Assay Development Report

Assay	CNTO 5825 PK
Developer	Surekha Gangakhedkar
Date released	

A) ASSAY DEVELOPMENT

1. Analyte background

The analyte for this assay is the therapeutic asthma drug, CNTO 5825 provided by Centocor. This assay is a two antibody sandwich ELISA using an anti-therapeutic antibody from Centocor as the capture reagent and an anti-human IgG as the detection antibody.

2. Assay specifications

The assay is designed to detect CNTO 5825 in buffer, human whole blood, plasma and serum. The assay has a reportable range of 2000 ug/mL – 0.2 ug/mL, in the above mentioned matrices. The assay is specific for human sample types, and has not been tested in other species.

3. Reference assays

N/A

4. Antibody screening for matched pair

The capture antibody, CNTO 8584 was tested against two anti- human IgG antibodies to determine the pair with the best dose response for use in this immunoassay.

		Dab 1		Dab 2	
		NH2 conj.	SH conj.	NH2 conj.	SH conj.
CNTO 8584	NH2 conjugate				
	SH conjugate				

Dose response	
	Moderate
	Good
	Best

5. Assay Reagents

A. Capture Antibody

Vendor	Centocor
Catalog #	CNTO 8584

Current lot #	RLG1031-08-099
Type	N/A
Epitope	Anti-CNTO 5825
Specificity	N/A
Stock Conc.	2.37 mg/mL
Data sheet	N/A
Storage	-70C, Avoid freeze thaw cycles

B. Detection Antibody

Vendor	Novus Biologicals
Catalog #	NB 100-2046
Current lot #	08/05-G2-C11
Type	IgG1
Epitope	Fc region specific
Specificity	Subclass specific for human IgG
Stock Conc.	5 mg/mL
Data Sheet	[HYPERLINK "Data%20sheets/Dab_NB100-2046_Novus.pdf"]

C. Analyte

Vendor	Centocor
Catalog #	CNTO 5825 (Therapeutic drug)
Current lot #	FV02K08A
Type	IgG1, kappa Light chain
Stock Conc.	52.5 mg/mL
Storage	-70C, Avoid Freeze thaw cycles
Data sheet	N/A

D. Analyte Diluent

Composition	50 mM TBS, 3% BSA, 0.05% Thimerosal, pH 8.0
Storage	2 -8 C

6. Reagent Handling and Storage

For the analyte and capture antibody, Centocor suggested storage at -70°C and after thaw to store at 2-8°C with an expiration of one year after the date of thaw. Stock analyte and capture antibody was aliquoted into smaller volumes sufficient for a single use, flash frozen and stored at -80°C.

Biotin and Alkaline Phosphatase conjugates were stored at 4°C in Dojindo storage buffers. Since Dojindo specifies that the conjugates are stable for at least two months at 0-5°C; the conjugates were qualified at the end of three months either against a new conjugate or compared to historical data.

7. Protocols

Protocol for tip coating can be found at

[HYPERLINK "T:\\Assay_Systems\\Assay Development Reports\\Completed Assays\\Centocor Assays\\CNTO 5825\\CNTO_5825 Protocol.doc"]

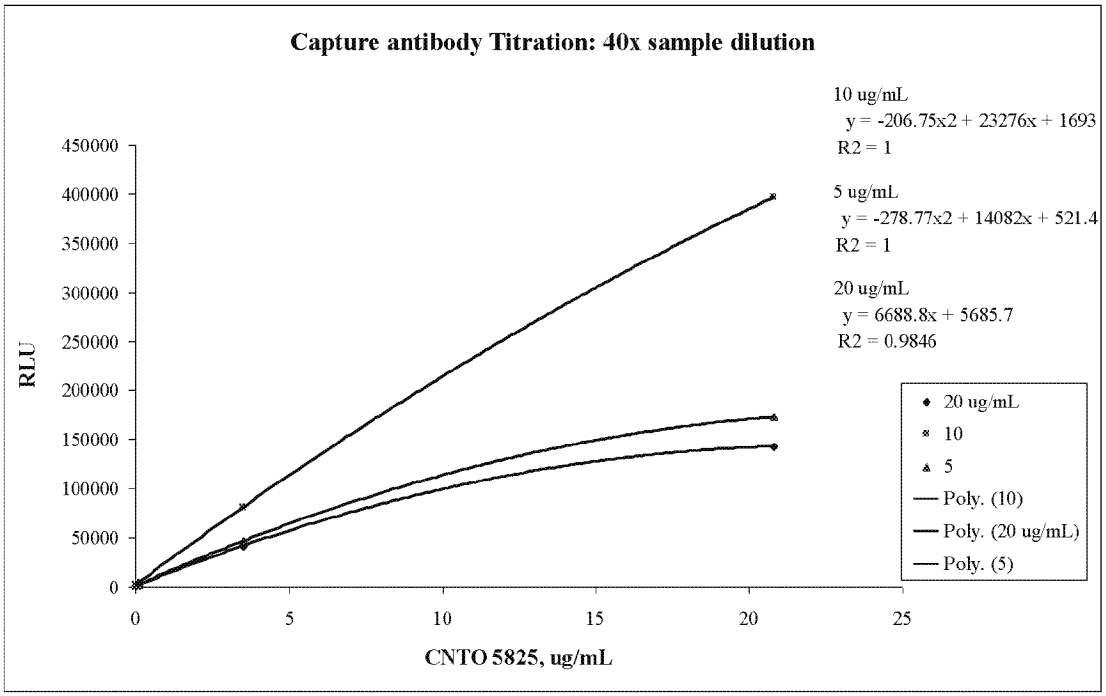
8. Capture Antibody Titration

Capture antibody was titrated at 5, 10, 20 ug/mL. Capture antibody at 10 ug/mL gave a higher signal than seen before, both for analyte and background. Each titration was coated on a separate tray. Sensitivity and CVs are the similar for all of the Capture concentration tested; S/B was the highest for 20 ug/mL CAB. A retest of the 20 ug/mL CAB gave results similar to S/B and sensitivity similar to 10 ug/mL. 10 ug/mL of capture antibody is considered to be optimum.

		40x sample dilution			4000x sample dilution		
[Cab]	**CNTO 5825 ug/mL**	**Avg**	**Stdev**	**%CV**	**Avg**	**Stdev**	**%CV**
20 ug/mL	2000	242052	43473	18	154032	29889	19
	500	224722	9406	4	59288	6342	11
	20.8	142771	13361	9	2139	109	5
	3.5	41371	9845	24	618	51	8
	0.1	1357	100	7	396	100	25
	0	450	54	12	379	48	13
10 ug/mL	2000	634647	37320	6	413589	23121	6
	500	594461	57762	10	131676	5456	4
	20.8	396390	57418	14	5721	941	16
	3.5	80625	4371	5	2369	647	27
	0.1	4077	566	14	1563	212	14
	0	1636	167	10	1500	426	28
5 ug/mL	2000	244178	35724	15	184512	2977	2
	500	242819	18749	8	81633	4008	5
	20.8	172815	22989	13	3349	304	9
	3.5	46398	6250	13	1354	431	32
	0.1	1757	162	9	988	422	43
	0	686	175	25	980	307	31

40x dilution			
CAB, ug/mL	**20**	**10**	**5**
S/B	538	388	356
S/B_Std5/6	3.0	2.5	2.6
Avg CV	12	10	14

4000x dilution			
CAB, ug/mL	**20**	**10**	**5**
S/B_std 1/6	406	276	188
S/B_std 5/6	1.6	1.6	1.4
Avg CV	14	16	20



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9. Detection Antibody Titration

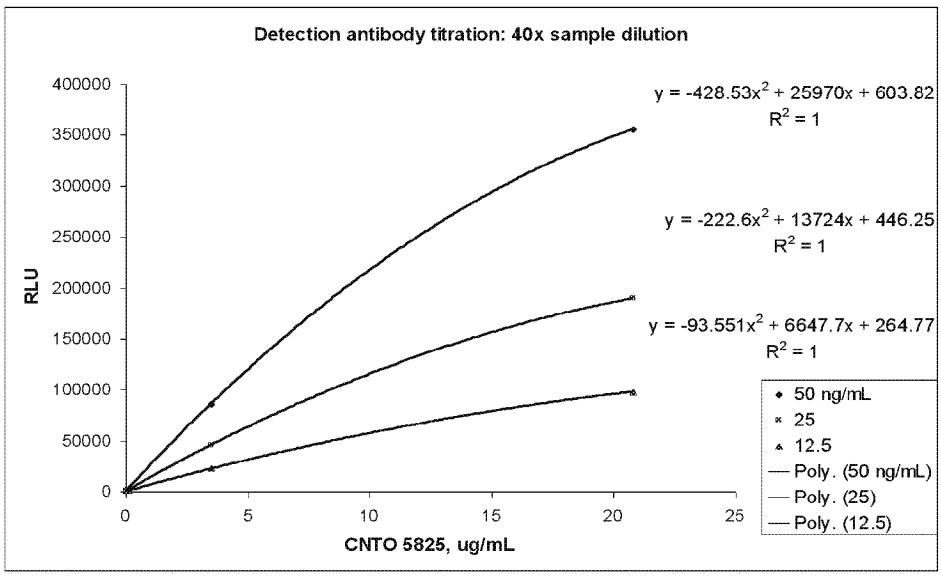
Detection antibody was titrated at 50, 25 and 12.5 ng/mL in stabilzyme with capture antibody at 10 ug/mL. As seen from the data, 25 ng/mL of detection antibody is optimum.

