Performance of a FRET-Based Point-of-Care Immunoassay for the Quantitation of Fecal Calprotectin

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ABSTRACT

A fast (~5 min), time-resolved fluorescence resonance energy transfer based immunoassay (Procise FCP™) was developed for the point-of-care quantitative detection of fecal calprotectin (FCP) using 15 mg of fecal specimen eluted in collection fluid from the Procise Stool Collection Device™. Studies were performed to characterize analytical performance of the Procise FCP assay on the ProciseDx™ analyzer.

The Procise FCP assay showed good analytical performance with respect to sensitivity, specificity, linearity, and precision suitable for routine clinical use in a point-of-care setting as well as excellent analytical agreement with a current commercial FCP measurement method.

Results indicate that the Procise FCP assay is sensitive, specific, and precise yielding results in less than 5 minutes. This indicates the Procise FCP assay is useful for obtaining fast and accurate FCP

quantitation, thus avoiding delays inherent to current methods and enabling immediate clinical assessment to be made during a single patient visit.

INTRODUCTION

Calprotectin is a small calcium-binding protein and is a member of the S100 family of zinc-binding proteins. It consists of a heterodimer of S100A8/A9. It was first discovered in 1980 and was found to contribute ~60% of the protein content of the cytosol in neutrophils.[1] It is a marker for inflammation derived from activated neutrophils, macrophages, monocytes, and epithelial cells.[2,3] When measured in human stool, calprotectin has shown utility in assessing inflammatory bowel disease (IBD) activity and has demonstrated ability to assess disease in both ulcerative colitis (UC) and Crohn's disease (CD).[3]

Since the symptoms of IBD are nonspecific and are seen in other inflammatory or functional gastrointestinal disorders, endoscopic, radiological and histological tests are often needed to confirm or exclude the final diagnosis. Thus, the use of biological markers capable of differentiating between inflammatory and functional diseases is useful identifying patients with suspected IBD who may need follow-up procedures such as colonoscopy.[4]

The Procise FCP™ is a homogeneous assay that uses a fluorescence resonance energy transfer (FRET) signal to detect the presence and quantity of the target analyte. FRET is a process in which a donor molecule (Lumiphore®), in an excited state, transfers excitation energy through dipole-dipole coupling to an acceptor fluorophore when the two are brought into close proximity. Upon excitation at a characteristic wavelength the energy absorbed by the donor is transferred to the acceptor, which in turn emits light energy. The level of light emitted from the acceptor fluorophore is directly proportional to the degree of donor/acceptor complex formation.

The Procise FCP assay requires two separate binding events to ensure specificity. The first binding event is a rabbit monoclonal antibody (labeled with a donor fluorophore) to the fecal calprotectin (FCP) protein. The second binding event uses a mouse monoclonal anti-FCP antibody labeled with an acceptor fluorophore which forms a binding pair with the donor labelled anti-FCP antibody. As the concentration of FCP increases, a proportional increase in signal is observed. A schematic of the operation of FRET assay is shown in Figure 1.

Point-of-care (POC) testing of FCP can provide convenience as well as enhanced speed of result turn-around for improved patient management. Thus, the purpose of this study is to validate the performance of the Procise FCP to determine its suitability for use as a POC assay.

MATERIALS AND METHODS

Analyzer

The ProciseDx™ analyzer (Figure 2) is a small desktop analyzer weighing about 4.5 kg. It uses a UV LED to excite a proprietary terbium cryptate fluorophore, which serves as a donor that is covalently bound to an antibody or antigen used to detect the analyte of interest present in the assay. The donor, when in proximity to an acceptor bound to a different antibody used to detect the analyte of interest, excites the acceptor causing light to emit at a specific wavelength. The analyzer measures

the signal at specific wavelengths of interest to analyze the reaction (donor and acceptor) by filtering the light through specific band pass filters and determining the signal at each wavelength of interest with a silicon photomultiplier (SiPM). The intensity of the fluorescent signal emitted from the Reaction Cartridge is converted to Relative Fluorescence Units (RFUs) and the RFU then undergoes final analysis and determination of a test result based on an algorithm described within assay specific method file and reported as FCP concentration in units of µg/g of stool.

