Automation of Creatinine and Calprotectin Assays on the ELISA NIMBUS®

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Introduction

New advances in the quantification of protein biomarkers facilitates diagnostic and prognostic testing in the clinical setting, providing solutions for therapeutic treatment. Immunoassays, such as enzyme-linked immunosorbent assays (ELISA), are still considered one of the gold standard methodologies for detection of biomarkers from various biological fluids in the clinical laboratory. Automation of immunoassays has shown to overcome limitations of manual methods by allowing for parallel processing of multiple assays, minimize variation from different laboratory technicians, and increase walk away time for the user, making ELISAs less time consuming than when performed manually. In this study, we evaluated two well-known biomarkers, creatinine and calprotectin, to determine automation performance of the ELISA NIMBUS® platform from Hamilton Robotics.

Creatinine is a critical biomarker for renal function. Creatinine is found in muscle tissues and is transported through blood and excreted into urine. The MicroVue™ Creatinine Assay Kit is a quantitative, colorimetric assay where alkaline picrate forms a colored solution in the presence of creatinine in urine samples.

Calprotectin is a biomarker for several diseases and conditions including cystic fibrosis and rheumatoid arthritis. Calprotectin is released from activated leukocytes which results in high concentrations in plasma, stools, etc.

The calprotectin assay kit is designed for quantitative determination of myeloid-related protein MRP8/14 (calprotectin) in human plasma. The test allows for the selective measurement of MRP8/14 antigen by sandwich ELISA with detection done using horseradish peroxidase conjugate and substrate tetramethylbenzidine (TMB).

Myriad RBM is a CLIA (Clinical Laboratory Improvement Amendments) certified laboratory providing researchers with reproducible, quantitative immunoassay data for hundreds of proteins. Creatinine and calprotectin assay kits were automated on the ELISA NIMBUS, which is based on the Microlab® NIMBUS liquid handling platform from Hamilton Robotics. The system has four independent pipetting channels, a microplate washer, four shaking incubators, and an absorbance reader. Results from this study showed that the automated methods performed well for both kits and produced quantitative results comparable to the manual methods. Consistency was obtained across replicates for both the high level and low level quality controls. The average percent recovery of analytes in serially diluted urine and human plasma samples for creatinine and calprotectin, respectively was within range as per the validation criteria set by the Myriad RBM lab. Thus, the ELISA NIMBUS can be used for automating the quantification of protein biomarkers in different biological fluids.

Materials and Methods — System Configuration

The ELISA NIMBUS, Figure 1, is a compact workstation for processing assay plates and can fully automate an ELISA workflow without being restricted to a particular kit or kit manufacturer.

Figure 1: ELISA NIMBUS system – a complete ELISA workstation. The system has four independent pipetting channels, a microplate washer, four shaking incubators, and an absorbance reader.

The instrument has a removable deck rack for simplified loading and unloading of reagents and ELISA plates, Figure 2.

