, inflammatory bowel disease, faecal calprotectin, faecal biomarkers*

Introduction

Inflammatory bowel disease (IBD), a lifelong gastrointestinal disorder that is characterized by prolonged symptomatic episodes of risk of relapse and remission, is categorised majorly as Crohn's disease (CD) and Ulcerative colitis (UC). Unexpected frequent flare–ups and poor clinical management cast significant burden on quality of life of IBD patients.[1,2] Crohn's disease (CD) is transmural, and affects not just the colon and small intestine but any segment of the gastrointestinal tract, existing or occurring across the entire wall of any organ or blood vessel from the mouth to the anus. Ulcerative colitis (UC), however, affects only the colon and the rectum.[3–5] Inflammatory bowel disease (IBD) symptoms may include but not restricted to diarrhoea, nausea, vomiting, constipation, fever, sweats, malaise, fatigue, arthralgia, reduced appetite, abdominal pain, cramping, blood in stool and weight loss. The complications could lead to severe bleeding and dehydration, bowel obstruction, anal fissure, colon cancer, ulcers, fistulas, osteoporosis, liver disease etc.[6–8]

Current diagnosis of IBD rely heavily on results of faecal biomarkers as heterogeneous groups of biomolecules that either drip from, or are actively released by inflamed mucosal cells, activated neutrophils or fast separating cells following the divergent episodes of inflammation of the gastrointestinal tract.[1,2,9,10] S100A12 and calprotectin (S100A8/A9 heterodimer) are calcium–binding, low molecular weight S100 proteins that are predominantly expressed in activated granulocytes under conditions of chronic inflammation.[11–13] S100 protein family are soluble in 100% saturated solution of ammonium sulphate at neutral pH.[14] Both S100A12 and calprotectin, released by neutrophils in the gut of IBD patients, are pro–inflammatory proteins that trigger important extracellular activities that contribute towards immune responses.[15–17]

Faecal calprotectin (fCAL) has been used in IBD studies as the 'gold standard' against which most faecal biomarkers are

benchmarked.[1] Some studies showed that fCAL has higher sensitivity and specificity, and compared to serum C–reactive protein (CRP) and erythrocyte sedimentation rate (ESR), fCAL is consistently a better diagnostic biomarker in the assessment of IBD.[18–20] IBD may be distinguished from irritable bowel syndrome (IBS) by using fCAL and faecal S100A12 (fA12).[19] These biomarkers avoid invasive and expensive endoscopy as the latter is not required for patients with suspected IBS. However, the fact that an ideal biomarker that is simple, easy to perform, non–invasive, inexpensive, quick and reproducible is non–existent implies that relying on fCAL as the 'gold standard' is doubtful for wide application in the assessment of IBD.[15,21]

Like most biomarkers[2], fCAL is not immune to certain limitations,[20–23] lacking specificity, a validated and an optimal cut–off threshold to characterize active inflammatory disease, distinguish IBD from IBS, forecast clinical remission, mucosal healing and assess response to treatment. This encourages a default to different application of the specific assay based on clinical situations.[1,2,9] Thus, fCAL levels are dependent on age and clinical comorbidities that could vary considerably every 24 hours. The problem of significant overlap that exists in fCAL levels (50–150 µg/g) in IBD and IBS patients presents an ambiguous situation regarding the decision to refer or not to refer a patient to endoscopy. The reluctance with which patients are receptive of providing stool samples for analyses limits the robust application of fCAL in assessing intestinal inflammatory diseases.

Quantitative determination of levels of fCAL through the application of enzyme–linked immunosorbent assay (ELISA) technique is well–established[24] and commercially prepared ELISA kits are now available for routine clinical laboratory investigations. Diagnostic accuracy of these kits are still subject to manufacturers' claim to the validity of their performance characteristics which must be confirmed by a user–laboratory prior to routine use. Some variations in kit performance characteristics have been reported.[25] Immunoassays that employ monoclonal testing technology posted superior performance in terms of accuracy, imprecision, sensitivity, recovery, linearity, assay drift, stability, interference etc compared to those with polyclonal technology in screening patients to identify those with organic intestinal disease of abdominal discomfort.[25,26]

High inter–individual biological variation has been reported for levels of fCAL and there are issues of spot variability during multiple sampling from the same faecal collection.[26] These issues may be overcome by using serum samples.[11–15] However, further work is needed to ascertain whether serum may be an alternative matrix for measuring concentrations of fCAL and fA12 in assessing IBD. Therefore, the primary goal of this study was to develop and validate S100A12 ELISA (Immunodiagnostik™ AG, Stubenwald–Allee 8a, D–64625 Bensheim, Germany) for the determination of S100A12 in serum (sA12), and to validate MRP8/14 Calprotectin S100A8/A9 ELISA (Bühlmann Laboratories AG, Baselstrasse 55, CH–4124 Schönenbuch, Switzerland) and IDK® Calprotectin ELISA

(Immunodiagnostik™ AG, Stubenwald–Allee 8a, D–64625 Bensheim, Germany) for the determination of calprotectin in serum (sCAL). When validated, both sA12 and sCAL will provide a prologue for evaluating their utility as alternative biomarkers to fA12 and fCAL in current and future IBD studies.

Materials and methods

Patient recruitment and study design

Forty patients included in this study were those that presented at the twice weekly IBD Clinic at New Cross Hospital Wolverhampton, West Midlands, United Kingdom scheduled for fCAL measurement. Inclusion criteria were based on symptoms contained in the guidelines set out in New Cross Hospital's standard operating procedure for requesting fCAL measurement to exclude IBD. These are symptoms associated with long–term diarrhoea and rectal bleeding (> 6 weeks) in patients > 45 years old, unplanned weight loss, presence of abdominal or rectal mass and anaemia. Exclusion criteria were current therapy with non–steroidal anti–inflammatory drugs (NSAIDs), intestinal infection accompanied by bloody diarrhoea and clinical signs indicative of IBD and IBS.

Healthy controls (HC) were included in the study. These were twenty patients that are asymptomatic of the criteria listed above and in whom there were no chronic diseases that have been known or postulated to increase systemic concentrations of calprotectin or S100A12. HC were selected from among those requests from primary care practices scheduled for routine annual general check–up that met the exclusion criteria. In addition, their CRP results were < 4 mg/L.

Sample collection

Serum samples used in this study were obtained from patients (n = 40) who were confirmed as positive IBD cases based on the result of their fCAL assay and from HC patients (n = 20). New Cross Hospital Wolverhampton uses Bühlmann fCAL™ ELISA kit for in–house measurement of fCAL. This method reports a result of greater than one hundred microgram per gram stool (> 100 µg/g) for a positive IBD case.

Five millilitres (5 mL) of serum were collected from both patient groups for this study. Unless indicated otherwise, all serum samples were stored away at –80°C on reception until analyses on them were required. They were however, allowed to thaw slowly and equilibrate at ambient (room) temperature for at least two hours prior to being assayed for sCAL or sA12 on the Diamedix™ Dynex DS2™ Automated ELISA (DS2) System (Diamed Corporation, Hialeah, Florida, USA). Although variations in ambient temperature in the laboratory did not occur throughout the duration of this study, it was still important to define ambient temperature as 18–25°C. All serum samples used in this study met the above stated criteria.