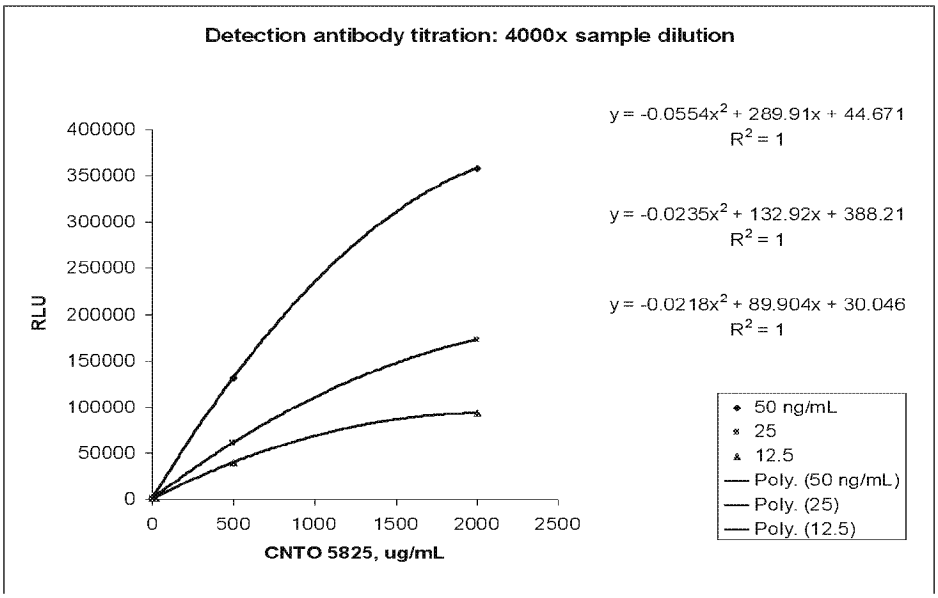
		40x sample dilution			4000x sample dilution		
[Dab]	**CNTO 5825 ug/mL**	**Avg**	**Stdev**	**%CV**	**Avg**	**Stdev**	**%CV**
50 ng/mL	2000	533794	35124	7	358202	38720	11
	500	504011	51039	10	131229	22952	17
	20.8	355390	45412	13	4535	235	5
	3.5	86264	11088	13	1167	83	7
	0.1	2817	383	14	668	82	12
	0	971	202	21	781	0	0
25 ng/mL	2000	311230	33397	11	172281	14022	8
	500	309006	34082	11	60974	7970	13
	20.8	189594	7242	4	3190	918	29
	3.5	45758	5102	11	750	300	40
	0.1	1670	272	16	406	82	20

	0	588	103	17	441	16	4
12.5 ng/mL	2000	147138	36762	25	92834	0	0
	500	146346	21982	15	39572	6031	15
	20.8	98063	3170	3	1382	139	10
	3.5	22390	1523	7	409	51	12
	0.1	804	19	2	252	21	8
	0	385	24	6	236	4	2

40x dilution			
DAB, ng/mL	**50**	**25**	**12.5**
S/B_std 1/6	550	530	382
S/B_std 5/6	2.9	2.8	2.1
Avg CV	13	12	10

4000x dilution			
DAB, ng/mL	**50**	**25**	**12.5**
S/B_std 1/6	458	391	394
S/B_std 5/6	1.5	1.7	1.7
Avg CV	9	19	8





10. Whole Blood screen

10 human blood samples were screened to check for sample interference, since the assay uses a generic anti-human IgG as the detection antibody. To avoid potential cross-reactivity when using whole blood, plasma or serum, the Theranos reader protocol includes a post sample wash feature. An assay buffer standard curve was used to calculate sample concentrations. None of the samples tested showed detectable cross reactivity to the matrix.

BLOOD SCREEN				
	Blood Tube #	20X	8000X	Blood
		RLU	RLU	Calc. ug/mL
1	W070509 111029, 2/25/09	1455	1129	0.00
2	W070509 111031, 2/25/09	2101	1509	0.03
3	W070509 111195, 2/25/09	1951	1095	0.02
4	W070509 111027, 2/25/09	1216	979	0.00
5	W070509 111028, 2/25/09	4571	1191	0.14
6	W070509 111197, 2/25/09	826	684	0.00

7	W070509 111026, 2/25/09	1733	1204	0.01
8	W070509 111030, 2/25/09	1521	1147	0.00
9	W070509 111055, 2/26/09	1701	1103	0.01
10	W070509 111058, 2/26/09	1382	1312	0.00

11. Precision

A 7 point assay buffer standard curve was assayed on replicate cartridges for 3 lots to determine precision. The cartridge used a 40x and 4000x fold dilution of the sample.
Total CV (any cartridge, any instrument): 7 %
Average Intra-Cartridge CV: 11%

40x Dilution	RLU	RLU	RLU			
CNTO 5825 ug/mL	**Lot 1**	**Lot 2**	**Lot 3**	**Avg RLU**	**Stdev**	**%CV**
2000	248438	305874	293164	282492	30168	11
500	229216	239340	253544	240700	12221	5
20.8	165556	209020	204015	192864	23781	12
3.5	59380	59958	57091	58810	1516	3
0.9	14017	12351	13225	13198	834	6
0.1	2284	2301	2367	2317	44	2
0	889	1069	1094	1017	112	11
					Total CV	**7**

4000x Dilution						
CNTO 5825 ug/mL	**Lot 1 RLU**	**Lot 2 RLU**	**Lot 3 RLU**	**Avg RLU**	**Stdev**	**%CV**
2000	188321	258915	238478	228571	36325	16
500	120836	117160	119573	119189	1868	2
20.8	5315	4691	5041	5016	313	6
3.5	1047	1031	997	1025	26	3
0.9	589	649	604	614	31	5
0.1	541	602	602	581	35	6
0	504	638	580	574	67	12
					Total CV	**7**

A) <u>Calculated concentrations using the Average RLU (of 3 cartridges) for each Lot</u>

40x Dilution				
CNTO 5825 ug/mL	**Lot 1**	**Lot 2**	**Lot 3**	**Avg**
2000	OORH	OORH	OORH	
500	OORH	OORH	OORH	
20.8	20.80	20.80	20.80	20.80
3.5	3.50	3.50	3.50	3.50
0.9	0.90	0.90	0.90	0.90
0.1	0.10	0.10	0.10	0.10
0	0.00	0.00	0.00	0.00

4000x Dilution

CNTO 5825	Lot 1	Lot 2	Lot 3	Avg
ug/mL				
2000	1999.99	2000.00	2000.07	2000.02
500	499.99	500.00	500.01	500.00
20.8	20.88	20.89	20.90	20.89
3.5	2.81	2.63	2.50	2.65
0.9	OORL	OORL	OORL	
0.1	OORL	OORL	OORL	
0	OORL	OORL	OORL	

B) Calculated concentrations for each cartridge for the 3 lots

40X	Nominal ug/mL	Calc. Run 1	Calc. Run 2	Calc. Run 3	Avg	Stdev	%CV
Lot 1	2000						
	500						
	20.8	16.49	24.02	22.57	21.03	4.00	19
	3.5	3.90	3.43	3.19	3.51	0.36	10
	0.9	0.79	0.70		0.75	0.06	8
	0.1	0.12	0.09	0.10	0.10	0.01	13
	0	0.04	0.00	0.00	0.01	0.02	

4000x	Nominal ug/r	Calc. Run 1	Calc. Run 2	Calc. Run 3	Avg	Stdev	%CV
Lot 1	2000	1574.4	1801.3	1613.6	1663.12	121.23	7
	500		894.8	641.5	768.17	179.13	23
	20.8	20.1	20.3	22.2	20.86	1.19	6
	3.5	3.0	2.5	3.0	2.81	0.26	9
	0.9						
	0.1						
	0						

40X	Nominal ug/mL	Calc. Run 1	Calc. Run 2	Calc. Run 3	Avg	Stdev	%CV
Lot 2	2000						
	500						
	20.8	18.78	21.25	22.52	20.85	1.90	9
	3.5	3.32	3.48	3.70	3.50	0.19	5
	0.9	0.89	0.99	0.82	0.90	0.08	9
	0.1		0.08	0.12	0.10	0.03	29
	0	0.00	0.00	0.01	0.00	0.02	

	Nominal ug/r	Calc. Run 1	Calc. Run 2	Calc. Run 3	Avg	Stdev	%CV
Lot 2	2000	1676	2533	1872	2026.79	448.81	22
	500	498	489	513	500.06	11.85	2
	20.8	20.18	18.44	24.05	20.89	2.87	14
	3.5	2.32	2.78	2.80	2.63	0.27	10
	0.9						
	0.1						
	0						

40X	Nominal ug/mL	Calc. Run 1	Calc. Run 2	Calc. Run 3	Avg	Stdev	%CV
Lot 3	2000						
	500						
	20.8	20.48	20.70	21.22	20.80	0.38	2
	3.5	3.37	3.39	3.73	3.50	0.20	6
	0.9	0.83	0.96	0.90	0.90	0.07	7
	0.1	0.12	0.08	0.09	0.09	0.01	11
	0	0.00	0.00	0.01	0.00	0.01	

	Nominal ug/r	Calc. Run 1	Calc. Run 2	Calc. Run 3	Avg	Stdev	%CV
Lot 3	2000	1804.17	2587.30	1697.24	2029.57	485.96	24
	500	490.73	451.86	563.47	507.67	78.92	16
	20.8	24.39	22.42	15.84	20.88	4.48	21
	3.5	2.26	2.47	2.77	2.50	0.26	10
	0.9						
	0.1						
	0						

One sample in the mid range of the assay (50 ug/mL) was assayed in a total of 24 cartridges on 24 different instruments to determine the mid-range Total CV.
Total CV (any cartridge, any instrument) at mid range: 15%

