

Assay Development Report

Assay	ENA-78/CXCL5
Developer	Hong Li
Date released	3/23/08

1. Analyte background

Epithelial neutrophil activating peptide 78 (ENA-78) is a 8 kDa pro-inflammatory member of the CXC subfamily of chemokines that was originally isolated from media conditioned by the growth of human lung type-II alveolar epithelial cell line (A549) stimulated by IL1 beta or TNF-alpha. The full-length cDNA encodes a 114 amino acid (aa) residue precursor protein with a 36 aa residue signal peptide that is cleaved to generate the 78 aa residue secreted protein. Human ENA-78 shares 57% significant amino acid sequence identity with mouse and rat. Among other human ELR chemokins, it shares 77%aa sequence identity with CXCL6/GCP-2 and 35% -51% with CXCL1 /GRO alpha, CXCL2/GRO beta, CXCL3/GRO gamma, CXCL7/NAP-2 and CXCL8/IL8. The gene for ENA-78 has been mapped to chromosome 4q13-q21.

ENA is a neutrophil attractant and activator in vitro. It has been suggested that ENA-78 activity can be mediated through the IL-8 receptor system. ENA-78 is expressed in human platelets. The expression of ENA-78 has also been detected in neutrophils and monocytes/macrophages following LPS stimulation. In addition, ENA-78 expression is highly inducible in endothelial cells, vascular smooth muscle cells, epithelial cells and pulmonary fibroblasts by pro-inflammatory cytokines such as IL-1 beta or TNF-alpha. CXCL5 exerts its effects primarily through interaction with CXCR2 receptor. It also binds Duffy antigen receptor for chemokines, which can limit CXCR2-mediated responses.

2. Assay specifications

The Theranos assay for ENA-78 is a sandwich ELISA specific to the recombinant and natural human ENA-78 in buffer, whole blood, plasma and serum. The antibody has been selected for its ability to neutralize human CXCL5 bioactivity. This antibody shows less than 20% cross-reactivity with rhGCP-2 and less than 5% cross-reactivity with rmKC, rrCINC-1, rhGRO alpha, rhGRO gamma and rmGCP-2. Additionally, in direct ELISA, this antibody shows no cross-reactivity with other chemokines tested.

Reportable ranges are:

Sample type	Low, pg/mL	High, pg/mL
Human plasma	23	24000
Human blood	23	24000

ENA-78 assay initially started with a range of 2000-23 pg/ml, and then optimized the range to 6000-23pg/ml and 24000-23pg/ml.

3. Reference assays

R&D ELISA kit

Cat# DX000

4. Antibody Screening

Antibody screening was carried out in 384 well Corning micro-titer plates.

Table 1: Antibody Screening Summary

		Dab1	Dab2	Dab3	Dab4	Dab5
	Cab1					
	Cab2					
	Cab3					
	Cab4					
	Cab5					

Number of Capture antibody tested: 5

Number of Detection antibody tested: 5

Total Number of antibody pairs tested: 10

	Good dose response, good candidate pair
	Best dose response, best candidate pair

The antibody pair Cab3 & Dab4 was selected for the Theranos assay. The criteria were good dose response for the range of the assay and highest sensitivity for the final assay conditions. The backup pair is: Cab2 & Dab4

Table 2: Best Antibody Pairs on 384 well microtiter plates.

	Cab3			Cab2		
ENA78	Dab4			Dab4		
pg/ml	Avg.	Stdev	CV	Avg.	Stdev	CV
2000	170110	3489	2	69658	993	1
500	39433	2359	6	16669	598	4
100	11910	396	3	4017	96	2
31	2489	245	10	1221	114	9
0	175	61	35	217	5	2
	Slope		121	Slope		32
	Avg Stdev		234	Avg Stdev		270
	LOD pg/ml		1	LOD pg/ml		16.5
	S/B (1/6)		970	S/B (1/6)		321
	S/B (5/6)		14	S/B (5/6)		6

Table 3: Best Antibody Pairs on Reader

	Cab3 (MAB654) R&D			Cab2 (MAB254) R&D		
ENA78	Dab4 (AF254) R&D			Dab4 (AF254) R&D		
pg/ml	Avg.	Stdev	CV	Avg.	Stdev	CV
2000	352408	3821	1	46530	4885	10
667	121968	435	0	14904	410	3
222	43863	2547	6	5908	218	4
74	16744	10	0	2215	51	2
25	6554	363	6	1269	35	3
0	936	19	2	763	63	8
	Slope		213	Slope		24
	Avg Stdev		131	Avg Stdev		101
	LOD pg/ml		3	LOD pg/ml		8.4
	S/B (1/6)		130	S/B (1/6)		20
	S/B (5/6)		7	S/B (5/6)		2

5. Capture Antibody Titration with Dab 100ng/ml

Capture antibody was tested at 2.5, 5, 10ug/mL. A concentration of 5ug/mL was determined to be optimum.