Laboratory methods

The in–house method at New Cross Hospital Wolverhampton was used to measure fCAL. The Immunodiagnostik™ method for the determination of fA12 was adapted to measure sA12 in this study. Storage and preparation/reconstitution of reagents was performed according to manufacturers' instructions. Although the assay protocol from each kit varied slightly in terms of the wash buffer, sample/incubation buffer, incubation timings, washing steps, choice of

conjugate, substrate and stop solutions, they were nevertheless based around the same principle framework detailed in the kit inserts made available for this study by Biohit Healthcare UK Limited.

Development and Optimisation of a two–site sandwich ELISA technique

A two–site sandwich ELISA was developed. The principle of this assay allows the binding of the antigen or antibody to a solid surface or a latex particle. ELISA plates were coated with the capture antibody, and non–specific binding sites were blocked identically for all plates. First, 96–well flat–bottom ELISA plates (manufactured by Bühlmann Laboratories AG, Baselstrasse 55, CH–4124 Schönenbuch, Switzerland and Immunodiagnostik AG, Stubenwald–Allee 8a, D–64625 Bensheim, Germany) were coated with 100 microlitre (µL) per well affinity–purified monospecific anti–calprotectin/anti–S100A12 and 200 nanogram (ng) per well carbonate–bicarbonate buffer (pH 9.4). The assay was run on the DS2 System. All routine maintenance of the instrument was performed as recommended in the owner's operating manual provided by Diamed Corporation, Hialeah, Florida, USA.

The assay protocol used disposable tips for sample and reagent pipetting steps. The rest of sample loading and on–board dilution steps used 5 mL fraction collection tubes (Sarstedt Aktiengesellschaft & Co., Germany). All the results of sCAL and sA12 were reported in nanogram per millilitre serum (ng/mL). The standard and control materials were prepared by dissolving different lyophilised calprotectin and S100A12 standards (calibrators) and control materials in 500 µL of deionised water. However, Immunodiagnostik™ recommends the use of Ultra–Pure Water (Water Type 1; ISO 3696), which is free of undissolved and colloidal ions and organic molecules free of particles > 0.2 micrometre (µm) with an electrical conductivity of 0.055 µS/cm at 25°C (≥ 18.2 MΩ cm). The vial content were allowed to stand for 10 minutes at 18–25°C and mixed thoroughly by gentle inversion to ensure complete reconstitution before use.

Sample/incubation buffers supplied by Bühlmann and Immunodiagnostik™ were used for blank preparation. Serum samples of unknown concentrations of calprotectin and S100A12 were initially prepared in a 1:100 (calprotectin) and 1:10 (S100A12) dilution with sample/incubation buffer before measurement.

For calprotectin measurement using the Immunodiagnostik™ assay (IDK®-Cp), a two–step dilution process was carried out, i.e., 50 µL sample + 450 µL SAMPLE BUFFER (Cat. No. K 6935 SAMPLEBUF) = Dilution I (1:10); followed by 50 µL Dilution I + 450 µL SAMPLE BUFFER = Dilution II (1:10), to give a final dilution factor of 1:100.

For calprotectin measurement using the Buhlmann assay (BMN®-Cp), a single step dilution of 10 µL sample with 990 µL INCUBATION BUFFER (Code: B–MRP8/14–IB) gave a dilution factor of 1:100.

For S100A12 measurement using the Immunodiagnostik™ assay (IDK®-A12), a single–step dilution of 50 µL sample with 450 µL SAMPLE BUFFER (Cat. No. K 6938 SAMPLEBUF) gave a dilution factor of 1:10.

During optimization the influence of several parameters was analysed. Different concentrations of primary antibody, secondary antibody and horseradish–peroxidase–labelled streptavidin were compared. Different wash buffers and washing protocols were analysed and the effects of using different buffers to dilute the reagents were studied. Various incubation times and protocols for standards and samples were also evaluated. In the interest of space none of the results of these experiments are presented here, and only the optimized ELISA procedure is described.

Measurement of calprotectin and S100A12 in serum with ELISA technique

All the plates were set up in the same fashion. Each well of the microtitre plate was loaded with 100 µL of the designated solution. For S100A12 only, the plate was first washed 5 times with IDK® ELISA Wash Buffer (Cat. No. K 6938 WASHBUF) prior to application of standards, controls and samples. Standard (or calibrator) solutions were applied in duplicates beginning with the highest to the lowest concentration of calprotectin or S100A12 as appropriate. This was followed by application of blank samples, then two control samples with different concentrations of calprotectin (or S100A12), and finally test samples. The plates were then incubated for 40 minutes (60 minutes for S100A12 with shaking at medium speed) at 18–25°C and washed 5 cycles, plate–wise with constant timing by purging the washer with 9999 µL of IDK® ELISA Wash Buffer (Cat. No. K 6935 WASHBUF for IDK®-Cp assay and Cat. No. K 6938 WASHBUF for IDK®-A12 assay) or a 3–cycle wash, plate–wise, with constant timing by purging the washer with 3000 µL of BMN® EK–Cal Wash Buffer (Code: B–MRP8/14–WB for BMN®-Cp assay).

For the detection of captured antigen in the standards, controls and samples, microtitre plates were incubated with antibody solution containing 100 µL per microtitre well of secondary antibody–enzyme conjugate. The respective antibody–enzyme conjugate include a monoclonal detection antibody (anti–MRP8/14 Ab) conjugated to horseradish peroxidase (HRP) streptavidin for BMN® EK–Cal Enzyme Label (Code: B–MRP8/14–EL for BMN®-Cp assay), monoclonal human anti–calprotectin peroxidase–labelled conjugate (Cat. No. K 3695 CONJ for IDK®-Cp assay) and polyclonal horseradish peroxidase–labelled anti–S100A12 Ab conjugate (Cat. No. K 6938 CONJ for IDK®-A12 assay)

After incubation for 40 minutes (60 minutes for S100A12 with shaking at medium speed) at 18–25°C, the plates were washed 5 cycles as described previously and then developed for 12 minutes (14 minutes for BMN®-Cp assay with shaking at low speed) with 100 µL per well of 3,3’,5,5’–tetramethylbenzidine (TMB) dihydrochloride substrate solution in citrate buffer with hydrogen peroxide (Cat. No. K 3695 SUB for IDK®-Cp assay, Code: B–TMB for BMN®-Cp assay and Cat. No. K 3698 SUB for IDK®-A12 assay). The reaction was stopped by promptly adding to each microtitre well 100 µL of a solution of 0.25M Sulphuric Acid (Cat. No. K 3695 STOP for IDK®-Cp assay, Code: B–STS for BMN®-Cp assay and Cat. No. K 3698 STOP for IDK®-A12 assay).

Following an initial orbital shaking at medium speed that lasted three seconds, the absorbance of each well was measured immediately by the microtitre plate reader on the DS2 System at two wavelengths. Absorbance measured at 450 nm served as the primary test filter wavelength while the absorbance measured at 620 nm served as the primary reference filter wavelength. It was important that the absorbance was read within 5 minutes of developing the colour taking into consideration that the intensity of the colour change is temperature sensitive.

ELISA calibration curve representation

Standard curves were calculated with the use of a 4–parameter curve fit: $y = (A – D)/[1 + (x/C)^B] + D$, where D is the *y* value corresponding to the asymptote at high values on the *x* axis, A is the *y* value corresponding to the asymptote at low values on the *x* axis, C is the *x* value corresponding to the mid–point between A and D, and B describes how rapidly the curve makes its transition from the asymptotes in the center. All 4–parameters were calculated with an algorithm based on the Levenberg–Marquardt method (SOFTMAX PRO; Molecular Devices).