12. Spike recovery from Whole blood

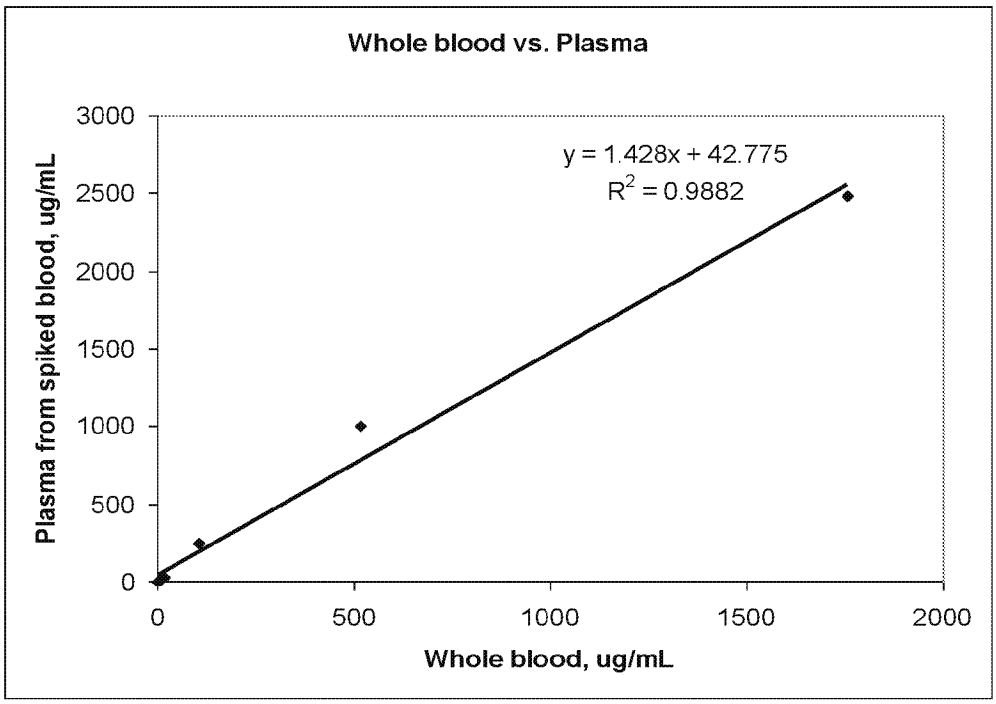
CNTO 5825 was spiked at 8 levels ranging from 2000 – 0.1 ug/mL, into human whole blood and the recovery calculated. The recovery ranged from 73 -104%, with an average of 85%. Recovery was calculated against an assay buffer standard curve. Plasma spun down from the spiked blood was tested to check for hematocrit effect. Recovery for this plasma ranged from 115 - 236 %, with an average recovery of 169%. A hematocrit correction factor of 1.4 will be required to estimate whole blood vs. plasma recovery. A Theranos reader protocol that uses 20 and 8000-fold sample dilution was used for the test. Percent recovery = observed ÷ expected × 100%.

SPIKE INTO WHOLE BLOOD					
	CNTO 5825 Nominal, ug/mL	RLU		Spiked Blood Calc. ug/mL	% Recovery Spiked Blood
		20X	8000X		
1	2000	314929	119279	1756	88
2	500	275556	35540	518	104

3	125	294211	7569	104	83
4	20.8	198382	1574	15	74
5	3.5	65678	682	3.10	88
6	0.9	14601	626	0.78	87
7	0.1	1784	549	0.07	73
8	0	723	818	0.01	
				Average	85

PLASMA FROM SPIKED BLOOD

	CNTO 5825	**RLU**		**Plasma**	**% Recovery**
	Nominal, ug/mL	**20X**	**8000X**	**Calc. ug/mL**	
1	2000	334456	168418	2482	141
2	500	312839	68268	1002	194
3	125	273295	17161	246	236
4	20.8	210028	2499	29	189
5	3.5	93801	1149	4.03	115
6	0.9	29871	902	1.55	173
7	0.1	2908	762	0.14	137
8	0	1021	1015	0.03	
				Average	169



13. Spike recovery from plasma

CNTO 5825 was spiked at 10 levels ranging from 2000 – 0.1 ug/mL, into human plasma and recovery calculated against an assay buffer curve. The recovery ranged from 66 -140%, with an average of 111%. A reader protocol that uses a 40 and 4000-fold sample dilution was used for the test. Percent recovery = observed ÷ expected × 100%.

SPIKED PLASMA RECOVERY					
	CNTO 5825 Nominal, ug/mL	**40x Dilution RLU**	**4000x Dilution RLU**	**Spiked Plasma Calc. ug/mL**	**% Recovery**
1	2000	140808	108365	1317	66
2	1000	138544	106265	1253	125
3	500	136915	71164	552	110
4	166.7	138441	31582	246	148
5	41.7	127380	6228	54.3	130
6	10.4	94624	1586	10.40	100
7	2.6	30738	554	2.64	102
8	0.7	6383	367	0.53	76
9	0.1	1094	259	0.14	137
10	0	428	205	0.09	
				Average	111

14. Matrix effect

The effect of spiking the analyte into various matrices like hemolyzed blood, lipemic plasma and serum from asthma patients was tested to evaluate assay response.

A) Spike into hemolyzed blood

CNTO 5825 was spiked at 4 levels ranging from 2000 – 0.1 ug/mL into two different blood samples that exhibited hemolysis. In blood sample 1, range of recovery was 78 -140%, with an average of 99% and the assay modulation was as expected from the assay buffer standard curve. In blood sample 2, which showed far greater hemolysis than sample 1 and was stringy, an increase in background was seen impacting the recovery at the 0.1 ug/mL analyte level for the 20-fold sample dilution. The effects of the sample condition were not observed with the higher dilution. Average recovery was 80% after excluding the analyte spike at the 0.1 ug/mL level.

Sample # 1					
	CNTO 5825	**Spiked Blood RLU**		**Spiked Blood**	**% Recovery**
	Nominal, ug/mL	**20X**	**8000X**	**Calc. ug/mL**	**Spiked Blood**
1	2000	622391	300927	1608	80
2	125	502955	15079	97	78
3	3.5	83072	3316	3.42	98
4	0.1	5839	3626	0.14	140
5	0	3739	3784	0.00	
				Average	99

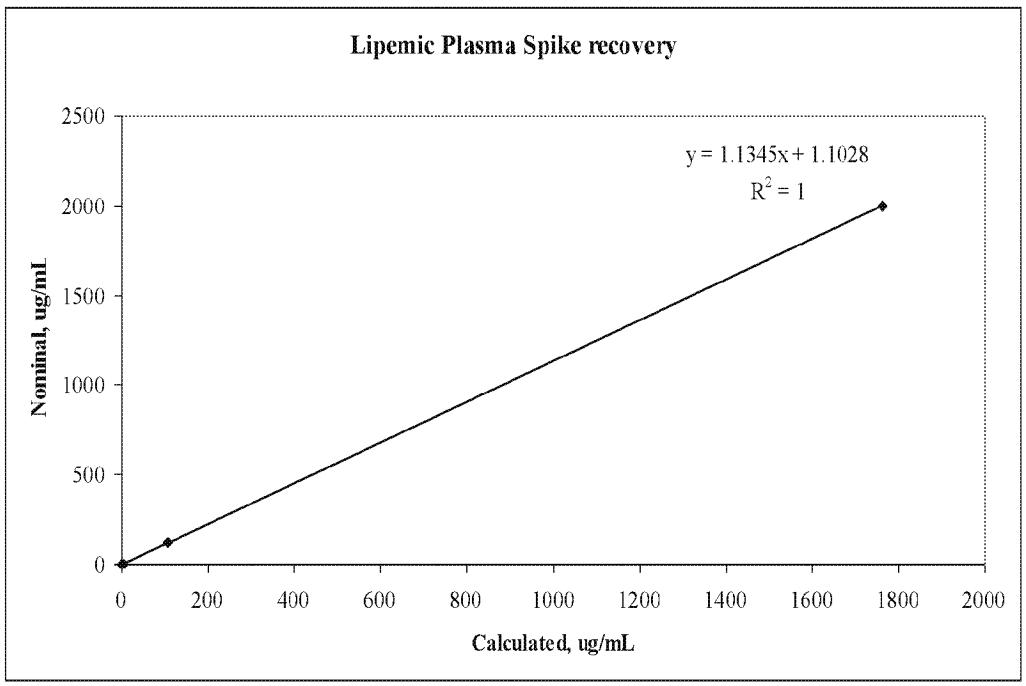
Sample # 2					
	CNTO 5825	**Spiked Blood RLU**		**Spiked Blood**	**% Recovery**
	Nominal, ug/mL	**20X**	**8000X**	**Calc. ug/mL**	**Spiked Blood**
1	2000	565090	238671	1279	64
2	125	500489	14812	96	77
3	3.5	103013	3037	3.49	100
4	0.1	18053	3630	0.97	973

5	0	17025	3185	0.91	
				Average	80

B) Spike into lipemic plasma

CNTO 5825 was spiked at 4 levels ranging from 2000 to 0.1 ug/mL into lipemic plasma. Recovery of the analyte ranged from 85 – 102%.

Spike into Lipemic Plasma					
	CNTO 5825 ug/mL	20x RLU	8000x RLU	Calculated ug/mL	% Recovery
1	2000	498250	224859	1762	88
2	125	501348	16320	107	85
3	3.5	99239	3554	3.50	100
4	0.1	5322	3626	0.10	102
5	0	3169	2821		
				Average	94



C) Spike into Asthma serum

CNTO 5825 was spiked at 200 and 20 ug/mL into the three asthma serum samples to calculate recovery. The levels of the asthma biomarkers were obtained by assaying those samples on the respective R&D ELISA kits. The three serum samples chosen to spike the analyte into showed different levels of the biomarkers. Recovery ranged from 80 – 130%, with an average of 105%. A sample dilution of 8000-fold only, on the instrument was used for this test.