The analyzer has been designed with fail-safe features including use of sensors, 1-D and 2-D barcodes, controlled access via Operator or Supervisor user levels, periodic calibration check, GUI interface messaging and Power on Self-Test (POST). The checks are conducted automatically at POST and during operation to ensure the analyzer is operating to its specifications. If any of these safety features fail, the analyzer will alert the user and prevent tests from running. The analyzer ensures all safety features are tested at least once a week and most are run during each test.

Reagents and Assay Operation

The primary reagents for both the Procise FCP assay consist of a single-use Reaction Cartridge and a premeasured buffer solution in a plastic bulb. The cartridge cap is designed to hold a lyophilized bead containing assay specific donor and acceptor fluorophore labeled reagents (Figure 3.). The Procise FCP assay format is designed as a traditional sandwich assay format. A rabbit monoclonal anti-FCP antibody and a mouse monoclonal anti-FCP antibody are labeled with donor and acceptor fluorophores respectively. The donor molecule has three usable spectrally distinct peaks: 490, 550 and 620 nm. For the FCP FRET assay the energy transfer occurs using the 490 nm peak to the AlexaFluor 488 acceptor as depicted in Figure 1. Similar to other sandwich assay formats, as the concentration of FCP increases, a proportional increase in the amount of signal is observed.

Specimen Collection

Fecal specimens were deidentified leftover clinical laboratory specimens from IBD patients. Specimens were sampled using the Procise Stool Collection Device™. The device plastic probe was removed from the transfer/storage vial containing collection fluid and inserted into the stool specimen three times in different locations to collect the specimen. The probe was then returned to the collection medium vial which automatically eluted the specimen into the collection fluid. Configuration and operation of the Procise Stool Collection Device™ is represented in Figure 4.

Assay Operation

The assay reaction was initiated by using a fixed-volume transfer pipette to transfer 200 µL of the eluted fecal specimen collection fluid, dispensing the premeasured contents of a buffer bulb into the reaction cartridge, and mixing by inversion. The cartridge was then inserted into the analyzer for automatic incubation and reading, which takes less than 5 minutes. The instrument automatically measures and corrects for buffer volume addition differences (created by dispensing differences caused by operator dispense) and reports results.

Establishment of Assay Calibration

As no international standards are available for FCP, calibration was established at ProciseDx by preparation of an in-house reference standard using a well characterized recombinant FCP protein linked to a US FDA cleared assay Inova Quanta Lite Calprotectin (reference US FDA K160447).

Determination of Analytical Sensitivity and Linearity
Analytical sensitivity was determined by limiting concentrations of FCP. Linearity was determined by testing FCP across the assay range. Testing followed CLSI Guideline, EP17-A2 *Evaluation of Detection Capability for Clinical Laboratory Measurement Procedures; Approved Guideline* – 2nd Edition and EP6-A *Evaluation of the Linearity of Quantitative Measurement Procedures: A Statistical Approach; Approved Guideline*.

Determination of Precision
Assay precision was evaluated by testing five FCP-spiked samples containing various concentrations of FCP. This study was performed with 1 operator for a period of 10 days. The operator ran the 5 samples in two replicates per run, with 2 runs per day and 2 lot of reagents (n=80 data points per sample). Coefficients of variation (%CV) were calculated consistent with CLSI Guideline, *EP05-A3 Evaluation of Precision Performance of Quantitative Measurement Methods.*

Method Comparison
The analytical agreement and correlation between the Procise FCP assay were also compared head-to-head with an FDA-cleared commercial assay: Inova Quanta Lite Calprotectin Extended Range (Inova, San Diego, CA, USA) using 46 fecal specimens. Both assays were run according to manufacturer's instructions.

Fecal specimens were deidentified leftover clinical laboratory specimens from IBD patients. Statistical analysis of the agreement was determined by Deming linear regression and Pearson correlation.

Analytical Specificity
The extent to which various potentially cross reacting and interfering substances influence the results of the Procise FCP assay was determined by testing specimens spiked with increasing concentrations of the potential interferents in the FCP quantitation. Significant interference was defined as $\pm$20% of the expected value.

RESULTS

The Procise FCP assay showed a Limit of Blank, Limit of Detection, and Lower Limit of Quantitation of 6.0, 17.6, and 33.6 µg/g, respectively. The linear assay range was determined to be 34 - 1500 µg/g. The analytical lot-to-lot, day-to-day, and run-to-run precision of the assay over 10 days is summarized in Table 1.