Assay working range

Samples with concentrations of the assay above the kit's measuring range (defined by the concentration range of the calibrators) were further diluted and re–assayed. The result obtained was multiplied by the dilution factor used. Samples with concentrations of the assay below the kit's measuring range cannot be clearly quantified. *However, the upper limit of the measuring range (ULMR) can be calculated as: highest concentration of the standard curve multiplied by sample dilution factor to be used while the lower limit of the measuring range (LLMR) can be calculated as: limit of the blank (LoB) multiplied by sample dilution factor to be used.*

Validation of ELISA for measurement of calprotectin and S100A12 in serum

Each assay was validated by determining sensitivity, linearity, recovery, intra– and inter–assay variability. Linearity, recovery, and intra– and inter–assay variability were determined with serum samples and calibrators provided by Immunodiagnostik™ and Bühlmann. All serum samples used for assay validation were stored at –20°C until used.

Analytical sensitivity: limit of the blank (LoB) and lower limit of detection (LLoD)

Assay sensitivity was determined by calculating the mean concentration of 10 sets of blank samples and evaluating the mean plus 2 standard deviations (i.e., mean + 2SD) on the standard curve.[27] The lower limit of the working range was defined as the sensitivity. The upper limit of the working range was determined by the apparent value of an absorbance, which equals the mean maximum absorbance minus 2SD, as determined from the mean absorbance in 10 duplicate wells containing approximately 100 µg/L of calprotectin or S100A12.

LoB is the highest analyte concentration expected to be found when replicates of a sample containing no analyte are tested.[28] LoB was derived by measuring replicates of a blank sample or dilution buffer (zero concentration of analyte) and calculating the mean result and SD. Five aliquots of sample/incubation buffer containing zero concentration of analyte (blank solution)

was each measured in duplicates (n = 10) on the same ELISA microtitre plate according to assay protocol. The mean concentration of analyte (calprotectin/S100A12) and SD was calculated and used to calculate the LoB according to the following formula: LoB = Mean($_{BLANK}$) + 2(SD_{BLANK})

LLoD was determined by using the calculated LoB and test replicates of a sample known to contain a low concentration of the analyte under consideration[28]. LLoD is estimated as the sum of the LoB and 2SD of low concentration of sample. Five aliquots of sample/incubation buffer containing zero concentration of analyte were spiked with a small known concentration of calprotectin/S100A12 taken from the manufacturer's supplied calibrators. Concentrations of calprotectin/S100A12 in the spiked samples were chosen based upon the manufacturer's claimed analytical sensitivity. These were 400, 3.9 and 0.66 ng/mL for BMN®-Cp, IDK®-Cp and IDK®-A12 assays respectively.

The spiked samples were analysed five consecutive occasions in duplicates (n = 10) on the same ELISA microtitre plate as per assay protocol used for LoB samples. Subsequently, the mean concentration of analyte and SD were calculated as described as per assay protocol used for LoB. The LLoD was calculated using the following formula: LLoD = LoB + 2($SD_{LOW\ CONCENTRATION\ OF\ SPIKED\ SAMPLE}$).

Functional sensitivity: Limit of quantitation (LoQ)

LoQ was determined as part of the imprecision experiments by evaluating the coefficient of variation expressed as a percentage (%CV) of the intra–assay and inter–assay imprecision experiments conducted with pooled HC samples and pooled serum samples from IBD patients whose concentrations of calprotectin and S100A12 were moderately and highly elevated respectively, to identify the lowest concentration of calprotectin or S100A12 at which the %CV was < 20%.

Linearity

Linearity was determined by evaluating each sample at its initial strength (1:1) and at serial dilutions of 1:2, 1:4, 1:8, 1:16, 1:32, 1:64 and 1:128.

Recovery

Recovery was determined for each assay by adding known concentrations of calprotectin or S100A12 to four (4) sample buffer aliquots in equal volumes to give the sample matrix and then each aliquot measured in duplicate runs (n = 8). Calprotectin or S100A12 was taken from the calibrators supplied by the manufacturer.

Imprecision

Intra–assay variability was determined by evaluating a minimum of 5 aliquots each of pooled serum samples from HC and IBD patients, a minimum of 10 times within the same assay run using the formular: *%CV* = (*SD/Mean*)*100, where *CV* = coefficient of variation. Inter–assay variability was determined by evaluating 2 levels of control material (low and high), aliquots of pooled serum samples (n ≥ 10) from HC and IBD patients severally (n ≥ 10) in consecutive assay runs using the formular: *%CV* = (*SD/Mean*)*100.

Assay carry over

In order to determine the concentration of calprotectin or S100A12 carried over into the blank sample (incubation or sample buffer), each of the serum samples with increased concentrations of calprotectin or S100A12 were individually placed in the microtitre well preceding the two neighbouring wells that contained the blank sample in the following sequence: HBB, where H is the serum sample with high calprotectin or high S100A12 concentration and B is the blank sample according to an established protocol.[29,30] Measurements were repeated in duplicate (n = 4). Consequently, any significant cross–contamination (carry over) of calprotectin or S100A12 between the wells during the ELISA washer–purge step and plate–wash cycle could be detected in the blank sample. Any cross contamination was regarded as being significant when the mean calprotectin or S100A12 concentration measured in the two blank replicates (i.e., BB) was greater than the previously calculated LoB for the assay.

Assay drift

To investigate assay drift, low– and high–quality control materials (supplied by ELISA kits manufacturers) used in this study were allocated to different positions at the beginning and towards the end of the microtitre plate for each particular run of calprotectin and S100A12.

Reference intervals

Reference intervals for serum calprotectin and S100A12 was not established as part of the study because enough HC ($n \geq 75$) that could meet the exclusion criteria were not recruited into the study. sCAL and sA12 values obtained in this study were compared with values obtained with previously described works.[31,32]

Statistical analysis

Data processing and statistical analyses were performed using SPSS version 26 (IBM SPSS Statistics Software, Armonk, New York, USA). Functional sensitivity was determined as the lowest concentration of calprotectin and S100A12 that could be measured with optimum reproducibility at <20%CV. fCAL assay was linear between 20 and 1932 µg/g and results <20 and >1932 µg/g were arbitrarily assigned a value of 20 and 1932 µg/g respectively, for statistical purposes. Since the data were non–parametric as determined by Kolmogorov–Smirnov (KS) and Shapiro–Wilk (SW) tests, data are expressed as medians with interquartile ranges. Spearman's rank–order coefficient of correlation (*r*) was used to measure the degree of association between variables, and *r*–values between 0.5 and 1.0 indicate a good correlation. Significant statistical relationships were defined as a *p*–value less than 0.05 (i.e., $p < 0.05$).

Result

The development of two–site sandwich ELISA resulted in a typical reproducible standard curve (figure 1). The upper limit of the measuring range for BMN®-Cp, IDK®-Cp and IDK®-A12 were 2.4 x 10^6, 2.5 x 10^4 and 5.4 x 10^2 ng/mL respectively.

Analytical sensitivity: LoB and LLoD

The calculated results of LoB and LLoD for the three assays are set out in table 1. Unlike the results of IDK®-Cp and IDK®-A12 assays, the LoB and LLoD values for BMN®-Cp assay were deliberately reported in µg/mL as against ng/mL for easy and quick comparison with the decision threshold provided by the assay manufacturer.