Sample	ID	Age	Gender	Medications	TARC* pg/mL	ENA-78* pg/mL	Eotaxin* pg/mL	Rantes* ng/mL
1	710	67	Female	Fosamax, Feosol, Lipitor, Nexium	197	642	123	47

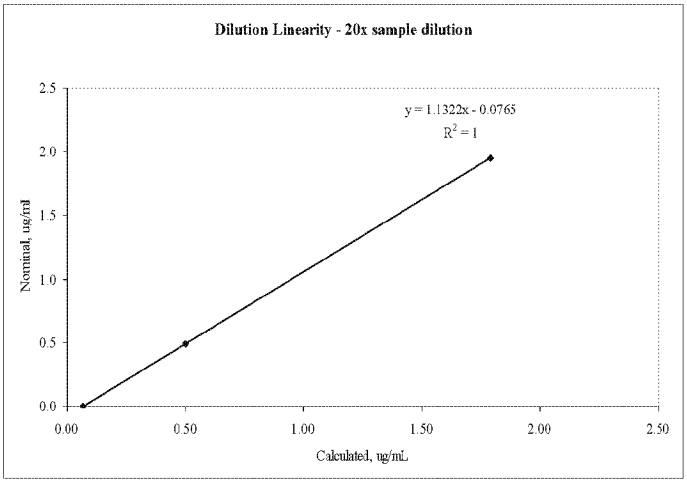
3	1247	46	Male	Advair, Lisinopril, Zocor	1021	4011	79	54
5	1442	20	Female	Albuterol	14	4	64	0.48

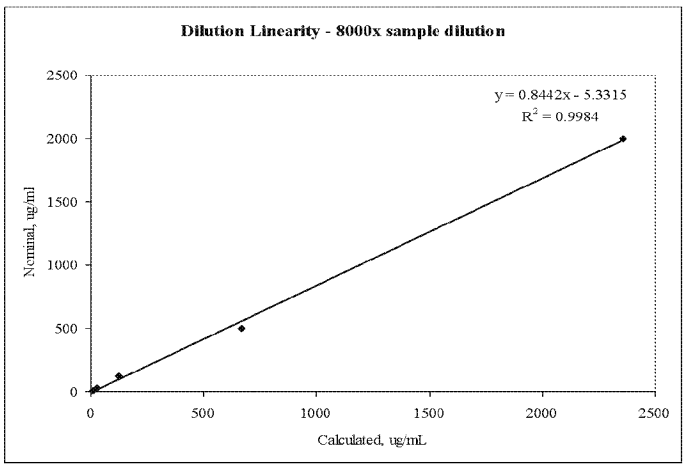
Sample #	Spike, ug/mL	1		2		Avg	Stdev	%CV	Calc.ug/mL	% Recovery
1	200	19968	19635	28971	28161	24184	5073	21	191	96
	20	2742	2579	3016	3287	2906	312	11	25	126
	0	1173	1409			1291	167	13	13	
3	200	23496	22824	17525	16652	20124	3534	18	160	80
	20	3144	3752	2784	3011	3173	414	13	27	137
	0	1206	1232			1219	19	2	12	
5	200	21398	22745	21073	20099	21329	1094	5	169	84
	20	2545	2703	2412	2098	2440	257	11	22	108
	0	891	1145			1018	180	18	11	
									Average	105

15. Dilution linearity

Dilution linearity of the assay was tested by spiking into plasma the analyte at 2000 ug/mL and serially diluting the sample to yield sample concentrations within the dynamic range of the assay. Recovery ranged from 88 - 134 %. Percent recovery = 100*[calculated conc. of standard] / ([calculated conc. of Std1] * [Nominal conc. of Std.]/[Nominal conc. of Std1]), assuming 100% recovery for Std 1.

Dilution Linearity					
	CNTO 5825 Nominal, ug/mL	**20X Dilution RLU**	**8000X Dilution RLU**	**Spiked plasma Calc. ug/mL**	**% Recovery**
1	2000	285650	167198	2357	100
2	500	297165	46298	668	113
3	125	244298	9005	125	85
4	31.3	216378	2397	27	75
5	7.8	138541	1150	9	98
6	2.0	34839	902	1.79	78
7	0.5	9464	846	0.50	87
8	0	1652	691	0.07	
				Avg	89



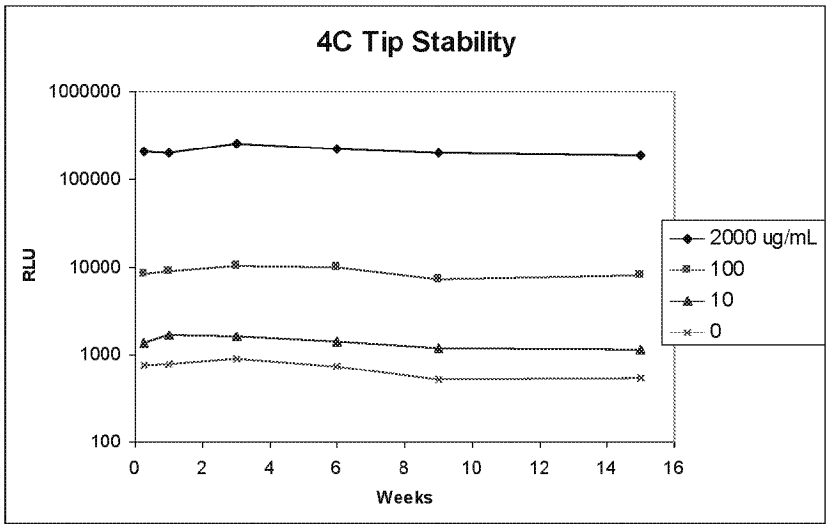


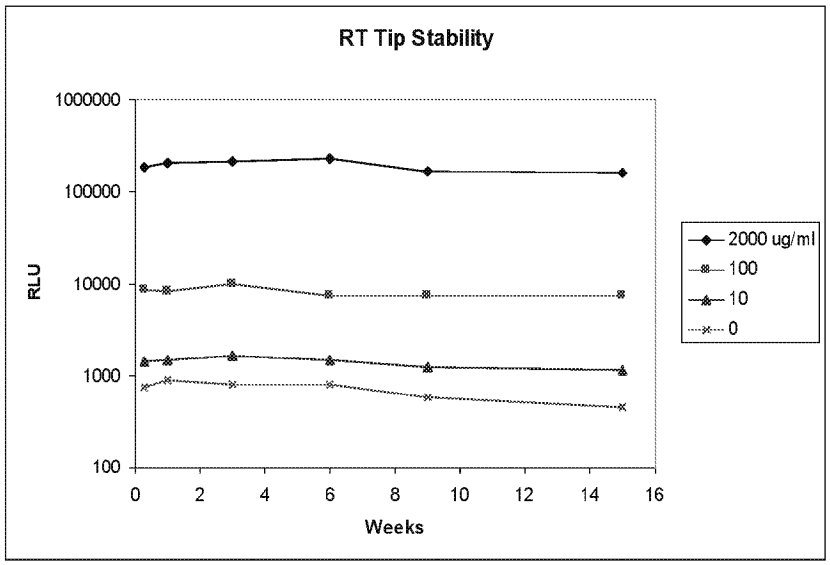
16. Stability

A) Capture antibody surface stability

Stability will be tested for a period of 12 weeks for storage at 4°C and room temperature with a 4-point assay buffer curve with an instrument protocol that allows for 8000-fold sample dilution. Analyte standards were pre-made for the entire study, aliquoted and flash frozen for single time use. A freshly made working concentration of detection antibody is made for each time point.

Summary		weeks					
Temp	ug/mL	0.3	1	3	6	9	15
4C	2000	207597	203481	252859	219996	200298	185699
	100	8283	8917	10032	9749	7330	7944
	10	1350	1673	1576	1417	1161	1148
	0	744	774	893	734	513	532
RT	2000	187518	203267	210900	229080	167076	158159
	100	8532	8315	9894	7504	7495	7266
	10	1415	1468	1629	1446	1225	1135
	0	757	900	788	805	582	455

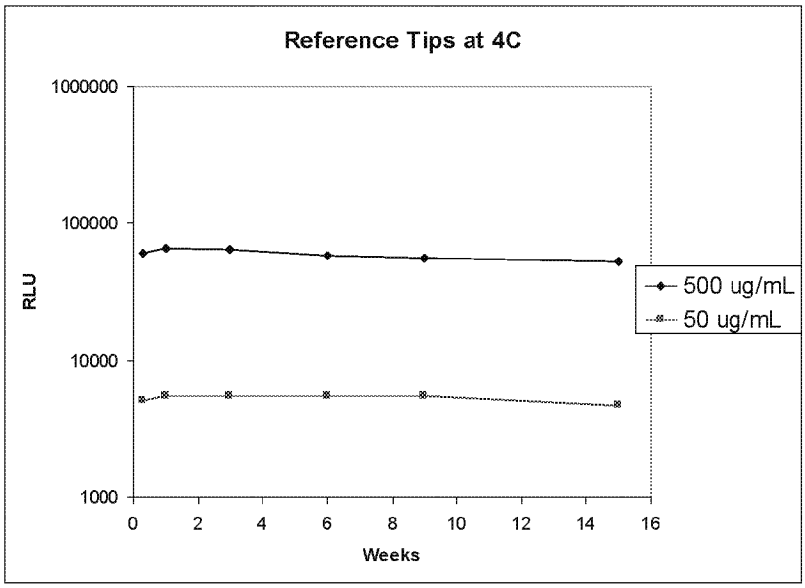




B) Reference surface stability

Reference or on-board instrument assay control stability will be tested for a period of 12 weeks for storage at 4°C and room temperature. Two levels of analyte, 500 and 50 ug/mL were used.