Figure 1: Capture Antibody Titration

[EMBED Excel.Chart.8 \s]

Table 1: Capture Antibody Titration

	10ul/ml			5ug/ml			2.5ug/ml		
pg/ml	Avg	Stdev	%CV	Avg	Stdev	%CV	Avg	Stdev	%CV
2000	304872	12414	4	333379	13540	4	236136	1910	1
667	116144	8025	7	126497	6739	5	88071	6902	8
222	40652	3719	9	49021	413	1	32690	2093	6
74	13996	650	5	15903	178	1	12255	689	6
25	5413	298	6	6017	120	2	4911	465	9
0	727	44	6	830	59	7	768	6	1

S/B_Std 1/6	419	S/B_Std 1/6	402	S/B_Std 1/6	307
S/B_Std 5/6	7.4	S/B_Std 5/6	7	S/B_Std 5/6	6
Avg CV	6	Avg CV	3	Avg CV	5
Slope	178	Slope	203	Slope	154
Avg stdev	330	Avg stdev	119	Avg stdev	386
LOD, pg/mL	4	LOD, pg/mL	1	LOD, pg/mL	5

6. Detection Antibody Titration with Cab 5ug/ml

Detection antibody was tested at 50ng/ml, 100ng/ml, 200ng/ml. A concentration of 100ng/mL was determined to be optimum.

Figure 1: Detection Antibody Titration

[EMBED Excel.Chart.8 \s]

Table 1: Detection Antibody Titration

	50ng/ml			100ng/ml			200ng/ml		
pg/ml	Avg	Stdev	%CV	Avg	Stdev	%CV	Avg	Stdev	%CV
6000	558701	28898	5	926132	44616	5	1922439	110533	6
1500	157759	25754	16	256878	47122	18	761721	39321	5
375	38731	4417	11	78345	8991	11	219768	11856	5
94	10207	1372	13	21647	1330	6	58400	9481	16
23	3002	156	5	7894	327	4	17390	5883	34
0	393	52	13	2446	446	18	3026	1767	58
	S/B_Std 1/6	1422		S/B_Std 1/6	379		S/B_Std 1/6	635	
	S/B_Std 5/6	8		S/B_Std 5/6	3		S/B_Std 5/6	6	
	Avg CV	11		Avg CV	11		Avg CV	21	
	Slope	104		Slope	203		Slope	589	
	Avg stdev	527		Avg stdev	701		Avg stdev	5710	
	LOD, pg/mL	10		LOD, pg/mL	7		LOD, pg/mL	19	

7. Whole blood screen

Numerous whole blood samples were screened to check for endogenous ENA-78 levels. The endogenous analyte levels were calculated from an assay buffer standard curve.

	Avg.	Std	CV	cal. Conc.
1	50754	1933	4	271
2	209556	5289	3	1176
3	89164	6640	7	489
4	97042	5644	6	534
5	81680	1426	2	447
6	155262	9908	6	866

7	68542	4453	6	372
8	165672	6880	4	926
9	106703	14480	14	589
10	95786	6952	7	527
11	231144	9299	4	1299
12	157257	3540	2	878

8. Validation

a) Analyte validation on ELISA kit:

Figure 1: Validation

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b) Analyte Validation on Theranos assay:

Figure 2: Validation

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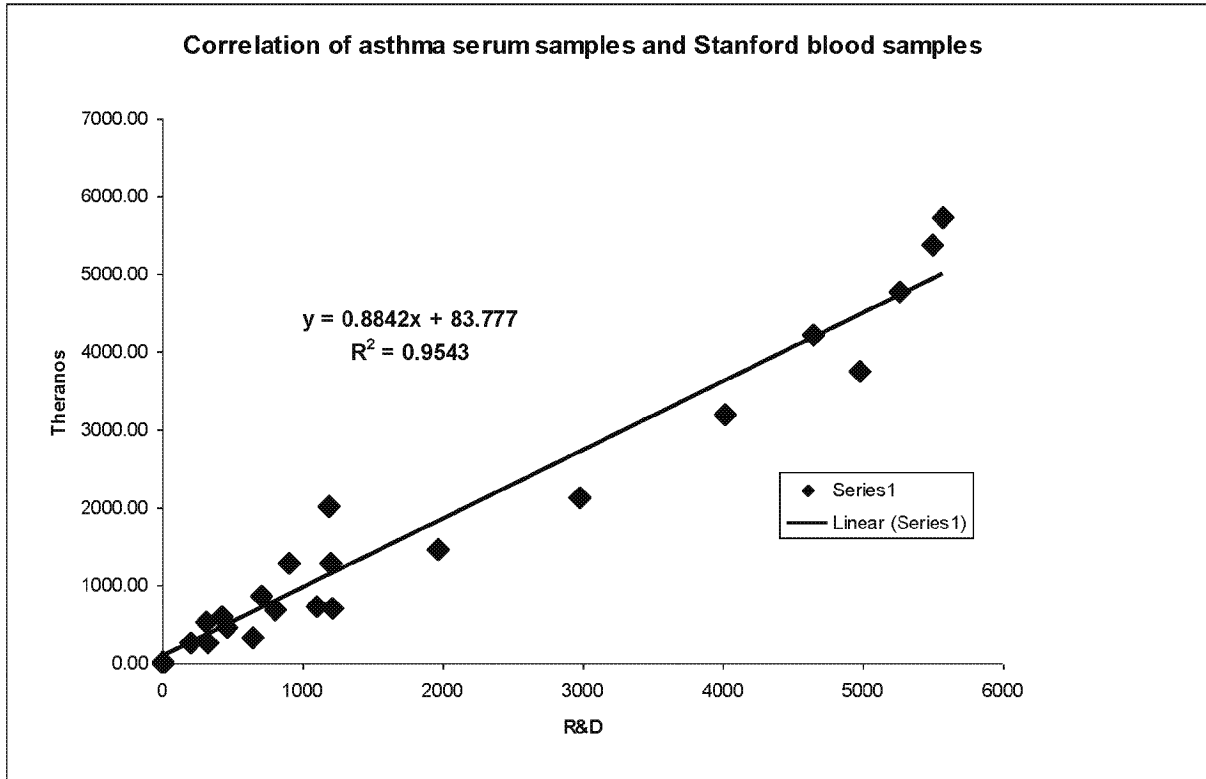
Table 1: Validation

pg/ml	Elisa analyte			Theranos analyte		
	Avg.	Stdev	CV	Avg.	Stdev	CV
2000	416251	29180	7	528669	46765	9
222	60187	2965	5	79217	6362	8
24.5	7721	517	7	11659	694	6
0	3243	730	23	3067	114	4

c) Assay Validation.

A set of clinical samples from 17 patients diagnosed with asthma and 4 Stanford blood samples (run with whole blood and plasma) were tested in reference ELISA kit and Theranos assay for clinical correlation in the Theranos assay. The correlation was good with $R^2 = 0.9544$ and slope = 0.8904.

Figure 3: Validation



9. Spike recovery test with analyte range 6000pg/ml-23pg/ml & 5X dilution protocol

Spike ENA-78 analyte into whole blood & plasma. Test the recovery of spiked whole blood, plasma from spiked blood and spiked plasma (spike into plasma directly).

The recovery was estimated with an assay buffer calibration curve. Good spike recovery with spiked whole blood (Average recovery 110%) and spiked plasma (Average recovery = 89%)

The spike recovery & the slope of plasma from spiked whole blood were much lower than those of spiked whole blood & spiked plasma. Also, endogenous ENA-78 was much higher in whole blood than in plasma. The red blood cell effect might be the cause (ENA-78 bound to red blood cells with high affinity) and platelet effect may play a role as well (ENA-78 is present in platelet granules and is released upon platelet activation).