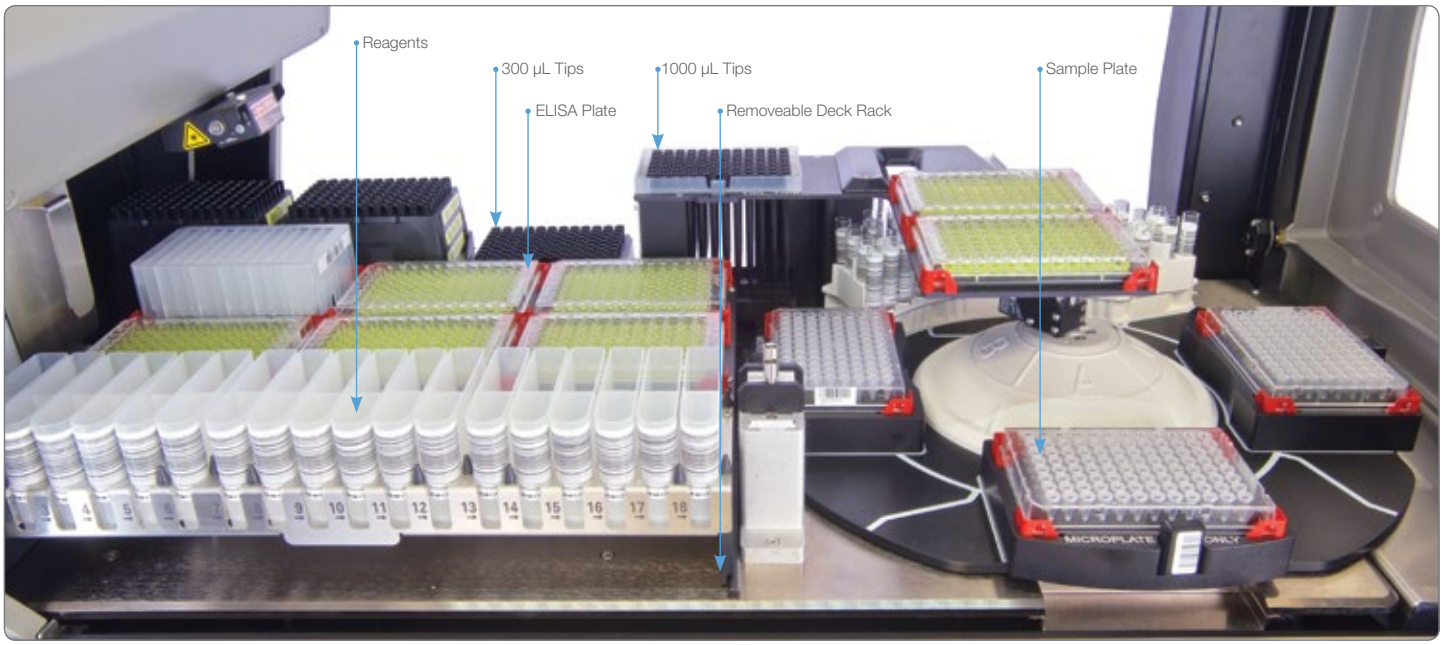

Figure 2: ELISA NIMBUS deck configuration with removable deck rack.

Materials and Methods — Creatinine Assay

The creatinine assay kit from Quidel was used with urine samples. The following items were used to perform the assay:

- MicroVue Creatinine Kit (#8009)
  - Low Control (82.58 mg/dL; Acceptable Range 70.14 to 93.89 mg/dL)
  - High Control (286.20 mg/dL; Acceptable Range 246.61 to 325.79 mg/dL)
  - Creatinine Standards
  - Color Reagent
  - Stop Solution
  - Microplate with 8-well Strips in a Frame
- 60 mL Hamilton Reagent Troughs (Hamilton P/N 56694-01)
- 1000 µL Non-filtered Non-sterile FTR Tips (Hamilton P/N 235904)
- 300 µL Nested Non-filtered, Non-sterile NTR Tips (Hamilton P/N 235950)

The creatinine assay protocol, following the kit manufacturer's instructions, was performed using the ELISA NIMBUS Template Designer software, Figure 3. The kit protocol was run both by hand and automated on the ELISA NIMBUS in order to compare the results of manual versus automated processing. Absorbance was read at 492 nm.

Figure 3: Simplified workflow for the Creatinine Assay.

The plate map for the creatinine assay was defined as shown in Figure 4. The creatinine standards (S1 – S5) were plated in duplicate in wells A1:E1 and A12: E12. Both the low (QC1) and high (QC2) quality controls were plated with 3 replicates (A2:C2 and D2:F2). The high control was diluted and placed in A11:H11. Eight urine samples were plated in columns 3 – 10 at 3 different dilution factors per sample.