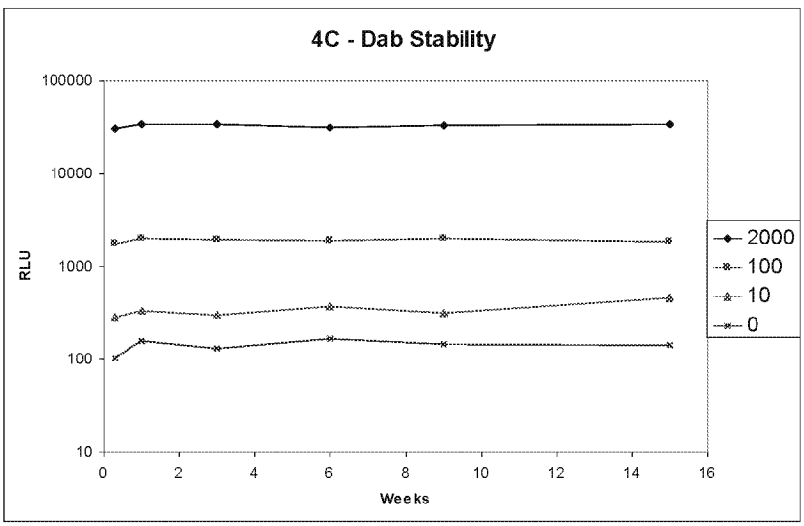
Summary		weeks					
Temp	ug/mL	0.3	1	3	6	9	15
4C	500	59978	65224	64199	58651	56213	52884
	50	5076	5524	5566	5505	5496	4642
RT	500	57081	61144	64435	58998	50551	49192
	50	5366	5416	5820	5118	4800	4169

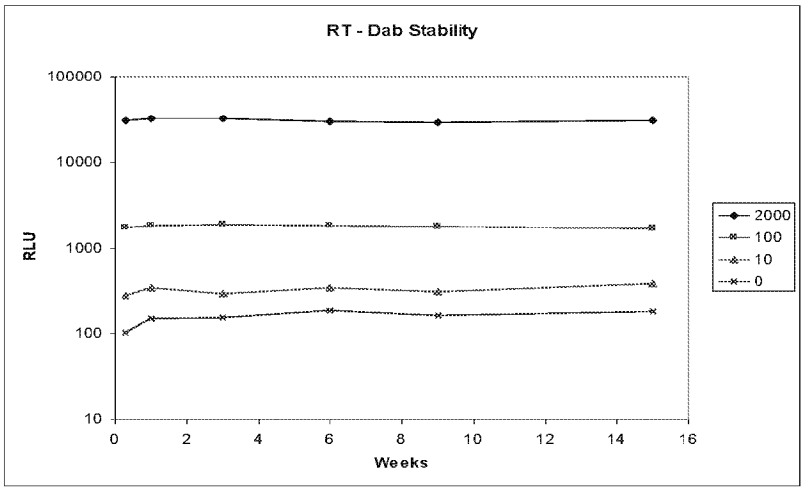


C) Detection antibody stability

Detection antibody stability at working concentration will be tested for a period of 12 weeks for storage at 4°C and room temperature in appropriate Alkaline Phosphatase stabilizer, with a 4-point assay buffer curve. These tests used an instrument protocol that allows for 8000-fold sample dilution. Analyte standards were pre-made for the entire study, aliquoted and flash frozen for single time use.

Summary		weeks					
Temp	ug/mL	0.3	1	3	6	9	15
4C	2000	30447	34582	34491	32008	33242	34057
	100	1748	2003	1930	1919	1985	1845
	10	281	335	296	371	317	460
	0	103	158	130	165	146	141
RT	2000	30447	32158	32133	29937	28929	30465
	100	1748	1813	1860	1807	1758	1710
	10	281	342	297	344	314	383
	0	103	149	156	187	163	181
CTRL	2000	30447	41532	41652	33838	37274	39130
	100	1748	2376	2217	2038	2127	2086
	10	281	441	342	400	328	456
	0	103	145	130	184	141	128

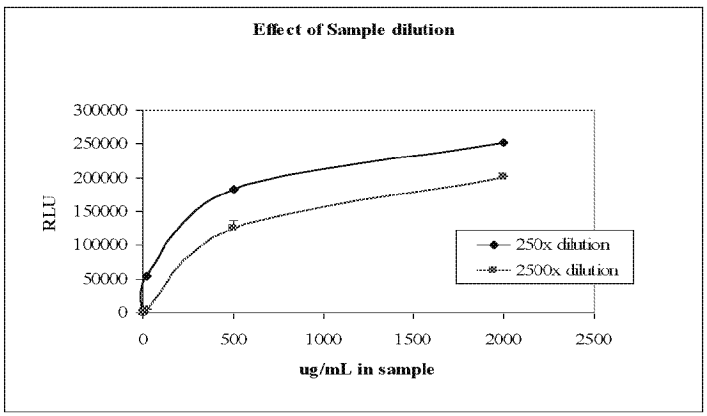




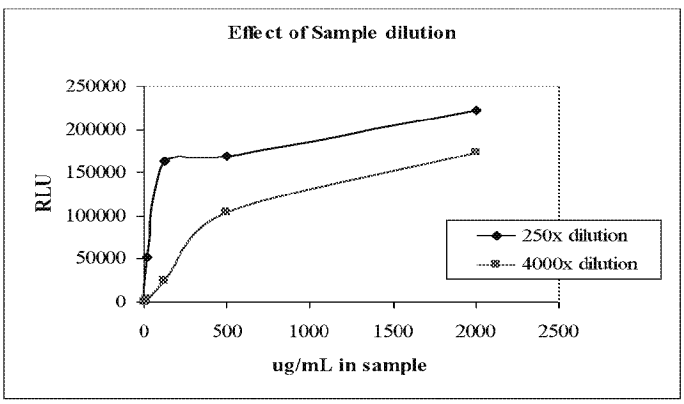
17. Optimizing for Sample dilutions on Theranos reader

Due to the wide dynamic range of the assay different sample dilution schemes were tested. The finalized instrument protocol uses a two dilution scheme (20x and 8000x) for the PK assay cartridge only and 8000-fold when used as a multiplex assay.

A) 250 and 2500x

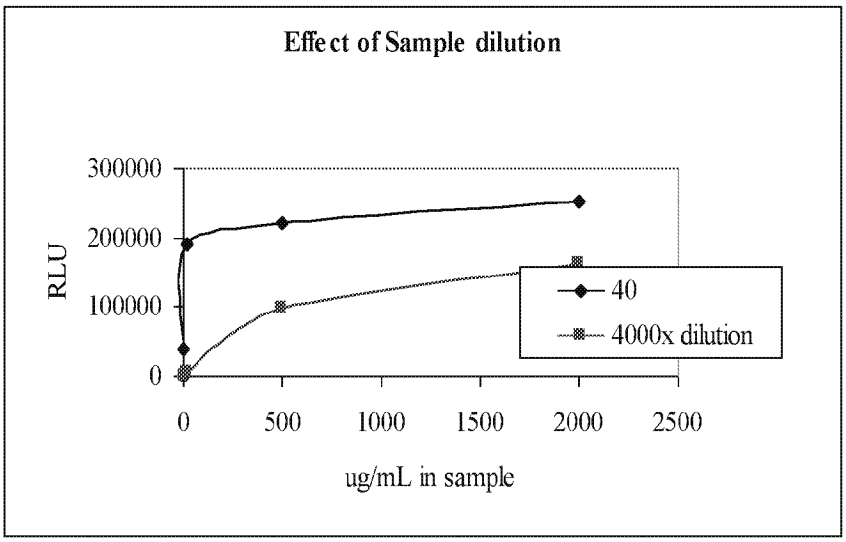


B) 250 and 4000x

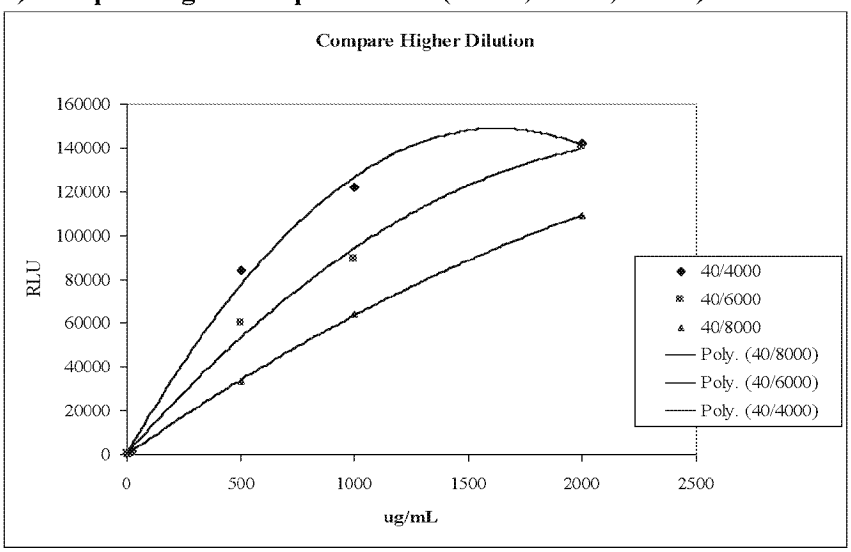


C) 40x and 4000x

	CNTO 5825	40x	4000x
1	2000	251017	162687
2	500	220942	99468
3	20.8	191728	5139
4	3.5	39875	777
5	0.1	2111	441
6	0	705	505
	Avg CV	13	13
	Slope	14058	88
	Avg stdev	245	39
	LOD, ug/mL	0.035	0.887



D) Compare higher sample dilution (4000x, 6000x, 8000x)



18. Calibration

This immumoassay was calibrated with analyte provided by Centocor. Calibration was performed with a 9-point assay buffer standard curve ranging from 4000 to 0.1 ug/mL, with duplicate cartridges for each standard point. A reader protocol that diluted the sample to 20 and 8000 fold was used. Given the wide dynamic range of the assay, a two dilution protocol is recommended.

Calibration rule
For Tips 3&4: If RLU >310000 use 8000x dilution Calibration equation
For Tips 5&6: If RLU <2000 use 20x dilution Calibration equation

Assay Buffer Standard curve:

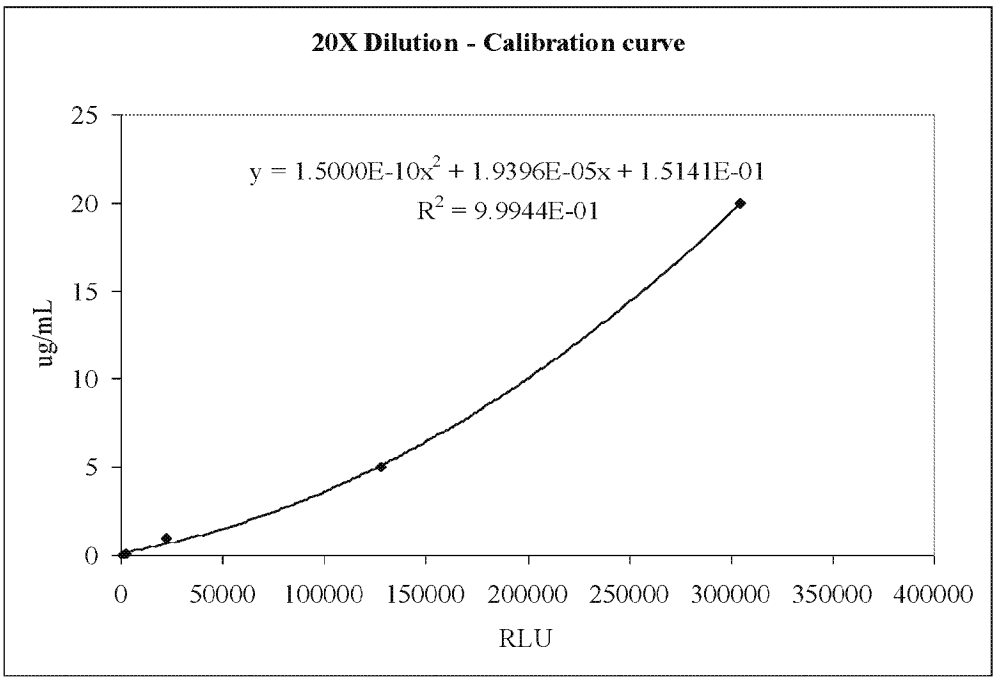
Dose response in RLU:

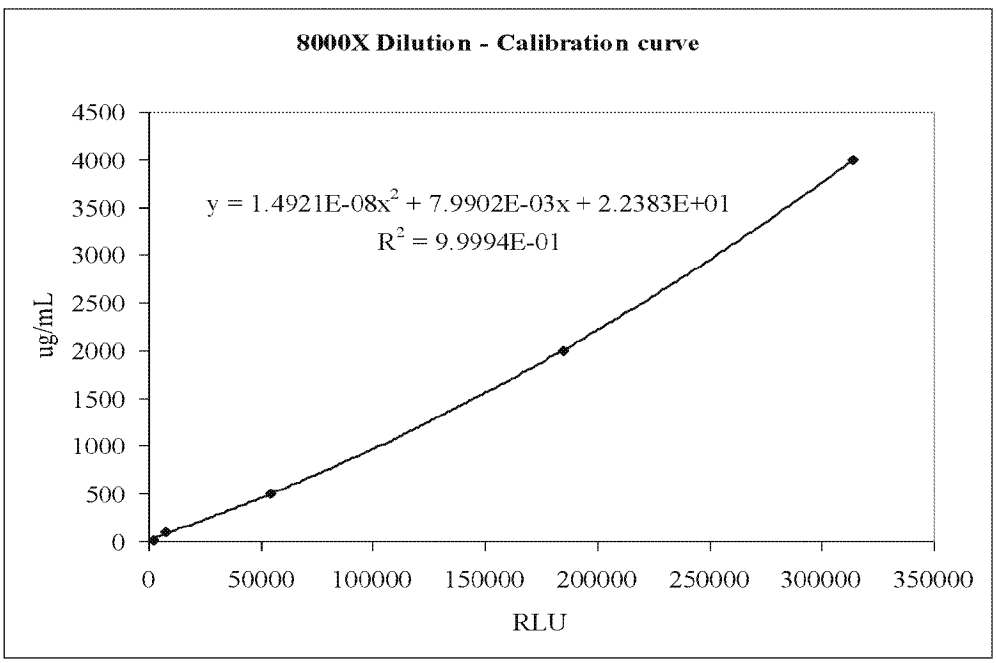
	CNTO 5825	20X					8000X				
	ug/mL	Run 1	Run 2	Avg	Stdev	%CV	Run 1	Run 2	Avg	Stdev	%CV
1	4000	471917	478350	491749	23155	5	334203	277224	313809	25802	8
		492818	523912				329379	314431			
2	2000	452892	394764	428558	27940	7	178799	185752	184369	12651	7
		416509	450066				201268	171657			
3	500	449088	433347	459426	25211	5	55266	52108	54004	1402	3
		462520	492747				54826	53816			
4	100	335954	392348	377371	33683	9	7156	7708	7424	350	5
		366987	414197				7743	7088			
5	20	295418	285939	304685	22903	8	1885	1971	1973	129	7
		337979	299403				1881	2156			
6	5	121550	128841	127976	4887	4	1087	1051	1094	116	11
		128097	133415				982	1255			
7	1	20629	22790	22248	1147	5	750	669	674	63	9
		22311	23263				597	681			
8	0.1	2222	2413	2512	238	9	617	757	744	88	12
		2681	2730				788	815			
9	0	697	799	728	58	8	781	828	766	74	10
		667	747				659	797			

Back calculated concentrations for Standard curve.

	CNTO 5825 Nominal, ug/mL	A.B Calc. ug/mL
1	4000	3999
2	2000	2003
3	500	497
4	100	83
5	20	20
6	5	5
7	1	1
8	0.1	0.20
9	0	0

	2000x	8000x
Average %CV	7	8
Slope	17839	73
Avg Stdev	148	85





19. Training set Correlation

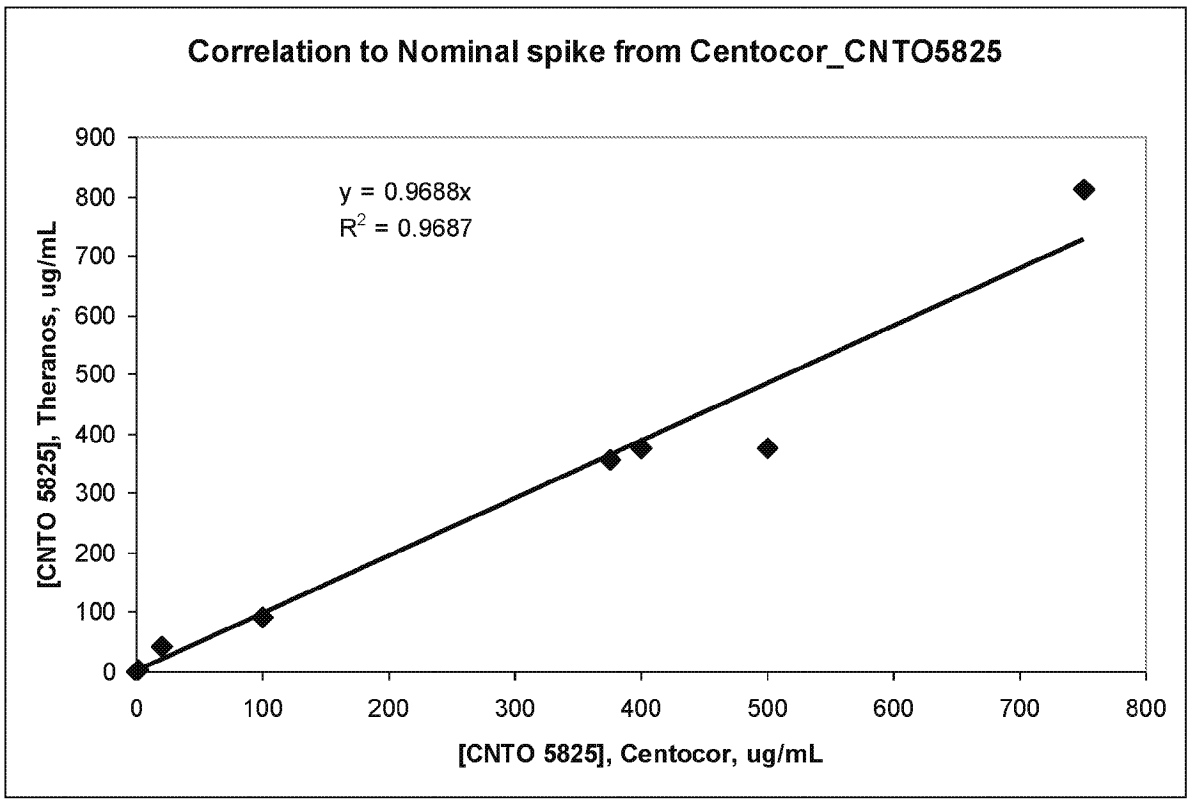
The assay was validated by testing 10 asthma serum samples spiked with different levels of CNTO 5825 provided by Centocor. The samples were run using a reader protocol that uses 20 and 8000-fold dilution of the sample. The aforementioned calibration was used to calculate sample concentration.

Samples from Centocor: RLU for duplicate cartridges

Sample	20X					8000X				
	Run 1	Run 2	Avg	Stdev	%CV	Run 1	Run 2	Avg	Stdev	%CV
1	383267	377331	371210	12594	3	2342	2350	2385	45	2
	354129	370113				2417	2431			
2	377534	380532	387572	18484	5	8240	9265	8444	553	7
	377022	415201				8066	8204			
3	480951	482948	494324	21450	4	41908	32790	38881	5220	13
	526266	487132				44337	36490			
4	472828	460230	466398	6637	1	70657	92975	85034	12416	15
	461132	471402				78979	97525			
5	488284	453613	463018	22295	5	44652	35638	41024	5484	13
	472957	437220				46725	37081			
6	436268	504740	467207	30519	7	38487	30305	41088	8448	21
	449653	478166				48651	46909			
7	1519	1714	1582	89	6	767	974	877	104	12
	1536	1560				811	956			
8	53905	62786	57897	4006	7	1031	1235	1170	115	10
	55462	59435				1126	1288			
9	1525	1916	1793	194	11	740	971	857	98	11
	1778	1955				890	827			
10	415430	431932	424294	9460	2	9109	6810	8544	1164	14
	432994	416819				8970	9288			

Calculated concentrations for above samples:

Sample	Run 1	Run 2	Avg. Conc.	Stdev	%CV	Centocor Nominal
	ug/mL	ug/mL	ug/mL			ug/mL
1	41	42	41.52	0.06	0.15	20
2	89	93	90.92	3.39	3.73	100
3	395	317	355.88	54.89	15.42	375
4	704	919	811.27	152.10	18.75	750
5	419	333	375.61	60.78	16.18	400
6	399	353	375.97	32.34	8.60	500
7	0.05	0.05	0.05	0.00	7.95	0.1
8	2.29	2.52	2.40	0.17	6.94	2
9	0.05	0.07	0.06	0.01	16.99	0
10	96	88	91.75	5.78	6.30	100


Correlation to Nominal spike from Centocor_CNTO5825
y = 0.9688x
R2 = 0.9687
[CNTO 5825], Theranos, ug/mL
[CNTO 5825], Centocor, ug/mL
0
100
200
300
400
500
600
700
800
900

B) ASSAY VALIDATION

20. Additional sample dilution

Though the assay development used two sample dilutions (20x and 8000x), Centocor had requested additional dilutions to be evaluated. Calibrations for the new dilution schemes tested were provided to Centocor and upon their approval were used for the following validation experiments. Since the 20x, 400x, 8000x dilution allows for better overlap across two successive dilutions, that was chosen as the final dilution scheme to proceed with. The highlighted analyte levels reflect the dose response overlap across multiple dilutions. Criteria for determining the assay limits for each dilution were that the % recovery of back calculated concentrations be within 20% of nominal and hold across two successive dilutions.