Figure 1: Spike recovery test with analyte range 6000pg/ml-23pg/ml

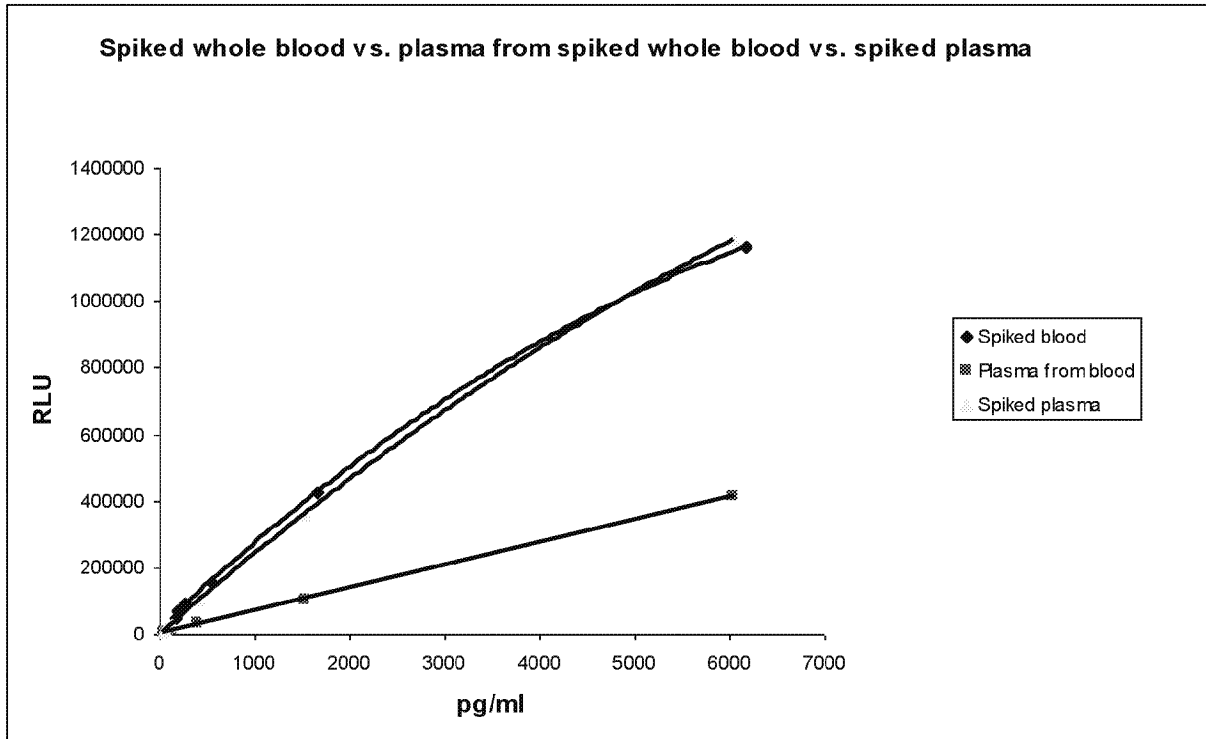


Table 1: Spike recovery test with analyte range 6000pg/ml-23pg/ml

Spiked whole blood				
pg/ml	Avg.	Stdev	%CV	Recovery
6165	1161096	96276	8	102
1665	426959	10914	3	102
540	156565	3254	2	100
259	93792	8797	9	122
188	72076	3041	4	128
163	51054	6231	12	
	Slope			417
	Avg %CV			7
	Analyte recovery %			111

Plasma from spiked whole blood				
pg/ml	Avg.	Stdev	%CV	Recovery
6030	413349	16652	4	27
1530	102628	2631	3	23
405	33989	539	2	28
124	12677	779	6	37
53	7791	510	7	58
30	7517	237	3	
	Slope			58

Avg %CV	4
Analyte recovery %	29

Spiked plasma				
pg/ml	Avg.	Stdev	%CV	Recovery
6031	1182847	110129	9	107
1531	361067	16526	5	91
406	100559	6653	7	83
125	27303	2428	9	74
54	11546	937	8	79
31	7880	607	8	
Slope				211
Avg %CV				7.5
Analyte recovery %				89

10. Spike recovery test with analyte range 24000pg/ml-23pg/ml & 10X dilution protocol
 Spike ENA-78 analyte into whole blood & plasma with anylyte range 24000pg/ml-23pg/ml. Recovery and dose responses of spiked whole blood, plasma from spiked whole blood and spiked plasma (spike into plasma directly) with a 10X sample dilution protocol.

The recovery was estimated with an assay buffer calibration curve. Good spike recovery and dose responses with spiked whole blood and with spiked plasma. Blocking buffer calibration curves remain linear with this wide analyte range