	1	2	3	4	5	6	7	8	9	10	11	12
A	S5	QC1	Urine U7995 1:40	Urine U7996 1:40	Urine U7997 1:40	Urine U7998 1:40	Urine U7999 1:40	Urine U8000 1:40	Urine U8001 1:40	Urine U8002 1:40	HQ 1:40	S5
B	S4	QC1	Urine U7995 1:40	Urine U7996 1:40	Urine U7997 1:40	Urine U7998 1:40	Urine U7999 1:40	Urine U8000 1:40	Urine U8001 1:40	Urine U8002 1:40	HQ 1:40	S4
C	S3	QC1	Urine U7995 1:40	Urine U7996 1:40	Urine U7997 1:40	Urine U7998 1:40	Urine U7999 1:40	Urine U8000 1:40	Urine U8001 1:40	Urine U8002 1:40	HQ 1:40	S3
D	S2	QC2	Urine U7995 1:40	Urine U7996 1:40	Urine U7997 1:40	Urine U7998 1:40	Urine U7999 1:40	Urine U8000 1:40	Urine U8001 1:40	Urine U8002 1:40	HQ 1:40	S2
E	S1	QC2	Urine U7995 1:80	Urine U7996 1:80	Urine U7997 1:80	Urine U7998 1:80	Urine U7999 1:80	Urine U8000 1:80	Urine U8001 1:80	Urine U8002 1:80	HQ 1:80	S1
F	empty	QC2	Urine U7995 1:80	Urine U7996 1:80	Urine U7997 1:80	Urine U7998 1:80	Urine U7999 1:80	Urine U8000 1:80	Urine U8001 1:80	Urine U8002 1:80	HQ 1:80	empty
G	empty	empty	Urine U7995 1:160	Urine U7996 1:160	Urine U7997 1:160	Urine U7998 1:160	Urine U7999 1:160	Urine U8000 1:160	Urine U8001 1:160	Urine U8002 1:160	HQ 1:160	empty
H	empty	empty	Urine U7995 1:160	Urine U7996 1:160	Urine U7997 1:160	Urine U7998 1:160	Urine U7999 1:160	Urine U8000 1:160	Urine U8001 1:160	Urine U8002 1:160	HQ 1:160	empty

Figure 4: Plate map for the creatinine assay.

Materials and Methods — Calprotectin Assay

The MRP8/14 – Calprotectin Assay Kit from Buhlmann Laboratories was used to quantitatively measured calprotectin in human plasma samples. The following items were used to perform the assay:

- Calprotectin Kit (#S100A8/A9)
  - Low Control (1.5 µg/mL; Acceptable Range 0 to 2 µg/mL)
  - High Control (5.9 µg/mL; Acceptable Range 4.7 to 9 µg/mL)
  - Calibrators A to E (5-point Curve)
  - Enzyme Label Conjugated to HRP
  - TMB-Substrate
  - Stop Solution
  - ELISA Microplate with 8-well Strips in a Frame
- 60 mL Hamilton Reagent Troughs (Hamilton P/N 56694-01)
- 1000 µL Non-filtered Non-sterile FTR Tips (Hamilton P/N 235904)
- 300 µL Nested Non-filtered, Non-sterile NTR Tips (Hamilton P/N 235950)

The calprotectin assay protocol, Figure 5, following the kit manufacturer's instruction was performed using the ELISA NIMBUS Template Designer software. The kit protocol was run both by hand and automated on the ELISA NIMBUS in order to compare the results of manual versus automated processing. Absorbance was read at 450 nm.