A) 20x and 8000x (original dilution scheme)

	In sample	20x dilution					6000x dilution				
	CNTO 5825	Avg	stdev	%CV	Calculated	% recovery	Avg	stdev	%CV	Calculated	% recovery
	ug/mL	RLU			ug/mL		RLU			ug/mL	
1	2000.000	521348	66960	13			206586	17857	9	2000.53	100
2	1000.000	493856	56111	11			98478	4186	4	990.40	99
3	500.000	513356	37789	7			50395	4188	8	532.44	106
4	250.000	489398	34525	7			18425	1706	9	206.40	83
5	125.000	501138	82962	17			10665	1260	12	123.63	99
6	62.500	440872	34296	8			4013	349	9	51.36	82
7	31.250	427453	65479	15	29.613	95	2612	230	9	35.99	115
8	15.625	314426	22741	7	15.626	100	1672	170	10	25.64	164
9	7.813	192297	21793	11	7.807	100	1292	45	4		
10	3.906	91871	4375	5	3.905	100	1078	47	4		
11	1.953	43700	3254	7	2.029	104	996	92	9		
12	0.977	17636	1897	11	0.871	89	956	64	7		
13	0.488	7682	1430	19	0.389	80	887	105	12		
14	0.244	4892	180	4	0.250	102	913	72	8		
15	0.122	2142	148	7	0.111	91	856	94	11		
16	0.061	1635	116	7	0.085	139	842	85	10		
17	0.031	1158	62	5	0.060	197	869	23	3		
18	0.015	1102	70	6	0.057		947	79	8		
19	0	867	74	9	0.045		968	127	13		

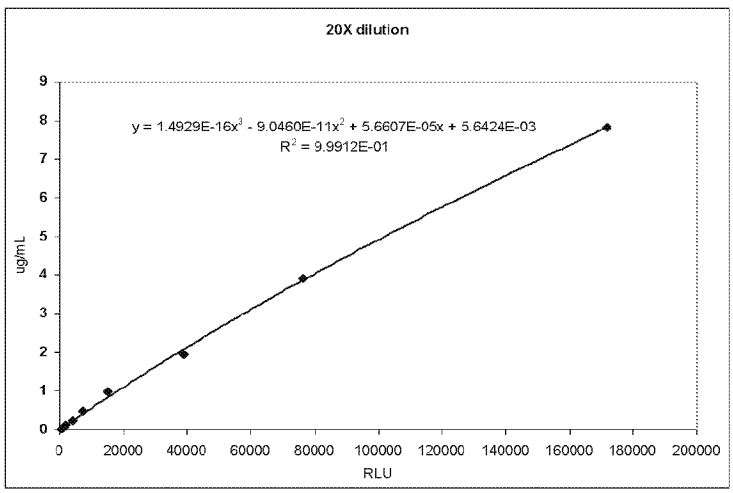
A) 20x and 6000x

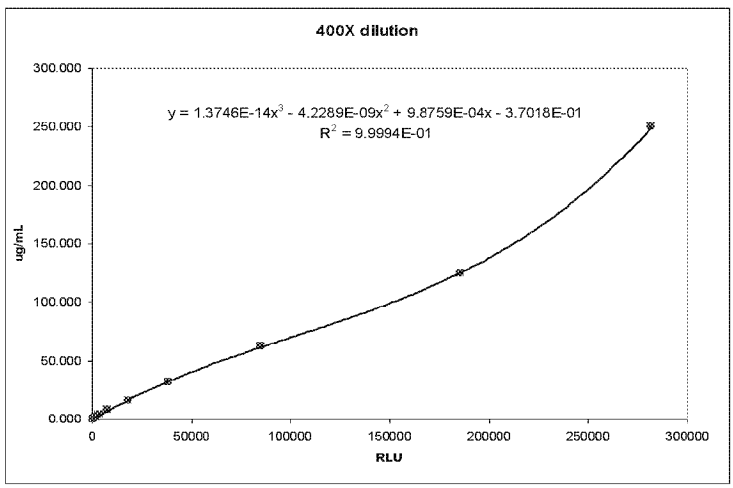
	In sample	20x dilution					6000x dilution				
	CNTO 5825	Avg	stdev	%CV	Calculated	% recovery	Avg	stdev	%CV	Calculated	% recovery
	ug/mL	RLU			ug/mL		RLU			ug/mL	
1	2000.000	526163	46082	9			234762	13316	6	1999.913	100
2	1000.000	596480	67539	11			138918	21579	16	1001.195	100
3	500.000	459010	18412	4			56066	2208	4	491.532	98
4	250.000	511449	28979	6			27473	3308	12	269.911	108
5	125.000	387688	69984	18			10290	1746	17	106.764	85
6	62.500	486157	34709	7			5934	917	15	60.967	98
7	31.250	373829	51326	14	23.626	76	2914	132	5	28.060	90
8	15.625	298064	9958	3	15.623	100	1876	86	5	16.525	106
9	7.813	177221	9757	6	7.828	100	1486	164	11	12.166	156
10	3.906	84194	3971	5	3.816	98	1116	73	7		
11	1.953	44534	1937	4	2.127	109	1089	89	8		
12	0.977	17630	1728	10	0.882	90	967	89	9		
13	0.488	7981	849	11	0.405	83	944	83	9		
14	0.244	3951	228	6	0.201	82	848	46	5		
15	0.122	2475	190	8	0.125	102	920	127	14		
16	0.061	1532	155	10	0.076	125	875	74	8		
17	0.031	1283	147	11	0.063	207	933	125	13		
18	0.015	1010	164	16	0.049		842	111	13		
19	0	826	110	13	0.040		878	104	12		

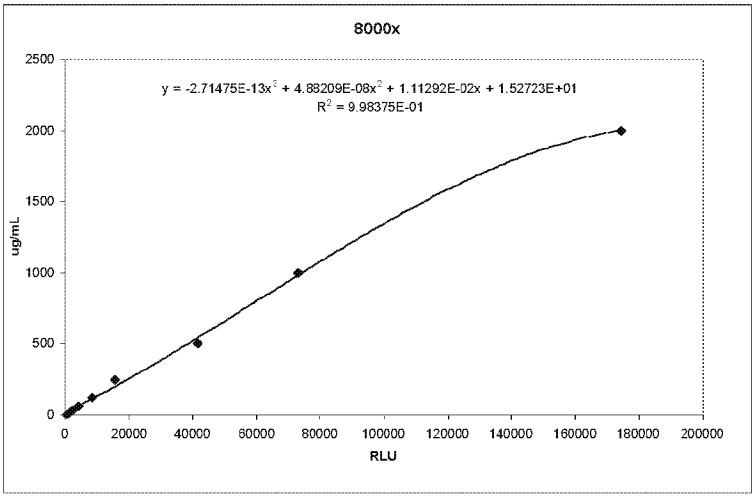
B) 20x, 400x and 8000x dilution

	In sample	20x dilution					400x dilution					8000x dilution				
	CNTO 5825	Avg	stdev	%CV	Calculated	% recovery	Avg	stdev	%CV	Calculated	% recovery	Avg	stdev	%CV	Calculated	% recovery
	ug/mL	RLU			ug/mL		RLU			ug/mL		RLU			ug/mL	
1	2000.000	492348	98262	20			429698	41657	10			158004	34600	22	2000.77	100
2	1000.000	487645	73685	15			391048	64984	17			85007	18566	22	992.19	99
3	500.000	481573	59940	12			452907	39263	9			43139	4834	11	524.38	105
4	250.000	509655	61424	12			287064	28639	10	250.00	100	17554	1176	7	229.08	92
5	125.000	380100	56339	15			153187	15549	10	125.05	100	7288	1151	16	102.78	82
6	62.500	436471	70864	16			74090	7150	10	61.92	99	3660	563	15	56.67	91
7	31.250	436089	38540	9			38453	6700	17	32.98	106	2451	160	7	41.13	132
8	15.625	282763	3467	1			16005	827	5	14.05	90	1668	206	12		
9	7.813	141049	29931	21			7766	455	6	6.91	88	1256	90	7		
10	3.906	84878	12662	15	3.907	100	4007	393	10	3.61	93	1111	149	13		
11	1.953	38566	3204	8	1.946	100	2502	241	10	2.29	117	1041	62	6		
12	0.977	15802	623	4	1.016	104	1400	223	16	1.31	135	918	45	5		
13	0.488	6218	1680	27	0.420	86	1086	138	13			835	87	10		
14	0.244	3667	775	21	0.232	95	1063	155	15			927	128	14		
15	0.122	2433	155	6	0.136	111	978	54	6			939	38	4		
16	0.061	1570	236	15	0.067	110	923	169	18			887	113	13		
17	0.031	1273	203	16	0.043	140	948	158	17			960	171	18		
18	0.015	956	163	17	0.017	109	858	154	18			866	123	14		
19	0	917	65	7	0.013		931	119	13			959	92	10		

Calibration curves for each dilution derived from Standard curve above:







21. Precision and Accuracy test for pooled plasma controls

Test the precision and accuracy of plasma controls for each of the dilutions. The 5 controls tested for each dilution spanned the assay range for that particular dilution. Plasma controls were generated by spiking stock analyte (CNTO 5825) into pooled plasma from normal donor samples obtained from the blood bank. The levels of the analyte for use as controls were recommended by Centocor.