Figure 1: Spike recovery test with analyte range 24000pg/ml-23pg/ml

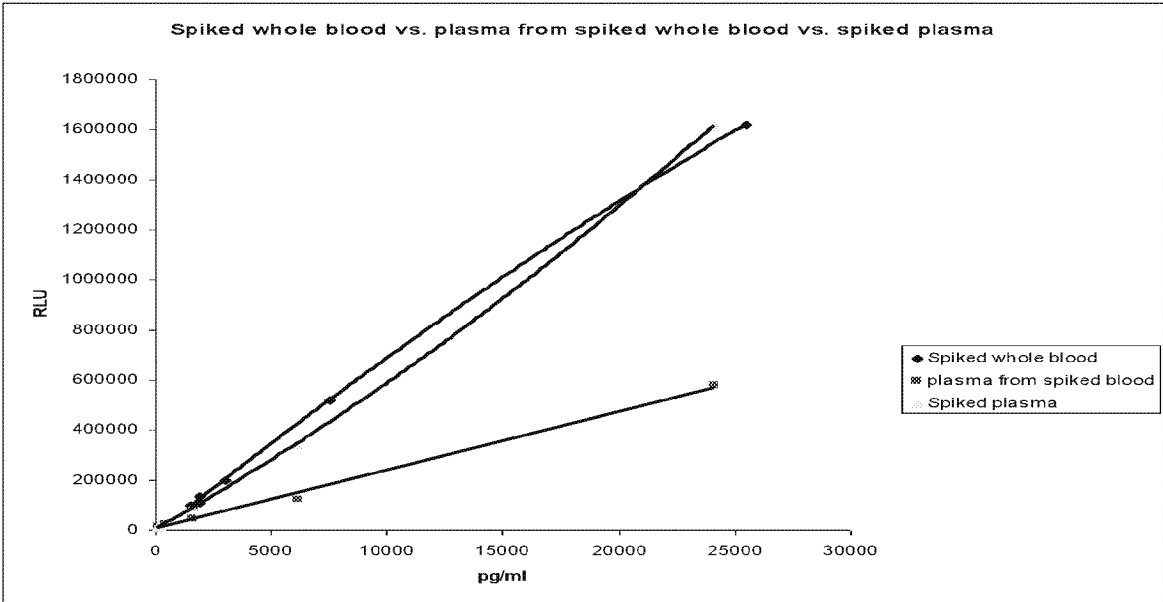


Table 1: Spike recovery test with this range 24000pg/ml-23pg/ml

Spike into whole blood				
pg/ml	Avg.	Stdev	%CV	Recovery
25506	1621544	90891	6	95
7506	518111	56079	11	98
3006	200461	21254	11	93
1881	137354	5214	4	100
1600	90021	6778	8	76
1529	97318	17351	18	86
1506	130439	7988	6	
	Slope			91
	Avg %CV			11
	Analyte recovery %			91

Plasma from spiked blood				
pg/ml	Avg.	Stdev	%CV	Recovery
24136	576462	13715	2	34
6136	122054	34330	28	27
1636	43714	1331	3	35
511	21467	1604	7	50
230	15761	1704	11	75
159	12686	1277	10	81
136	13144	2232	17	
	Slope			32
	Avg %CV			11
	Analyte recovery %			44

Spike into plasma				
pg/ml	Avg.	Stdev	%CV	Recovery
24093	1616270	104067	6	100
6093	344089	28300	8	80
1593	88031	1007	1	75
468	25141	7104	28	65
187	11694	1184	10	62
116	11451	666	6	96
93	10129	966	10	
	Slope			40
	Avg %CV			9.9
	Analyte recovery %			80

11. Dilution Linearity

Dilution linearity of the assay was tested by serially diluting the high level of ENA-78 serum sample with low level of ENA-78 plasma sample to yield sample concentrations within the dynamic range of the assay. Recovery ranged from 100 - 151 %. (one point excluded) 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						
Std.	Nominal pg/ml	Average	Stdev.	CV%	Calc. pg/ml	% Recovery
1	5369	1429735	42917	3	5369	107
2	1342	471513	20314	4	1225	100
3	336	132542	10174	8	300	103
4	84	36525	2700	7	90	141
5	21	13495	390	3	42	307
6	30	7568	142	2	30	151
					Average	152

12. Matrix effect

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

A) Spike into hemolyzed blood

ENA-78 was spiked at 4 levels ranging from 2400 – 375pg/mL into hemolysis blood sample. The recovery was estimated with an assay buffer calibration curve. The range of recovery was 73 -122%

ENA-78 pg/mL					Avg	Stdev	%CV	Calculated pg/mL	% Recovery
	1	2	3	4					
25227	914179	1404269	1288799.1	1390280.8	1249382	229316	18	18344	73
7227	478870	640321	568867.68	557451.51	561378	66109	12	8001	111
2727	277326	237858	248274.22	192640.72	239025	35145	15	3336	122
1602		135128	140330.11	130559.58	135339	4889	4	1860	116
1227	82412	78499	99200	102252	90591	11877	13		
Ave. %									105

B) Spike into lipemic plasma

ENA-78 was spiked at 4 levels ranging from 2400 – 375pg/mL into lipemic plasma. The recovery was estimated with an assay buffer calibration curve. Recovery of the analyte ranged from 60 – 120%.