The plate map for the calprotectin assay was defined as shown in Figure 6. The calprotectin calibrators (S1 – S5) were plated in duplicate in wells B1:F1 and B12:F12. Both the low and high controls for calprotectin were plated with 7 replicates (A10:G10 and A11:G11). Seven plasma samples were plated in columns 2 – 8 at 4 different dilution factors per sample. Incubation buffer was used as a blank in wells A1, A9:F9, and A12.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Incubation Buffer	P227 (1:100)	P234 (1:100)	S942 (1:100)	S936 (1:100)	HS930 (1:100)	BRH613221 (1:100)	BRH613227 (1:100)	Incubation Buffer	Low Control	High Control	Incubation Buffer
B	Calibrator A (S1)	P227 (1:100)	P234 (1:100)	S942 (1:100)	S936 (1:100)	HS930 (1:100)	BRH613221 (1:100)	BRH613227 (1:100)	Incubation Buffer	Low Control	High Control	Calibrator A (S1)
C	Calibrator B (S2)	P227 (1:200)	P234 (1:200)	S942 (1:200)	S936 (1:200)	HS930 (1:200)	BRH613221 (1:200)	BRH613227 (1:200)	Incubation Buffer	Low Control	High Control	Calibrator B (S2)
D	Calibrator C (S3)	P227 (1:200)	P234 (1:200)	S942 (1:200)	S936 (1:200)	HS930 (1:200)	BRH613221 (1:200)	BRH613227 (1:200)	Incubation Buffer	Low Control	High Control	Calibrator C (S3)
E	Calibrator D (S4)	P227 (1:400)	P234 (1:400)	S942 (1:400)	S936 (1:400)	HS930 (1:400)	BRH613221 (1:400)	BRH613227 (1:400)	Incubation Buffer	Low Control	High Control	Calibrator D (S4)
F	Calibrator E (S5)	P227 (1:400)	P234 (1:400)	S942 (1:400)	S936 (1:400)	HS930 (1:400)	BRH613221 (1:400)	BRH613227 (1:400)	Incubation Buffer	Low Control	High Control	Calibrator E (S5)
G	Empty	P227 (1:800)	P234 (1:800)	S942 (1:800)	S936 (1:800)	HS930 (1:800)	BRH613221 (1:800)	BRH613227 (1:800)	Empty	Low Control	High Control	Empty
H	Empty	P227 (1:800)	P234 (1:800)	S942 (1:800)	S936 (1:800)	HS930 (1:800)	BRH613221 (1:800)	BRH613227 (1:800)	Empty	Empty	Empty	Empty

Figure 6: Plate map for the calprotectin assay.

Material and Methods — Statistical Analysis

The data comparison tool that was used to create the correlation graph was a linear regression trend line. Additional standard curve trend lines were also used to compare known standards and controls. Percent correlation of variation (%CV) was determined for each assay as well.

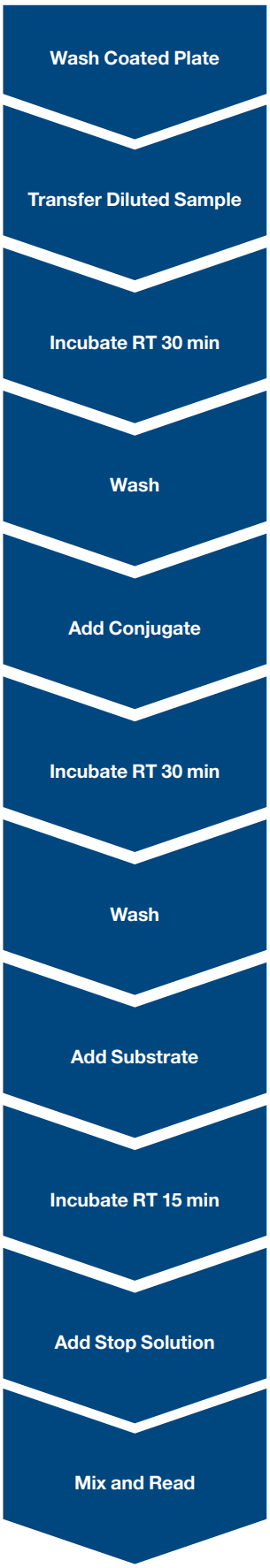

Figure 5: Simplified workflow for the calprotectin assay.

Results and Discussion — Creatinine Assay

The ELISA NIMBUS successfully processed a 96-well plate for the creatinine assay. Quality controls for both high and low levels were in the concentration range provided by the kit manufacturer, Table 1. The Coefficient of Variation (CV) of each control did not exceed 10%.