Functional sensitivity: LoQ

The intra–assay and inter–assay imprecision results for the moderately and highly elevated fractions for BMN®-Cp, IDK®-A12 and IDK®-Cp assays (tables 2 and 3) showed %CVs of <20%. The LoQ derived from the moderately elevated pooled concentrations were 3615 and 522 ng/mL for the BMN®-Cp and IDK®-A12 assays respectively, and from pooled HC samples to be 2880 ng/mL for the IDK®-Cp assay. All imprecision was at an acceptable %CV criterion of <20% and the LoQ > LLoD.

Linearity

The results from the serially diluted serum samples containing elevated concentration of calprotectin or S100A12 for each assay are shown in table 4. The mean of Measured to Expected ratios for linearity (n = 7) for the BMN®-Cp assay was 100.8% (range: 92.7–108.3%). For the IDK®-A12 assay, mean = 103.7% (range: 71.4 – 148.8%) and for the IDK®-Cp assay, mean = 98.4% (range: 89.2–112.3%). Linear regression was also carried out on the plot of the Expected and Measured results of the dilutions (n = 7) for each assay (figure 2). The slope, intercept and square of regression coefficient (r^2) were: 0.9390, +73.061, 0.98 (BMN®-Cp); 1.1103, –22.615, 0.98 (IDK®-Cp) and 1.5107, –20.998, 0.98 (IDK®-A12) respectively. In each case, the dilutions were linear over the range tested and the results met the >80% of target acceptance criterion for immunoassays. A summary of the linear regression fits characteristics of the assays are shown in table 5.

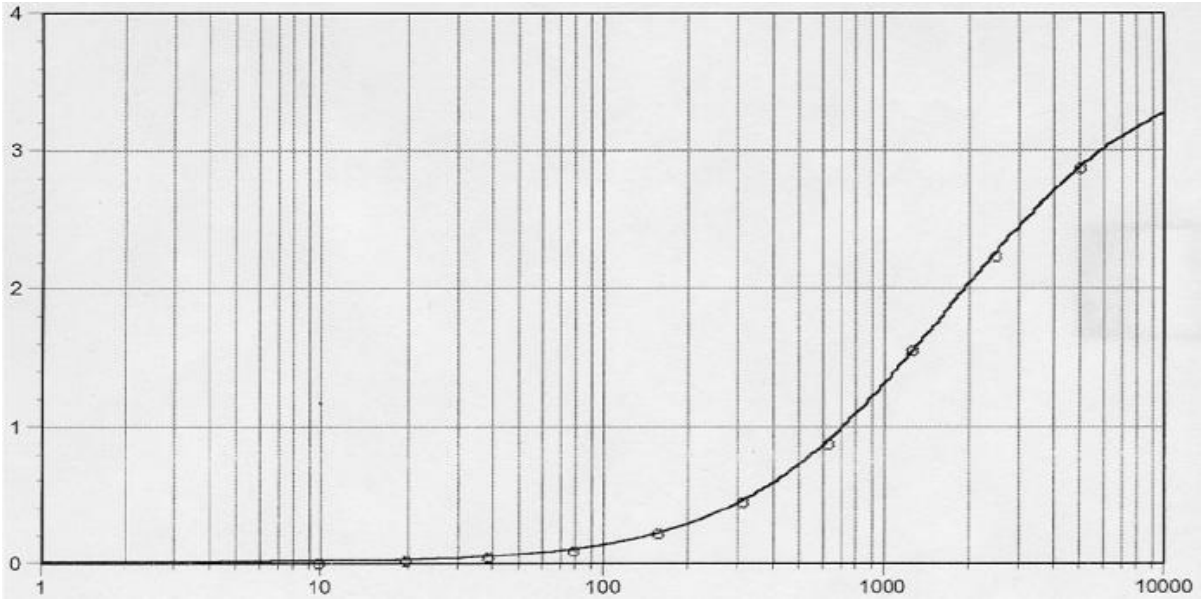


Figure 1 – A representative calibration curve for the estimation of calprotectin and S100A12 by the two–site sandwich ELISA. This method utilises two selected monoclonal antibodies that bind to human calprotectin and S100A12 respectively. The standard curve was calculated using the 4–parameter curve fit: $y = (A - D)/(1 + [x/C]^B) + D$; where A = 0.002, B = 1.055, C = 18.669 and D = 3.531. The y–axis displays the absorbance at a dual wavelength mode of 450 nm(s) and 620 nm(s). R–Squared (r^2) = 1.000

Table 1 – Analytical sensitivity data. Calculated values of the mean and SD for limit of the blank (LoB) and the lower limit of detection (LLoD); n = the number of replicates

Assay	n	Mean BLANK (S100A12 CALPROTECTIN)	SD BLANK	LoB (CALCULATED)	Mean (LOW CONC. OF SPIKED SAMPLE)	SD (LOW CONC. OF SPIKED SAMPLE)	LLoD (CALCULATED)
IDK®-A12 (ng/mL)	10	0.319	0.177	0.673	0.517	0.223	1.119
IDK®-Cp (ng/mL)	10	0.539	0.303	1.145	0.436	0.244	1.633
BMN®-Cp (µg/mL)	10	0.545	0.016	0.577	0.533	0.010	0.597

Table 2 – Intra–assay (within–batch) imprecision data for the ELISA for serum BMN®-Cp assay, IDK®-A12 and IDK®-Cp assays using pooled highly elevated IBD samples (#1), pooled moderately elevated IBD samples (#2) and pooled HC, i.e., non–IBD samples or controls (#3). * = Functional sensitivity (LoQ) for the assay indicated.

n = Number of replicates; SD = Standard Deviation; CV = Coefficient of Variation

Intra–assay Imprecision	**BMN®-Cp (ng/mL)**			**IDK®-A12 (ng/mL)**		**IDK®-Cp (ng/mL)**		
Sample #	#1	#2	#3	#1	#2	#1	#2	#3
N	10	10	10	10	10	10	10	10
Mean (ng/mL)	9691	4553	3940	1257	535	15184	3038	**2880***
SD (ng/mL)	278.8	86.3	179.4	115.8	25.8	156.1	119.9	111.3
CV (%)	2.8	1.8	4.6	9.2	4.8	1.0	3.9	3.8
Mean CV (%)	**3.1**			**7.0**		**2.9**		

Table 3 – Inter–assay (between–batch) imprecision data for the ELISA for serum BMN®-Cp, IDK®-A12 assay and IDK®-Cp assays using pooled highly elevated IBD samples (#1), pooled moderately elevated IBD samples (#2), low internal quality control (#3) and high internal quality control (#4) provided by the kit manufacturers. * = Functional sensitivity (LoQ) for the assay indicated. n = Number of replicates; SD = Standard Deviation; CV = Coefficient of Variation

Inter–assay Imprecision	**BMN®-Cp (ng/mL)**				**IDK®-A12 (ng/mL)**				**IDK®-Cp (ng/mL)**			
Sample #	#1	#2	#3	#4	#1	#2	#3	#4	#1	#2	#3	#4
N	10	10	8	8	12	12	8	8	10	10	8	8
Mean (ng/mL)	18748	**3615***	1526	5158	723	**522***	0.7	6.6	13489	2901	7.8	38.1
SD (ng/mL)	1109.9	245.9	2.2	42.7	16.6	6.1	0.1	0.5	874.8	122.4	0.3	1.5
CV (%)	5.2	6.8	0.1	0.8	2.3	1.2	4.3	7.6	6.5	4.2	4.1	3.9
Mean CV (%)	**3.2**				**3.8**				**4.7**			