ENA-78 pg/mL					Avg	Stdev	%CV	Calculated pg/mL	% Recovery
	1	2	3	4					
24746	1585361	1491778	1588334	1268455	1483482	150198	10	21982	89
6746	564198	583009	593804	683064	606019	52800	9	8657	128
2246			196594	169393	182993	19234	11	2537	113
1121	60693	74996	68540	72011	71849	3231	4	962	86
373	37874	34155	22962	25052	30011	7148	24		

13. Precision

6 points of calibrators were assayed on 18 instruments and replicate on each instrument for 3 lots

Ave. Intra CV: 6 %

Ave. Total CV: 16%

Ave. Lot to lot CV: 10%

ENA-78 pg/mL	Lot 1	Lot 2	Lot 3	Avg	Stdev	%CV
6000	991131	1026486	1087824	1035147	33197	3
1500	261534	314035	374185	316585	34015	11
375	75545	74313	100957	83605	13524	16
94	18851	20094	24552	21166	2327	11
23	5789	5963	7122	6291	597	9
0	865	1003	1223	1030	120	12
% CV						10

14. CV test

Middle point of calibrator were assayed 24 times on 24 instruments and replicate on each instrument

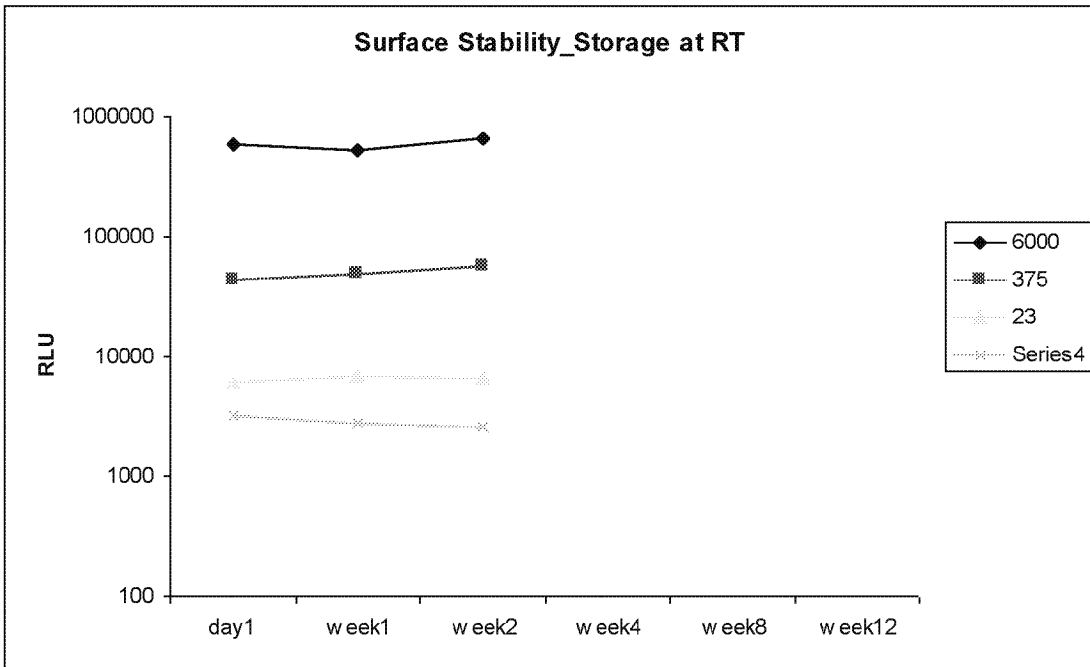
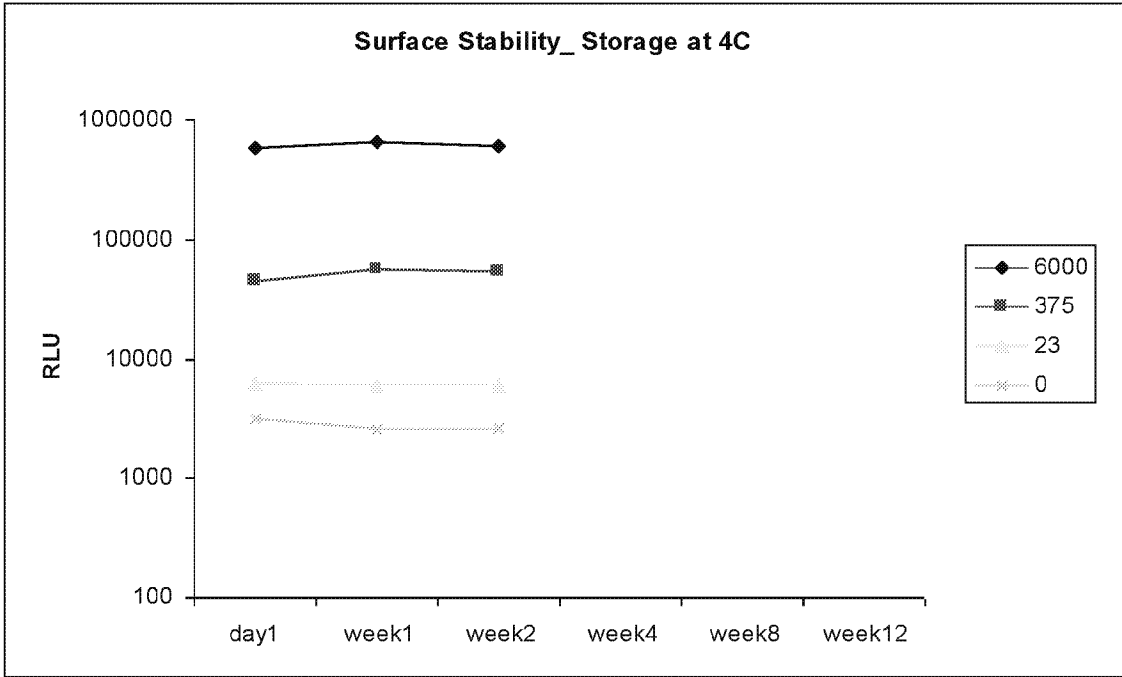
Ave. Intra CV: 5.3 %