Table 1: Summary of Results for Low Level QC and High Level QC with the Creatinine Assay

Control	Kit Manufacturer's Range (mg/dL)	Average (mg/dL)	%CV	Status
Low Level (QC1)	70.13 to 93.89	76.8	10	Pass
High Level (QC2)	246.61 to 325.79	306.7	3	Pass

For the creatinine assay, a linear regression was used to compare the results of sample concentration run on the ELISA NIMBUS versus the manually performed assay, Figure 7. The linear regression resulted in a slope of 1.16 and a correlation coefficient of 0.81, Table 2, indicating a strong correlation between the automated versus manual assay.

The sample ratio for the creatinine assay is defined as the ratio of the average ELISA NIMBUS concentrations compared to the average manual concentrations obtained. The concentration values are shown in Table 2. Since the values of the sample ratios were close to a slope of 1, the test performed was considered to pass, indicating the performance of the automated method is comparable to the manual method.

The preparation of the standard curve for the creatinine assay performed on the ELISA NIMBUS gave a R2 value of 0.98802, Figure 8. The results also demonstrated that the concentration of the high and low controls were quantitated to be in line with the concentration indicated by the kit for those controls, as shown by purple diamonds.

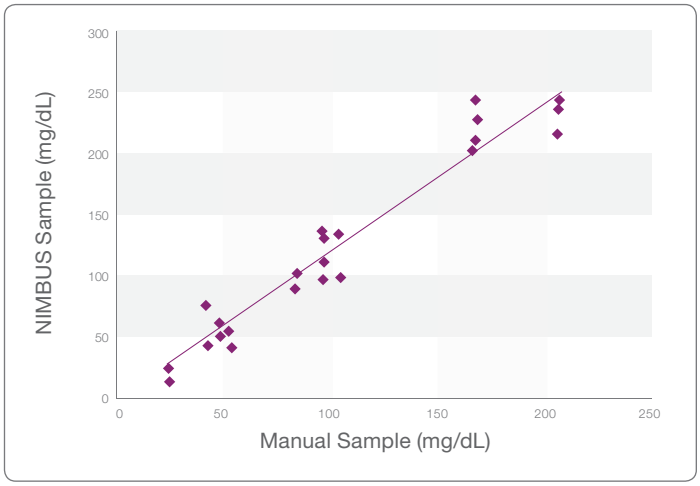

Figure 7: Correlation between manual ELISA and automated ELISA for the creatinine assay.

Table 2: Correlation Data Comparing Manual and Automated Methods for Creatinine

Correlation	0.81
Sample Ratio N:C (ELISA NIMBUS:Manual)	1.17
Slope 1	1.16
Status	Pass