Experimental criteria:

- Each control was run on 6 cartridges. Each cartridge has duplicate tests for each of the 3 dilutions.
- Inclusion of the 0.1 ug/ml control to test lower limit
- Precision data will be presented in concentration CV based on calibration.
- The acceptance criteria is that the average % bias and %CV (over 6 assays) must be less than 20% for the middle three concentrations at each dilution (0.6, 1, 4 at 20X; 12, 25, 75 at 400X; 240, 500, 1500 at 8000X) and less than 25% for the high and low concentrations at each dilution (0.2 and 5 ug/mL at 20X; 4 and 100 ug/mL at 400X; 80 and 2000 ug/mL at 8000X.

		Plasma	AVERAGE SIGNAL FOR EACH CARTRIDGE					
		Controls	Day 1			Day 2		
Dilution	STD	ug/mL	1	2	3	4	5	6
8000x	1	2000.0	200786	195164	158539	146789	155749	177965
	2	1500.0	114956	145033	111947	124863	151815	107818
	3	500.0	35410	37069	32735	35774	37297	32571
	4	240.0	17977	15640	17105	18602	17942	15142
	5	80.0	6172	5094	6236	4946	5598	4245
400x	1	100.0	150853	110368	117748	152680	147091	141233
	2	75.0	101662	111110	87821	110364	89259	66058
	3	25.0	32120	25150	23685	28456	31439	28580
	4	12.0	13183	11456	14329	8895	9764	10778
	5	4.0	4357	3949	3345	4063	5403	3493
20x	1	5.0	107869	96867	113568	117611	111187	104476
	2	4.0	99972	92236	85142	79135	111241	74032
	3	1.0	21387	17285	22900	19627	18104	16415
	4	0.6	13542	9829	10547	11460	12006	10599
	5	0.2	4347	4699	4031	4265	4025	4037
	6	0.1	2776	2209	2456	2812	2912	2838
	7	0.0	1225	1360	1437	1238	1541	1206

CALCULATED CONCENTRATIONS FOR EACH CARTRIDGE												
			Day 1			Day 2						
Dilution	STD	ug/mL	1	2	3	4	5	6	Average	Stdev	%CV	% Recovery
8000x	1	2000.0	2020.5	2028.7	1925.0	1842.2	1907.3	2011.9	1955.9	76	4	98
	2	1500.0	1527.4	1828.1	1492.2	1637.6	1880.2	1442.5	1634.7	182	11	109
	3	500.0	458.5	481.1	422.4	463.5	484.2	420.2	455.0	28	6	91
	4	240.0	229.5	200.2	218.6	237.5	229.1	194.1	218.2	17	8	91
	5	80.0	85.8	73.2	86.5	71.5	79.1	63.4	76.6	9	12	96
400x	1	100.0	99.6	75.6	79.7	100.8	97.1	93.5	91.0	11	12	91
	2	75.0	70.8	76.0	63.1	75.6	63.9	50.4	66.6	10	15	89
	3	25.0	27.4	22.0	20.8	24.6	26.9	24.7	24.4	3	11	98
	4	12.0	11.9	10.4	13.0	8.1	8.9	9.8	10.3	2	18	86
	5	4.0	3.9	3.5	2.9	3.6	4.8	3.0	3.6	1	19	90
20x	1	5.0	5.2	4.8	5.5	5.7	5.4	5.1	5.28	0.31	6	106
	2	4.0	4.9	4.6	4.3	4.0	5.4	3.8	4.48	0.60	13	112
	3	1.0	1.2	1.0	1.3	1.1	1.0	0.9	1.06	0.13	12	106
	4	0.6	0.76	0.55	0.59	0.64	0.67	0.60	0.64	0.07	11	106
	5	0.2	0.25	0.27	0.23	0.25	0.23	0.23	0.24	0.01	6	122
	6	0.1	0.16	0.13	0.14	0.16	0.17	0.17	0.16	0.02	10	156
	7	0.0	0.07	0.08	0.09	0.08	0.09	0.07	0.08	0.01	9	

21. Selectivity

Test the selectivity of the assay for 10 individual plasma (5 male 5 female) at unspiked, 0.1 and 0.2 ug/mL spike levels.

Experimental criteria

- Samples tested run in triplicate cartridges.
- Calibration for the test samples was back calculated t oan assay buffer calibration curve and must be within 25% of nominal to pass.
- The acceptance criteria for this experiment is that 8 out of 10 of the results from the individuals to be within 25% of the nominal concentration (0.15 – 0.25 ug/mL) for the 0.2 ug/mL spike and 8 of 10 of the unspiked have to have results below 0.2 ug/mL.

Since the % recovery at the 0.1 ug/mL spike level does not meet the acceptance criteria and the 0.2 ug/mL meets the requirements, the LLOQ of the assay is established at 0.2 ug/mL.

CNTO 5825	Sample #	Sample ID	AVERAGE RLU FOR EACH RU			CALCULATED CONCENTRATION (ug/mL)						
ug/mL			1	2	3	1	2	3	Average	Stdev	%CV	% Recovery
No Spike	1	F1	1315	866	1185	0.08	0.05	0.07	0.07	0.013	19	
	2	F2	950	1107	1162	0.06	0.07	0.07	0.07	0.006	9	
	3	F3	1067	959	820	0.07	0.06	0.05	0.06	0.007	12	
	4	F4	1372	1450	1564	0.08	0.09	0.09	0.09	0.005	6	
	5	F5	1018	1184	1115	0.06	0.07	0.07	0.07	0.005	7	
	6	M1	1589	1779	1829	0.10	0.11	0.11	0.10	0.007	7	
	7	M2	1187	1232	1208	0.07	0.08	0.07	0.07	0.001	2	
	8	M3	1042	1041	1119	0.06	0.06	0.07	0.07	0.003	4	
	9	M4	918	1083	1034	0.06	0.07	0.06	0.06	0.005	8	
	10	M5	1251	1392	1317	0.08	0.08	0.08	0.08	0.004	5	
0.1	1	F1	2691	2509	2524	0.16	0.15	0.15	0.15	0.006	4	151
	2	F2	2144	2644	1895	0.13	0.15	0.11	0.13	0.021	16	131
	3	F3	2437	2816	2598	0.14	0.16	0.15	0.15	0.011	7	153
	4	F4	2911	2856	3134	0.17	0.17	0.18	0.17	0.008	5	173
	5	F5	2911	2856	3134	0.17	0.17	0.18	0.17	0.008	5	173
	6	M1	3784	3522	3611	0.22	0.20	0.21	0.21	0.007	4	210
	7	M2	2836	1915	2970	0.17	0.11	0.17	0.15	0.032	21	151
	8	M3	2507	1861	2713	0.15	0.11	0.16	0.14	0.025	18	139
	9	M4	2924	2692	2522	0.17	0.16	0.15	0.16	0.011	7	159
	10	M5	2842	2643	2719	0.17	0.15	0.16	0.16	0.006	4	160
0.2	1	F1	3079	4393	4424	0.18	0.25	0.25	0.23	0.043	19	114
	2	F2		4270	4008		0.25	0.23	0.24	0.010	4	119
	3	F3	3899	3912	3036	0.22	0.23	0.18	0.21	0.028	13	105
	4	F4	4177	4902	4933	0.24	0.28	0.28	0.27	0.024	9	134
	5	F5	3992	3286	3589	0.23	0.19	0.21	0.21	0.020	9	105
	6	M1	5213	4950	5375	0.30	0.28	0.31	0.30	0.012	4	148
	7	M2	4382	3888	3453	0.25	0.22	0.20	0.23	0.026	12	113
	8	M3	4549	4011	4257	0.26	0.23	0.24	0.25	0.015	6	123
	9	M4	3874	3786	3855	0.22	0.22	0.22	0.22	0.003	1	111
	10	M5	4315	4224	2954	0.25	0.24	0.17	0.22	0.043	19	111

22. Spike recovery of control levels in Whole blood

Test accuracy of the assay with spiked whole blood. Two individual samples were used for this purpose. Experimental criteria are indicated below:

- Spike whole blood with 1 control for each dilution. Calculate recovery based on assay buffer calibration curve.
- Control levels: 0.2 ug/mL at 20x, 25 ug/mL at 400x and 2000 ug/mL at 8000x.
- Each control tested on 3 cartridges.

SAMPLE1:

		AVERAGE SIGNAL FOR EACH CARTRIDGE								
		20x			400x			8000x		
STD	ug/mL	1	2	3	1	2	3	1	2	3
1	**2000.0**	515863	477131	448689	420175	504941	443881	93022	185165	118832
2	**25.0**	228999	282677	338237	19461	25339	23429	1554	1953	1747
3	**0.2**	3453	3597	4133	1080	1071	1011	958	951	841
4	**0.0**	1011	1047	995	849	952	916	884	937	953

CALCULATED CONCENTRATIONS FOR EACH CARTRIDGE									
STD	ug/mL	Dilution	1	2	3	Avg	Stdev	%CV	% Recovery
1	**2000.0**	8000x	1254.5	2026.4	1571.7	1618	388	24.0	81
2	**25.0**	400x	17.3	22.2	20.6	20	2	12.3	80
3	**0.2**	20x	0.20	0.21	0.24	0.22	0	9.3	108
4	**0.0**	20x	0.06	0.06	0.06	0.06	0	2.4	

SAMPLE 2:

		AVERAGE SIGNAL FOR EACH CARTRIDGE								
		20x			400x			8000x		
STD	ug/mL	1	2	3	1	2	3	1	2	3
1	**2000.0**	573609	529642	431345	527672	442302	388176	171301	128869	133766
2	**25.0**	301077	348751	336201	25349	16335	22440	1942	1493	1779
3	**0.2**	2742	3371	3571	839	1128	830	794	1036	771
4	**0.0**	911	863	840	939	866	896	981	858	796

CALCULATED CONCENTRATIONS FOR EACH CARTRIDGE									
STD	ug/mL	Dilution	1	2	3	Avg	Stdev	%CV	% Recovery
1	**2000.0**	8000x	1989.7	1679.3	1727.8	1799	167	9.3	90
2	**25.0**	400x	22.2	14.7	19.8	19	4	20.2	76
3	**0.2**	20x	0.16	0.20	0.21	0.19	0	12.9	94
4	**0.0**	20x	0.06	0.05	0.05	0.05	0	3.7	