Ave. Total CV: 13.5 %

15. Stability test

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 10-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.

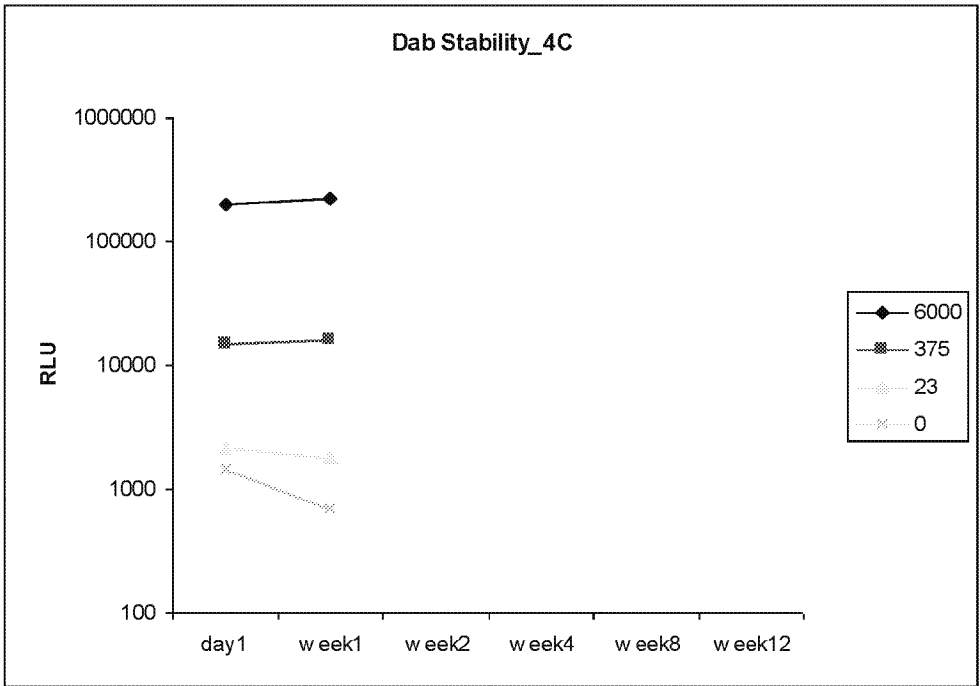


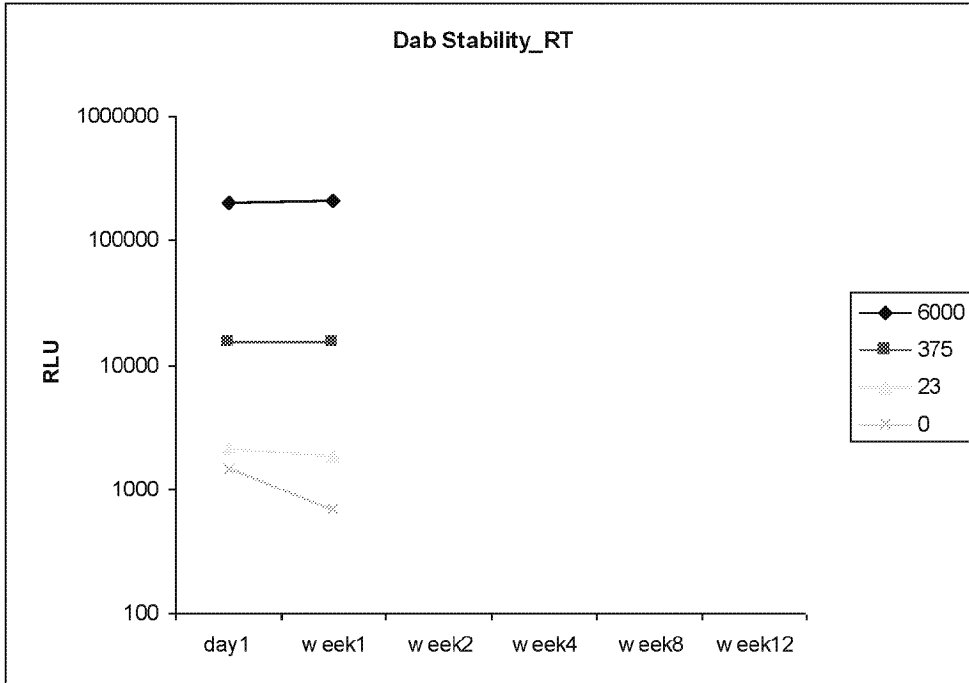
Summary							
Temp	pg/ml in sample	Day1	Week1	Week2	Week4	Week8	Week12
4C	6000	584587	649495	615816			
	375	44013	56898	53461			
	23.3	6142	5906	6068			

	0	3184	2559	2583
RT	6000	584587	530723	667348
	375	44013	47962	56244
	23.3	6142	6878	6668
	0	3184	2773	2545

b) Detection antibody stability

Detection antibody stability at working concentration are 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. Analyte standards were pre-made for the entire study, aliquoted and flash frozen for single time use.





Summary							
Temp	pg/ml in sample	Day1	Week1	Week2	Week4	Week8	Week12
4C	6000	197923	227080				
	375	14969	16099				
	23.3	2141	1820				
	0	1465	684				
RT	6000	197923	205275				
	375	14969	15347				
	23.3	2141	1845				
	0	1465	686				

16. Calibration

Calibration was carried out on a 7-point standard curve (24000-23pg/ml in sample) in blocking buffer.
 < Close to linear over a very wide dynamic range (1000-fold).

Figure1: Calibration

[EMBED Excel.Chart.8 \s]

Figure2: Calibration

[EMBED Excel.Chart.8 \s]

Table 1: Calibration

pg/ml	1	2	3	4	Ave.	STd	Total CV%	% recovery	
24000	1666624	1583285	1594907	1601704	1611630	37443	2	100	