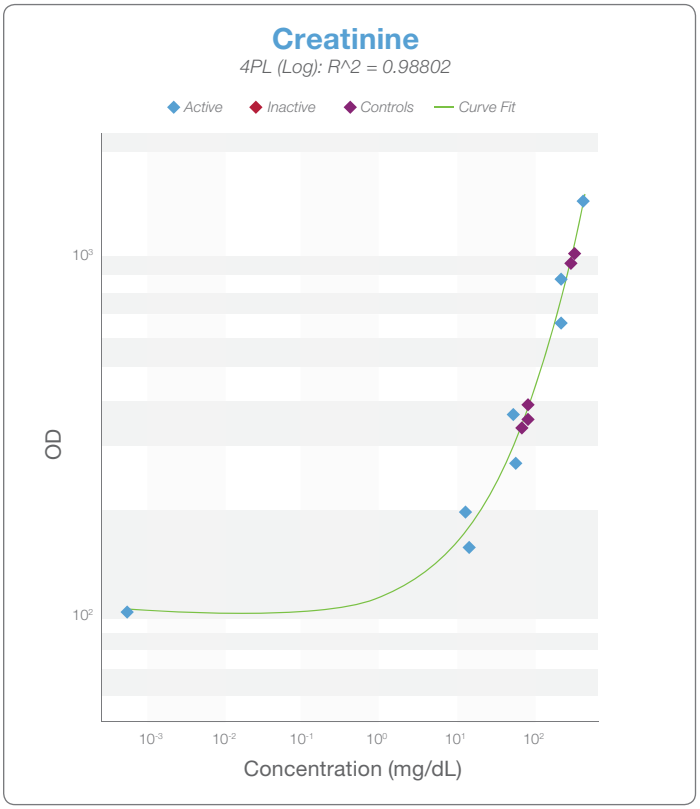

Figure 8: Standard curve of creatinine. The creatinine assay standard curve included three points (451 mg/dL, 264 mg/dL, and 42.5 mg/dL). Two additional points were added to the curve by performing a 1:3 dilution of the lowest concentration point and using DI water for the first point. Blue diamonds represent the standards and the purple diamonds represent the controls.

Linearity of the samples was determined by serially dilution and comparing the observed values with expected values. For Creatinine, the average percent recovery was within the range as specified by the validation criteria of 70 – 130%, set by the Myriad RBM lab, Table 3.

Table 3: Linearity Results for Creatinine

Creatinine	Dilution	Expected Concentration (mg/dL)	Observed Concentration (mg/dL)	% Recovery
Urine U7995	1:40	241.5	241.5	-
	1:80	120.75	116.85	97%
	1:160	60.38	48.6	80%
			Average % Recovery:	**89%**
Urine U7996	1:40	207	207	-
	1:80	103.5	96.4	93%
	1:160	51.75	60.4	117%
			Average % Recovery:	**105%**
Urine U7997	1:40	125	125	-
	1:80	62.5	57.4	92%
	1:160	31.25	19.8	63%
			Average % Recovery:	**78%**
Urine U7998	1:40	206.5	206.5	-
	1:80	103.25	83.75	81%
	1:160	51.63	70.9	137%
			Average % Recovery:	**109%**
Urine U8000	1:40	118.8	118.8	-
	1:80	59.4	63.1	106%
	1:160	29.7	15.2	51%
			Average % Recovery:	**79%**
Urine U8001	1:40	326.5	326.5	-
	1:80	163.25	138	85%
	1:160	81.63	118	145%
			Average % Recovery:	**116%**
Urine U8002	1:40	176	176	-
	1:80	88	84.9	96%
	1:160	44	28.3	64%
			Average % Recovery:	**80%**

Results and Discussion — Calprotectin Assay

The ELISA NIMBUS successfully processed one 96-well plate, and two 96-well plates in two separate runs. The single plate run was performed twice, and the 2 plate run was performed once. The calculated concentrations for both the high and low levels controls for this assay were within the range of values defined as acceptable by the kit manufacturer, Table 4. The %CV of each control did not exceed 5% within the individual runs (intra-run) and 11% between runs (inter-run) showing a high degree of consistency between runs performed on the ELISA NIMBUSs (Myriad RBM lab acceptance criteria is <20% CV intra-run and <20% CV inter-run).

Table 4: Summary of Results for Low Level QC and High Level QC with the Calprotectin Kit in Plates P1 and P2 as Compared to the Given Kit Manufacturer's Range

Control	Range (µg/mL)	Average (µg/mL)	%CV Run 1 P1	%CV Run 2 P1	%CV Run 3 P1	%CV Run 3 P2	%CV Inter Run	Status
Low Level (QC1)	0 to 2	1.60	1	2	2	3	7	Pass
High Level (QC2)	4.7 to 9	6.50	3	2	5	1	11	Pass

A linear regression was also performed for the calprotectin assay and was used to compare the results of the automated versus the manually performed assay. The linear regression resulted in a correlation coefficient of 0.9, indicating a strong correlation between the automated and manual ELISAs. The values for concentration obtained from using the ELISA NIMBUS compared to those obtained with the manual method are reported in Table 5 as sample ratios. Similar to the creatinine assay, the sample ratios were close to a slope of 1, which again indicates the performance of the automated method is comparable to the manual method and is considered a passed test.