Table 4 – Linearity of dilution (parallelism) data for the ELISA for serum BMN®-Cp assay, serum IDK®-A12 assay and serum IDK®-Cp assay shown for elevated concentration of neat serum at serial dilutions of 1 in 2, 1 in 4, 1 in 8, 1 in 16, 1 in 32, 1 in 64 and 1 in 128. The Measured to Expected ratios are given in %

Assay	n	Range: (Measured/Expected)*100	Mean	SD	%CV
BMN®-Cp (ng/mL)	7	92.7 – 108.3	100.8	6.2	6.1
IDK®-Cp (ng/mL)	7	89.2 – 112.3	98.4	9.3	9.4
IDK®-A12 (ng/mL)	7	71.4 – 148.8	103.7	29.6	28.6

Table 5 – A summary of the linear regression fits analysis of the results of Measured (M) and Expected (E) concentrations of serum BMN®-Cp assay (A), serum IDK®-Cp assay (B) and serum IDK®-A12 assay (C) from serial dilutions (n = 7) of the analyte for the respective ELISA kits. ¶The relationship between the Measured and Expected concentration of the analyte is expressed in the form of the equation: $y = mx + c$ where x and y represents the Expected and Measured result respectively. SEM = Standard error of the mean

Assay	Slope	Intercept	Linear Fit	Relationship of M to E	Line Equation (y = mx + c)¶	r^2	*P*	SEM
A	0.9390	+73.061	73.06 + 0.939*x*	M = 73.06 + 0.939E	y = 0.939*x* + 73.061	0.98	0.0001	68.9
B	1.5107	–20.998	–21 + 1.511*x*	M = –21 + 1.511E	y = 1.5107*x* – 20.998	0.98	0.0001	14.7
C	1.1103	–22.615	–22.62 + 1.11*x*	M = –22.62 + 1.11E	y = 1.1103*x* – 22.615	0.98	0.0001	29.9

Table 6 – Spiking recovery data for the ELISA for serum BMN®-Cp, IDK®-A12 and IDK®-Cp assays shown for 4 spiking concentrations of calprotectin and S100A12 in 4 serum matrices. The Measured to Expected ratios are given in %

Assay	n	Range: (Measured/Expected)*100	Mean	SD	%CV
BMN®-Cp (ng/mL)	4	65.5 – 95.5	82.1	10.7	13.0
IDK®-Cp (ng/mL)	4	84.7 – 93.8	89.5	3.2	3.6
IDK®-A12 (ng/mL)	4	107.7 – 155.9	126.5	18.4	14.5

Recovery

The results of recovery experiment for the assays were expressed as the ratios of Measured to Expected concentration of the analyte in the sample matrix. These are set out in table 6. The Measured to Expected ratios for recovery for the BMN®-Cp assay was: mean = 82.1%, range: 65.5–95.5%, SD: 10.7% and %CV: 13.0%. For the IDK®-Cp assay, mean = 89.5%, range: 84.7–93.8%, SD: 3.2% and %CV: 3.6%. For IDK®-A12 assay, mean = 126.5%, range: 107.7–155.9%, SD: 18.4% and %CV: 14.5%. Overall, the assays exhibited acceptable individual analytical performance judged by the mean %Recovery of 104 ± 22%. However, the BMN®-Cp assay demonstrated 65.5–68.9% under recovery at the lowest spiked concentration of 1200 ng/mL calprotectin in serum.

Intra–assay (within–run) Imprecision

The results of the intra–assay (within–run) imprecision experiment varied between 1.0 and 9.2% as set out in table 2. %CV for intra–assay variability for pooled highly elevated IBD samples, pooled moderately elevated IBD samples and pooled HC samples for the BMN®-Cp assay were 2.8, 1.8 and 4.6% respectively. %CV for intra–assay variability for pooled highly elevated IBD samples, pooled moderately elevated IBD samples and pooled normal samples for the IDK®-Cp assay were 1.0, 3.9 and 3.8% respectively. %CV for intra–assay variability for pooled highly elevated IBD samples and pooled moderately elevated IBD samples for the IDK®-A12 assay were 9.2 and 4.8% respectively. The observed mean of intra–assay imprecision for S100A12 and calprotectin was <10% for each assay: (BMN®-Cp: 3.1%; IDK®-A12: 7.0% and IDK®-Cp: 2.9%) to indicate a good performance characteristic. The IDK®-Cp assay posted the best overall intra–assay performance with a demonstrable lowest mean %CV and narrowest %CV range.

Inter–assay (between–run) Imprecision

The results of inter–assay (between–run) imprecision experiment varied between 0.1 and 7.6% as set out in table 3. %CV for inter–assay variability for pooled highly elevated IBD samples, pooled moderately elevated IBD samples, low IQC and high IQC for the BMN®-Cp assay were 5.2, 6.8, 0.1 and 0.8% respectively. %CV for inter–assay variability for pooled highly elevated IBD samples, pooled moderately elevated IBD samples, low IQC and high IQC for the IDK®-Cp assay were 6.5, 4.2, 4.1 and 3.9% respectively. %CV for inter–assay variability for pooled highly elevated IBD samples, pooled moderately elevated IBD samples, low IQC and high IQC for the IDK®-A12 assay were 2.3, 1.2, 4.3 and 7.6% respectively. The observed mean of inter–assay imprecision for S100A12 and calprotectin was <10% for each assay: (BMN®-Cp: 3.2%; IDK®-A12: 3.8% and IDK®-Cp: 4.7%) to indicate a good performance characteristic. The BMN®-Cp assay posted the best overall inter–assay performance with a demonstrable lowest mean %CV and narrowest %CV range.

Assay carry over

A significant level of assay carry over from one microtitre well to a neighbouring one is confirmed if the mean calprotectin or S100A12 levels in the blank (sample or incubation buffer) solution expressed as LoB of carry over assay (LoB^{C}) are greater than the LoB of sample or incubation buffer ($LoB^{§}$) for the particular assay. As

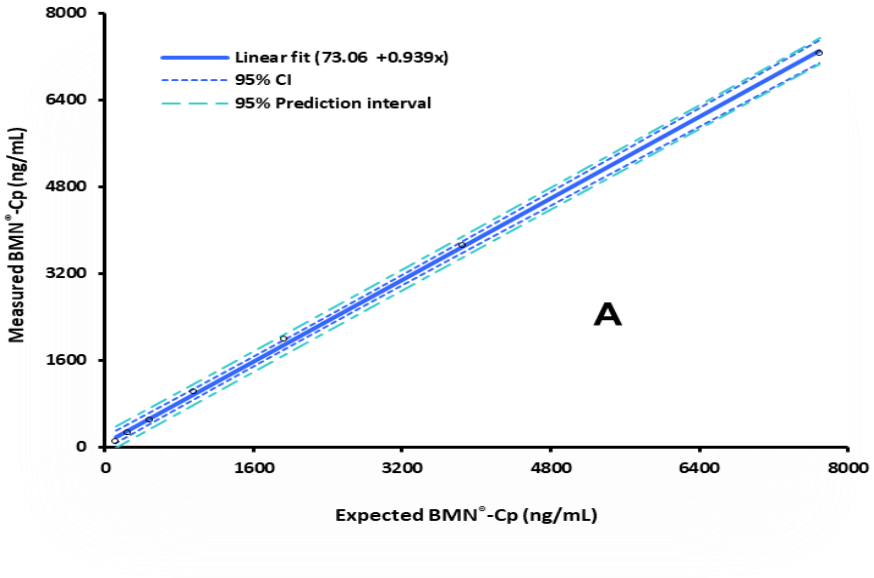
shown in table 7, no concentration of calprotectin or S100A12 was detected at or above the respective LoB[§] in the neighbouring wells that followed the elevated samples. This confirms that no significant carry over was detected (LoB[C] < LoB[§]) in the assays.