The method comparison by Deming linear regression between the Procise FCP assay and the Inova Quanta Lite Calprotectin Extended Range assay was Y = 0.995X – 0.171, r = 0.993 and is shown graphically in Figure 5.

The analytical specificity of the Procise FCP assay as measured by interference in assay quantitation of various potentially cross-reacting or interfering substances fell within acceptable limits and is shown in Table 2.

DISCUSSION

FCP has shown significant diagnostic accuracy for discriminating IBD from functional disorders such as irritable bowel syndrome. Gisbert *et al*[5] reported an overall sensitivity of 80% and specificity of 76% for the diagnosis of IBD, reaching a higher accuracy for CD (sensitivity 83%, specificity 85%) than for UC (sensitivity 72%; specificity 74%). In a meta-analysis, von Roon *et al*[6] assessed the diagnostic precision of FCP for IBD and showed higher FCP levels than non-IBD patients with a sensitivity of 95% and a specificity of 91%.

Although there is no universally established cut-off level to diagnose IBD, it is widely accepted that 50 µg/g is an accurate FCP level to rule out inflammatory intestinal disease with a high negative predictive value (NPV).[7] Higher cut-offs are not usually recommended for rule-out because that would result in more false negative results and in this clinical use the NPV needs to be high in order to prevent delays in diagnosis. A normal level of FCP makes the diagnosis of intestinal inflammatory disease unlikely. FCP with a cutoff of 50 µg/g is commonly used as a reasonable first step to exclude inflammatory disease in cases where the symptoms are only moderately suspicious of IBD. Similarly, an FCP level of 120 µg/g or higher is commonly used as a cut-off to indicate an abnormally elevated FCP indicating the likely presence of an inflammatory condition in the bowel.

The diagnostic accuracy of FCP for the diagnosis of IBD has been shown to be higher than other biomarkers such as C-reactive protein, erythrocyte sedimentation rate, anti-neutrophil cytoplasmic antibodies and anti-saccharomyces cerevisiae antibodies.[4] In addition, current practice guidelines for the management of IBD from the American College of Gastroenterology recommend the measurement of FCP as a method to confirm gastrointestinal inflammation.[9] POC testing of FCP can provide convenience as well as enhanced speed of result turn-around for improved patient management.

The quantitative Procise FCP assay and ProciseDx analyzer are designed for POC use. Specimen collection can be performed at home or in the clinic if patients can produce a suitable specimen. The primary workflow has been designed to be simple by following the workflow of CLIA-waived assays. This offers advantages in immediate access, ease of specimen collection, and quick turn-around of FCP results compared to traditional central laboratory ELISA or send-out laboratory developed tests.

The findings of this study have confirmed that the Procise FCP assay provides good analytical performance and excellent correlation to an existing commercial assay sufficient to support their use in POC laboratories and physicians' offices. This convenience of location, ease of use, and quick availability of assay results may increase the use of FCP leading to better management of IBD.

This study is primarily analytical and does not offer direct comparisons between the Procise assay results and the clinical state of patients. However, excellent analytical correlation to an assay already commercialized and in routine clinical use suggest that the Procise FCP assay will also prove to be clinically useful but with the added advantages of POC availability. Ongoing future retrospective and prospective clinical studies will focus on directly demonstrating clinical utility in IBD patients.

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DISCLOSURE

All authors are employees of ProciseDx Inc.