Table 5: Correlation Data Comparing the Manual and Automated Methods for Calprotectin

	Run 1 Plate 1	Run 2 Plate 1	Run 3 Plate 1	Run 3 Plate 2
Correlation	0.9	0.9	0.9	0.9
Sample Ratio N:C (ELISA NIMBUS:Manual)	1.0	0.9	0.8	1.0
Slope 1 (through zero)	1.0	0.9	0.8	1.0
Status	Pass	Pass	Pass	Pass

The preparation of the standard curve for calprotectin on the ELISA NIMBUS gave a R2 value of 0.9988, Figure 9. This also demonstrates that the concentration of the high and low controls and diluted high controls were quantitated to be in line with the concentrations indicated by the kit, as shown by purple diamonds.

For calprotectin, the average percent recovery was within the range of values specified by the validation criteria of 70 – 130% set by the Myriad RBM Lab, Table 6.

Conclusion

The creatinine and calprotectin kits were successfully automated using the ELISA NIMBUS system. The ELISA results obtained had high precision for concentrations of quality controls and correlations of greater than 0.8 compared to manual processing. The average percent recovery values on the ELISA NIMBUS were high, resulting in strong linearity for both urine samples and human plasma samples. Taken together, these data illustrate that the ELISA NIMBUS is reliable and robust system for automating ELISA methods.

Table 6: Linearity Results for Calprotectin

	Average % Recovery			
Plasma Samples	**Run 1**	**Run 2**	**Run 3**	**Status**
Plasma 1	104	96	104	Pass
Plasma 2	89	86	88	Pass
Plasma 3	107	101	107	Pass
Plasma 4	107	106	114	Pass
Plasma 5	105	100	106	Pass
Status	Pass	Pass	Pass	

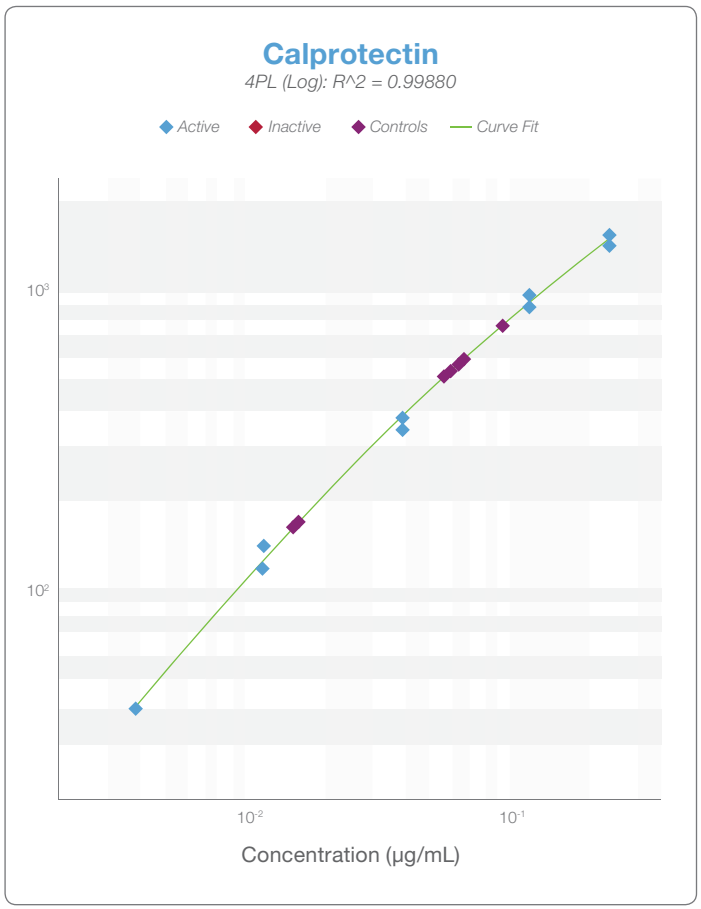

Figure 9: Standard curve of calprotectin. The calprotectin kit included five standard points in duplicate (0.226, 0.128, 0.0411, 0.0131, and 0.00401 µg/mL). Blue diamonds represent the standards and the purple diamonds represent the controls.

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