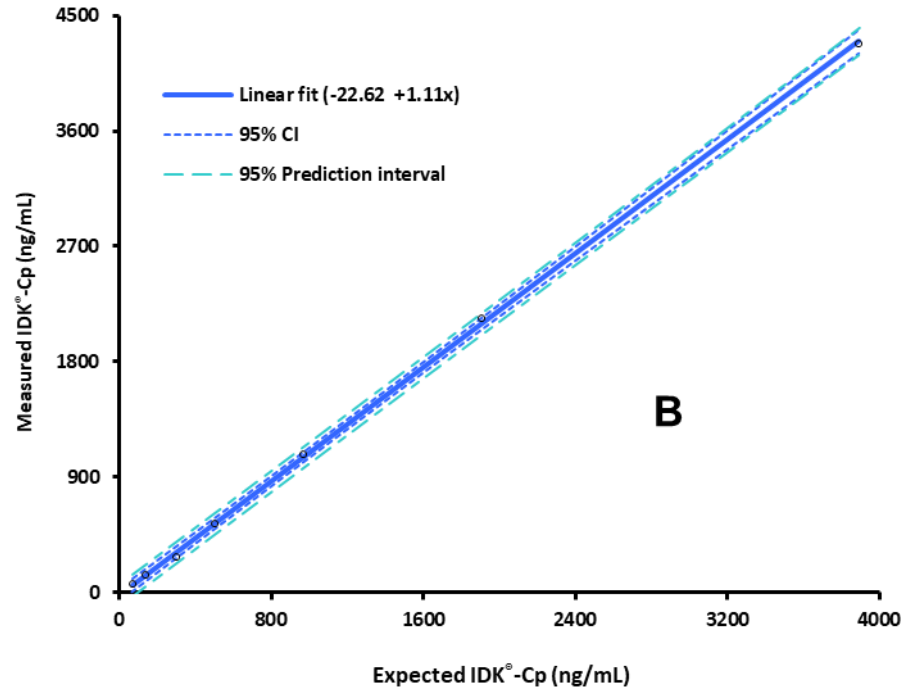
Assay drift

There was no drift in the results of both levels (low and high) control materials recorded after a particular run for calprotectin and S100A12.

Method comparison: BMN®-Cp versus IDK®-Cp

The results of the cross–kit comparison for fCAL, serum BMN®-Cp, IDK®-Cp and IDK®-A12 assays in 40 patients with IBD are presented in table 8. Median fCAL was 297 µg/g (IQR: 102–1454 µg/g). Median sCAL measured with the BMN®-Cp assay, 5428 ng/mL (IQR: 3728–8603 ng/mL) was higher (p = 0.0001) than median sCAL determined with the IDK®-Cp assay, 3254 ng/mL (IQR: 2085–4606 ng/mL). Median sA12 measured with the IDK®-A12 assay was 412 ng/mL (IQR: 321–565ng/mL). The Spearman's rank–order coefficient of correlation (r) test for non–parametric data showed close correlation between BMN®-Cp and IDK®-Cp values for the common set of 40 IBD samples (Spearman's, r^2 = 0.9852, p < 0.0001) (figure 3). The strong, positive linear correlation between the BMN®-Cp and IDK®-Cp assays is described by the line equation: $y = 1.6885x + 190.02$, where y = BMN®-Cp and x = IDK®-Cp as measured by both assays.


8000
6400
4800
3200
1600
0
Measured BMN®-Cp (ng/mL)
Linear fit (73.06 +0.939x)
95% CI
95% Prediction interval
A
0
1600
3200
4800
6400
8000
Expected BMN®-Cp (ng/mL)



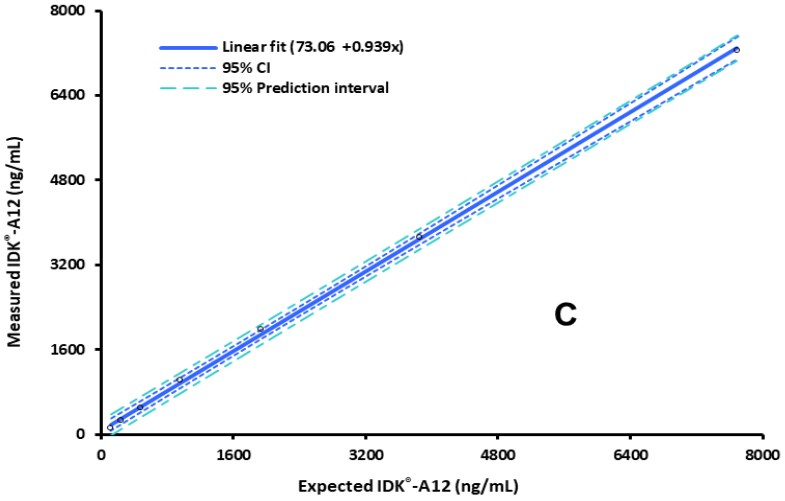


Figure 2 – Scatter plot with fit for serum BMN®-Cp, IDK®-Cp and IDK®-A12 assays. Measured and Expected results for the elevated sample dilutions (n = 7 for each assay) demonstrate reliability of:

(A) BMN®-Cp assay: 95% CI = –15.02 to 161.15 (Intercept), 0.913 to 0.965 (Slope) and t–statistic = 2.13 (Intercept), 92.00 (Slope);

(B) IDK®-Cp assay: 95% CI = –61.12 to 15.89 (Intercept), 1.09 to 1.13 (Slope) and t–statistic = –1.51 (Intercept), 125.63 (Slope);

(C) IDK®-A12 assay: 95% CI = –40 to –2 (Intercept), 1.408 to

Table 7 – Limit of the blank (calculated from sample/incubation buffer) versus limit of the blank (determined from the carry over assay). The mean concentration of serum calprotectin and serum S100A12 of low spiked sample and 'carry over blank', SD of 'carry over blank' (SD^C), their respective calculated LoB above and results. LoB^C = Limit of the Blank of carry over assay; $LoB^§$ = Limit of the Blank of sample/incubation buffer as was previously determined above for the particular assay protocol; n = the number of replicates.

Assay	n	Mean Concentration		SD^C	Calculated Value		Result
		LOW CONCENTRATION OF SPIKED SAMPLE	CARRY OVER BLANK (S100A12/CALPROTECTIN)		LoB^C	$LoB^§$	
IDK®-A12 (ng/mL)	4	0.517	0.251	0.086	0.424	0.673	$LoB^C < LoB^§$
IDK®-Cp (ng/mL)	4	0.436	0.778	0.095	0.968	1.145	$LoB^C < LoB^§$
BMN®-Cp (µg/mL)	4	0.533	0.517	0.026	0.569	0.577	$LoB^C < LoB^§$

Table 8 – Faecal calprotectin, serum calprotectin and serum S100A12 concentrations for 40 IBD patients. [a]In–house reference method. [b]Reference range supplied by kit manufacturer. [c]Reference range quoted in a published study by Larsen et al[35].