REFERENCES

1. Fagerhol M, Dale I, Andersson T. A radioimmunoassay for a granulocyte protein as a marker in studies on the turnover of such cells. *Bull Eur Physiopathol Respir*. 1980;16(Suppl):273–282.
2. Røseth AG, Fagerhol MK, Aadland E, Schjønsby H. Assessment of the neutrophil dominating protein calprotectin in feces. A methodologic study. *Scand J Gastroenterol*. 1992 Sep;27(9):793-798.
3. Heida A, Park KT, van Rheenen PF. Clinical Utility of Fecal Calprotectin Monitoring in Asymptomatic Patients with Inflammatory Bowel Disease: A Systematic Review and Practical Guide. *Inflamm Bowel Dis*. 2017 Jun;23(6):894-902.
4. Benítez JM, García-Sánchez V. Faecal calprotectin: management in inflammatory bowel disease. *World J Gastrointest Pathophysiol*. 2015;15;6(4):203-209.
5. Gisbert JP, McNicholl AG. Questions and answers on the role of faecal calprotectin as a biological marker in inflammatory bowel disease. *Dig Liver Dis*. 2009;41:56–66.
6. von Roon AC, Karamountzos L, Purkayastha S, Reese GE, Darzi AW, Teare JP, Paraskeva P, Tekkis PP. Diagnostic precision of fecal calprotectin for inflammatory bowel disease and colorectal malignancy. *Am J Gastroenterol*. 2007;102:803–813.
7. Yang Z, Clark N, Park KT. Effectiveness and cost-effectiveness of measuring fecal calprotectin in diagnosis of inflammatory bowel disease in adults and children. Clin *Gastroenterol Hepatol*. 2014;12:253–262.
8. Mendoza JL, Abreu MT. Biological markers in inflammatory bowel disease: practical consideration for clinicians. *Gastroenterol Clin Biol*.33 Suppl 3:S158–S173 2009.
9. Lichtenstein, GR, Loftus EV, SB , Isaacs, KL, Regueiro, MD, Gerson, LB, Sands, BE. "Management of Crohn's disease in adults." *Am J Gastroenterol* 113:481-51. 2018 doi: 10.1038/ajg.2018.27

FIGURES

Figure 1. A representation of the Procise FCP FRET assay format.

As depicted in Fig. 1 a FRET emission signal from the acceptor is only created in the presence of calprotectin within the sample.

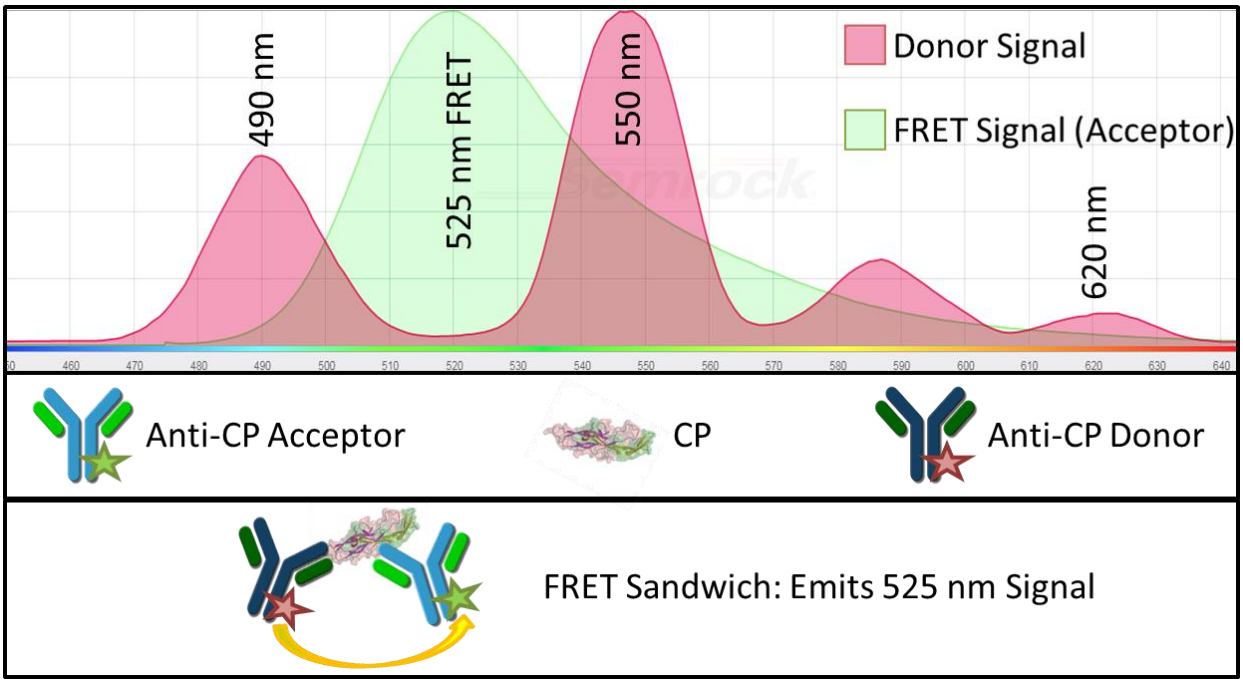


Figure 2 The ProciseDx Analyzer with Single-Use Reaction Cartridge.

Figure 3. The Reagent Cartridge – Exterior (Left) and Cut-Away View Showing Reagent Bead (right)

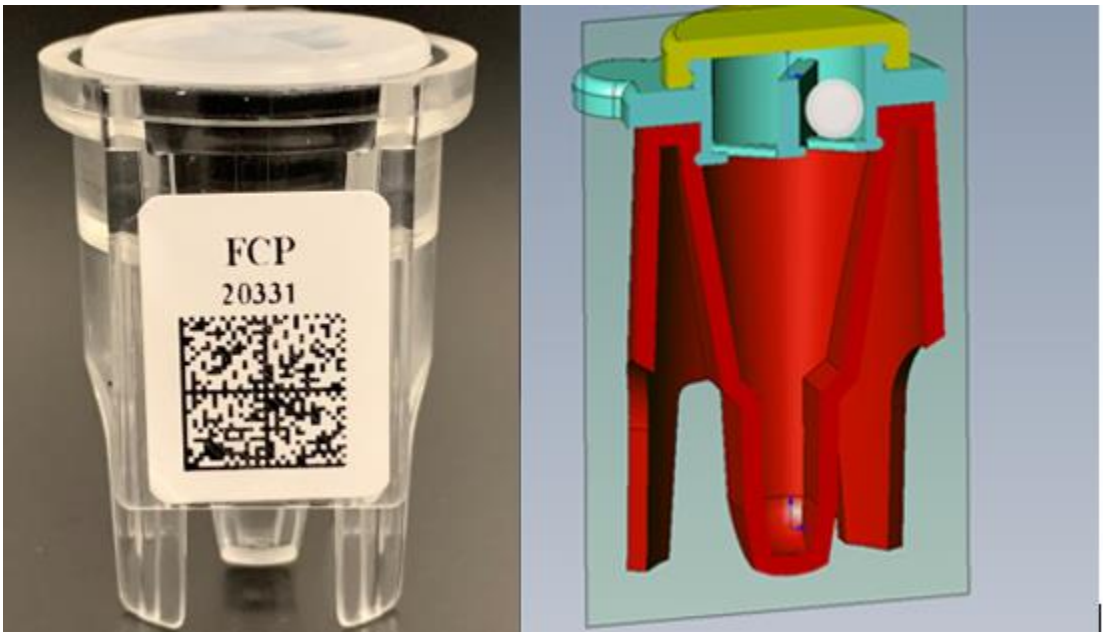


Figure 4. Configuration and Operation of the Procise Stool Collection Device™

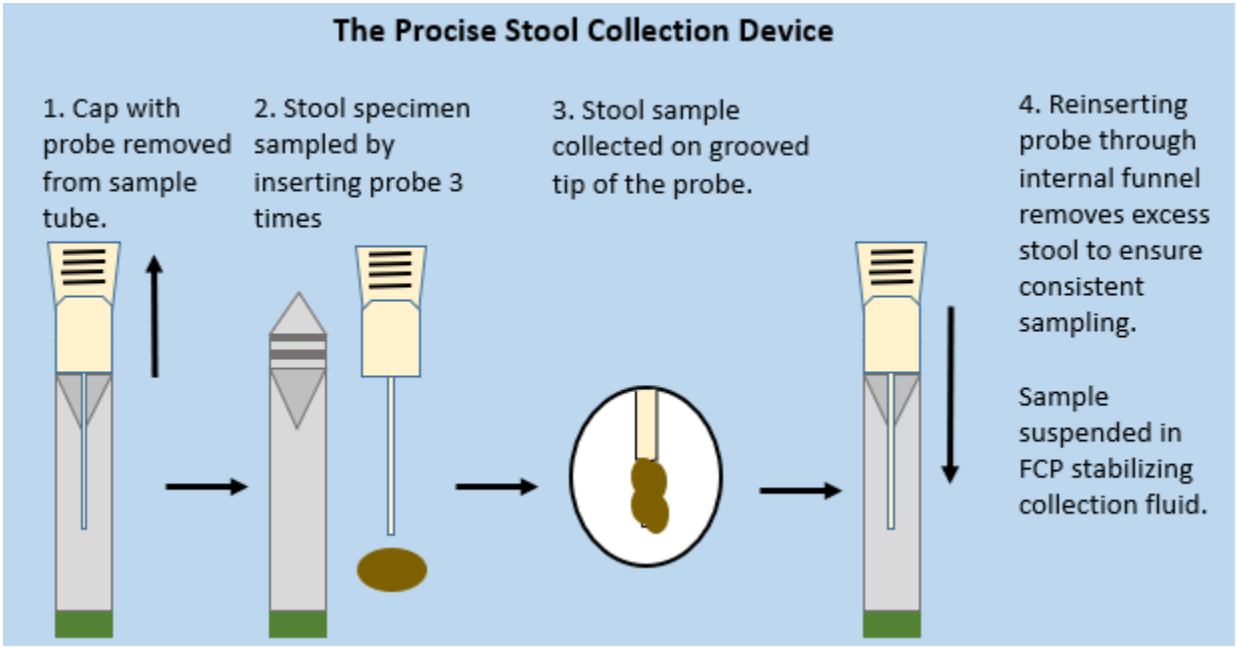
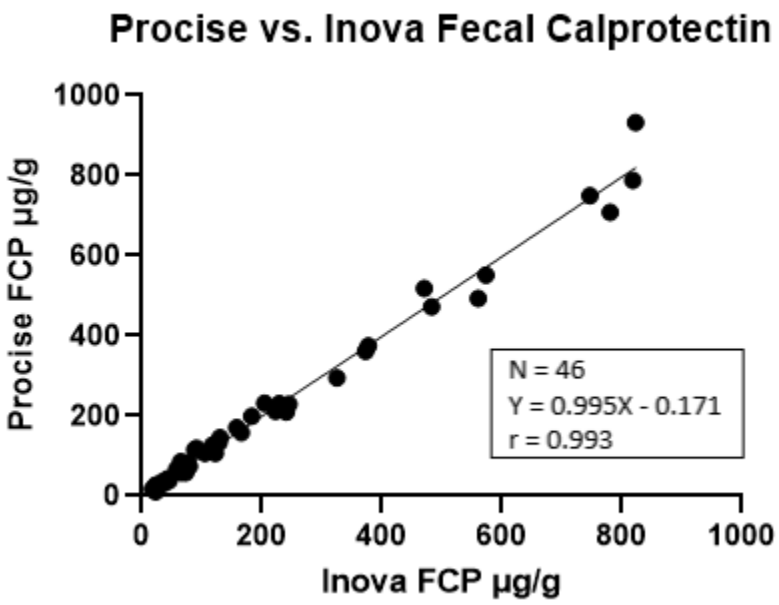


Figure 5. Deming linear regression and Pearson correlation between Procise FCP and Inova Quanta Lite Calprotectin Extended Range assays testing fecal specimens.



TABLES

Table 1. Results of FCP precision testing consisting of 2 reagent lots, 1 operator, 2 runs per day, 2 replicates per run across 10 days.

Sample	Mean (µg/g)	Within Run		Between Run		Between Day		Between Lot		Total	
		SD	CV (%)	SD	CV (%)	SD	CV (%)	SD	CV (%)	SD	CV (%)
1	68.9	8.0	11.6%	1.5	2.2%	3.8	5.5%	0.9	1.3%	9.1	13.2%
2	199.0	12.8	6.4%	1.6	0.8%	2.3	1.2%	1.6	0.8%	13.2	6.6%
3	1203.2	42.3	3.5%	5.5	0.5%	18.9	1.6%	6.2	0.5%	47.1	3.9%
4	138.0	10.4	7.5%	2.2	1.6%	3.6	2.6%	1.7	1.2%	11.4	8.3%
5	516.8	18.5	3.6%	3.1	0.6%	6.9	1.3%	2.3	0.4%	20.1	3.9%

Table 2. The analytical specificity of the Procise FCP assay as measured by interference in assay quantitation of various potentially cross-reacting or interfering substances. Significant interference was defined as ± 20% of the expected value.

Interferents	Maximum Concentration at Which No Significant Interference was Observed (Quantity per 50 mg stool-equivalent)
Prednisone	0.01 mg
Prevacid	0.02 mg
Vancomycin	0.67 mg
Asacol	1.33 mg
Azathioprine	0.07 mg
Ciproflaxin HCl	0.50 mg
Ferrous Sulfate	50 mg
Vitamin D	1.1 IU
Vitamin E	0.10 mg
Hemoglobin	5.83 mg