Parameters	fCAL™ (µg/g)	BMN®-Cp (ng/mL)	IDK®-Cp (ng/mL)	IDK®-A12 (ng/mL)
N	40	40	40	40
Minimum	47	1290	675	202
Maximum	1932	22743	12808	1285
Median	297	5428	3254	412
Interquartile range (IQR)	102–1454	3728–8603	2085–4606	321–565
Reference range	> 200[a]	400–3900[b]	< 3000[b]	35–1570[c]

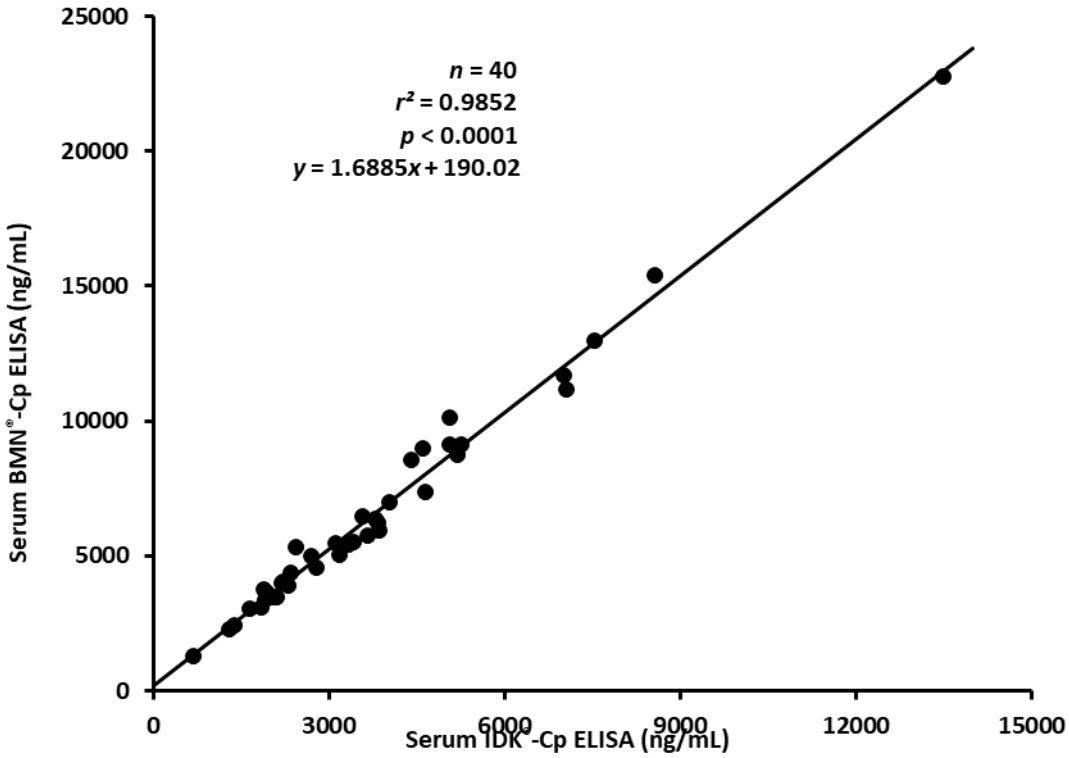


Figure 3 – Serum BMN®-Cp versus serum IDK®-Cp in IBD. Calprotectin concentrations measured in 40 serum samples from patients presenting with IBD using the IDK®-Cp ELISA (Cp^{Ω}) and the BMN®-Cp ELISA (Cp^{ϕ}). Both assays showed a strong, positive correlation (Spearman's, r^2 = 0.9852, p < 0.0001) as given by the equation of the straight line: $y = 1.6885x + 190.02$, for the data set. A gradient of 1.6885 indicates a good relationship between the two assays where Cp^{ϕ} [ng/mL] = 190 + 1.689 Cp^{Ω} [ng/mL].

Discussion

In this study, a two–site sandwich ELISA for the quantitation of sCAL and sA12 was developed, and optimised. During optimization, it was established that incubation of standards and samples with orbital shaking at low–to–medium speed did not result in much lower and more variable reactions. It may be that there was not higher density of macromolecules in serum samples than in standard solutions and that with orbital shaking at low–to–medium speed, no other macromolecules push for antigen molecules from a favourable position for antibody binding before a bond can be determined. This meant that shaking during incubation of controls, standards and samples was not discontinued.

The three assays: BMN®-Cp, IDK®-Cp and IDK®-A12 that were developed, optimised and validated for use in serum were analytically sensitive and specific, linear, precise, accurate, reproducible with a wide working range, and have the ability to discriminate between patients with or without an inflammatory disease such as IBD. All three assays demonstrated good analytical performance on validation.

It may appear counterintuitive that concentrations of sCAL measured by the BMN®-Cp and IDK®-Cp assays are significantly different. However, as noted elsewhere[27–30,33,34], immunoassay methods are not truly analytic in that different immunological procedures for the same analyte or substance could produce different results. More importantly in this study, the results from the BMN®-Cp and IDK®-Cp assays correlated closely, with a Spearman's rank–order coefficient of correlation, r^2 = 0.9852, $p < 0.0001$, indicating that both assays do evaluate the same function (figure 3).

Concentrations of sCAL measured with the BMN®-Cp assay were 1.7–fold higher than those measured with the IDK®-Cp assay. This is consistent with other studies that reported between–assay variability of ELISA kits[31,32]. Reasons for this include a possible difference in assay antibodies and assay format. The BMN®-Cp and IDK®-Cp assays use the same type of capture antibody made of monoclonal anti–human antibody. Whilst the detection antibodies are monoclonal in structure, they are however, of different origins (horse vs. human respectively) and different assay format (sandwich vs. two–site sandwich respectively). The lack of agreement in the results of sCAL determined by the BMN®-Cp and IDK®-Cp assays indicate that absolute results of sCAL are not interchangeable. The upper reference ranges of the BMN®-Cp (>3900 ng/mL) and IDK®-Cp (>3000 ng/mL) assays, however, reflect this 1.7–fold difference and therefore, sCAL results from both assays relative to their reference ranges may be usefully compared.

Measurement of S100A12 in serum was adapted from the S100A12 ELISA kit for the in–vitro determination of S100A12 in stool. Concentrations of sA12 in this study was not compared with fCAL levels as part of assay application in clinical studies of IBD since there was no reference range provided by Immunodiagnostik™ AG, Bensheim, Germany. We did not determine reference range for sA12 either as this was beyond the scope of this study. The performance characteristics of the IDK®-A12 assay was however, validated against other criteria provided by Immunodiagnostik™ AG, Bensheim, Germany.

Analytical sensitivity or LLoD for IDK®-A12 (1.119 ng/mL), IDK®-Cp (1.633 ng/mL) and BMN®-Cp (597.0 ng/mL) were calculated with a working range of 6.73 – 540 ng/mL for IDK®-A12, 114.5 – 25000 ng/mL for IDK®-Cp and 57700 – 2400000 ng/mL for BMN®-Cp. The lowest standard of 0 ng/mL, however, was not consistently detectable by IDK®-Cp and IDK®-A12 assays. The next higher standard for IDK®-Cp (3.90 ng/mL) and IDK®-A12 (0.66 ng/mL) were consistently measurable. Thus, the practical sensitivities of the assays were set at 4.0 ng/mL for IDK®-Cp and 1.0 ng/mL for IDK®-A12, and the working ranges were defined as 4 – 250 and 1 – 54 ng/mL for IDK®-Cp and IDK®-A12 respectively.

Taking into account the dilution of serum samples to a factor of 1:100 for IDK®-Cp and 1:10 for IDK®-A12, this translates into a practical working range of 400 – 25000 ng/mL (IDK®-Cp) and 10 – 540 ng/mL (IDK®-A12) for serum samples. Similarly, the lowest standard of 4 ng/mL was not consistently detectable by the BMN®-Cp assay, while the next higher standard of 12 ng/mL was consistently measurable and therefore the practical sensitivity of the BMN®-Cp assay was set at 12 ng/mL and the working range was defined as 12 – 240 ng/mL for serum samples. Considering that the serum samples were diluted to a factor of 1:100, this translates into a practical working range of 1200 – 24000 ng/mL for serum samples.

These adjusted or practical working ranges for the IDK®-Cp, IDK®-A12 and BMN®-Cp assays show a wide range suitable for routine clinical laboratory practice. The LLoD for the IDK®-Cp and BMN®-Cp assays are adequate when considered against their respective upper limit of the manufacturer's provided reference intervals (>3000 ng/mL for IDK®-Cp and >3900 ng/mL for BMN®-Cp). As previously stated, the reference interval for the IDK®-A12 assay was neither supplied by the kit manufacturer nor determined as part of the analytical validation process for the ELISA kits in this study.

The standards (calibrators) used in this study include the same lyophilised materials in five ampoules of varying concentrations of calprotectin (i.e., 0.0, 3.9, 15.6, 62.5 and 250.0 ng/mL for the IDK®-Cp assay; 0.4, 1.2, 4.0, 12.0 and 24.0 µg/mL for the BMN®-Cp assay) that had been used to calibrate the BMN®-Cp and IDK®-Cp assays, and the practical working range in this study is comparable to that reported by their respective manufacturers, making the data comparable, thus facilitating data analysis and interpretation.

Functional sensitivity or LoQ is the lowest concentration of an analyte that may be discriminated from zero with a high degree of confidence and it is reported as the lowest analyte value whose %CV is < 20.[28,33] In all cases and at an acceptable criterion of < 20 %CV, the LoQ is greater than LLoD (LoQ > LLoD). In this study, the LoQ was reported as 3615, 2880 and 522 ng/mL for the BMN®-Cp, IDK®-Cp and IDK®-A12 assays respectively, and this satisfied the 'fit for purpose' criteria of analytical methods as the corresponding values for LLoD were 597.0 , 1.633 and 1.119 ng/mL for the BMN®-Cp, IDK®-Cp and IDK®-A12 assays respectively. All three assays were linear between 840 and 15380 ng/mL, which will provide robust comparison within the analytical range of between 10 and 25000 ng/mL that cut across the linearity bracket provided by the

commercially available ELISA kit manufacturers.

A mean value of 103.7%, 98.4% and 100.8% for Measured to Expected ratios for linearity for the IDK®-A12, IDK®-Cp and BMN®-Cp assays respectively, compared favourably with the kit manufacturers provided mean data of 91.8%, 95.8% and 97.8% for the IDK®-A12, IDK®-Cp and BMN®-Cp assays respectively, and these values are within the targeted Measured to Expected ratios of between 80% – 120% acceptance criteria for immunoassays.

Measured to Expected ratios for linearity ranged from 71.4% to 148.8% for the three assays. Some values were outside the range of 80% – 120% acceptance criteria for immunoassays. The two highest values of 148.8% and 134.8% were observed for the sample with the lowest sA12 concentration of 840 ng/mL (i.e., 1:1 or neat dilution), suggesting that the IDK®-A12 assay has a limited linearity in the lower limit of the working range (i.e., 10 – 540 ng/mL). Nevertheless, for the assessment of IBD, it would be expected that sA12 values would be in the upper rather than the lower area of the working range and therefore, would not affect the clinical usefulness of sA12. In conclusion, the assays are linear within the analytical range for clinical application, with decreased linearity for extremely low and extremely high concentrations of sCAL and sA12.

Recovery for the IDK®-Cp (89.5%) and BMN®-Cp (82.1%) assays were within accepted target criteria of 80% – 120% for immunoassays. Recovery for the IDK®-A12 assay (126.5%) was acceptable even though just outside the target criteria (table 6). These recoveries compare favourably to those provided by the assay manufacturers. Recovery of calprotectin with the IDK®-Cp assay was adequate at all concentrations studied. Recovery at low concentrations of calprotectin with the BMN®-Cp assay was just outside the target criteria but acceptable because it would not affect the clinical utility of the assay in detecting inflammation due to IBD. Similarly, over–recovery of sA12 at low concentrations with the IDK®-A12 assay would not affect the clinical utility of the assay in detecting inflammation due to IBD.

It is generally recognized that intra–assay (within–run) and inter–assay (between–run) variability for immunoassay of less than 20% are acceptable. In the present study, intra–assay and inter–assay variability were all less than 10% and compared favourably to manufacturers' supplied imprecision values. This reinforces the accuracy of the three assays for clinical usefulness.

Serum samples may be haemolysed, icteric or lipaemic. The potential effects of these were not investigated but icteric, lipaemic and haemolysed samples were excluded. The assay protocols, however, require dilution of samples which may provide protection from these interferences. The shelf–life of the ELISA kit components used in this study was over 12 months of refrigerated storage. The fact that the values of the calibrator for calprotectin and S100A12 remained constant during the course of the over 12–month shelf–life of the assays indicated that the reagent and assays were stable. The effect of sample stability and repeated freeze–thaw cycles on calprotectin and S100A12 assays was not investigated in this study. Previous studies have been reported that calprotectin and S100A12 are stable in serum samples when stored frozen at –20ºC for at least 6 months.[31,32,35] However, storage at ambient

temperature may give a 6–fold or greater increase in calprotectin and S100A12 concentrations[35]. Caution must, therefore, be exercised in analysing old samples not appropriately stored. Furthermore, repeated freeze–thaw cycles did not alter the analytes concentrations in blood[35]. This was supported from good inter–assay (between–run) imprecision reported in this study.

A limiting factor in the evaluation of performance characteristics of the assays was the smaller than desired number of samples available for measuring sCAL and sA12, and high ELISA kit costs. All assays, however, were fully validated for the quantitation of sCAL and sA12, and showed good performance characteristics that compared favourably with assay parameters of commercially prepared kits. Linearity, recovery and imprecision studies indicate the assays to be linear, accurate and reproducible. Due to unavailability of calprotectin and S100A12 analogues, analytical specificity of the ELISA kits for calprotectin and S100A12 assays could only be demonstrated by linearity and recovery of calprotectin and S100A12. However, as more S100 protein family and/or analogues may become available in future, analytical specificity of calprotectin and S100A12 ELISA assays may need to be further evaluated.

Conclusion

This study has shown that the BMN®-Cp, IDK®-Cp and IDK®-A12 assays are suitable serum assays for routine application in a clinical laboratory. The performance characteristics were robust and sensitive from LoB, LLoD or LoQ and carry over studies. The three assays are reliable from the results of imprecision, reproducibility, recovery and linearity experiments, and compared favourably to manufacturer's provided performance characteristics. The large difference in numerical values between sCAL concentrations measured with the BMN®-Cp and IDK®-Cp assays indicate that the results and any derived cut–offs between the assays are not directly inter–changeable. Further studies are required to evaluate the clinical utility of the validated BMN®-Cp, IDK®-Cp and IDK®-A12 assays in serum in the assessment of inflammatory disorders, in particular IBD.

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