6000	360318	359994	487194	491030	424634	74469	18	100	
1500	96901	105394	123784	102531	107153	11635	11	97	
375	31189	32762	33067	35557	33144	1807	5	111	
94	10355	9605	10405	9734	10025	414	4	98	
23	5280	4799	4712	4766	4889	263	5	86	
0	2828	3399	3260	2511	2999	406	14		
Slope							75		
LOD pg/ml in sample							9.70		
S/B (1/6)							537		
S/B (5/6)							2		
Ave. % analyte recovery							99		
Ave. Tatal %CV							8.5		

17. Centocor samples

20 Centocor samples were assayed with Theranos system.

Table1 Centocor samples

Sample #	1	2	1	2	Ave.	Stdev	CV%	Calcu. Conc pg/ml
1	29787	39613	30640	27086	31782	5436	17	398
2	489795	424073	379657	422216	428935	45464	11	6071
3	213340	232999			223169	13901	6	3110
4	62663	67109	56974	54414	60290	5705	9	799
5	29703	37892			33797	5791	17	426
6	37647	44049	32078	33291	36766	5412	15	468
7	64151	73683	61159	63534	65632	5520	8	875
8	26060	32827	28758	29850	29374	2799	10	364
9	819950	862590	1003118	1070423	939020	117466	13	13613
10	14681	15323	15479	16961	15611	964	6	171
11	106826	96540	100348	116667	105095	8806	8	1432
12	59586	56734	43530	55014	53716	7047	13	707
13	21293	23656	16094	16207	19312	3777	20	223
14	211049	222176	212353	195670	216613	7868	4	3016
15	16262	19089	16238	14533	16531	1888	11	184
16	765477	956494	829679	947176	874707	92915	11	12646
17	223483	233132	172906	171683	200301	32582	16	2784
18	456384	432297	403924	358564	412793	42032	10	5837
19	1321806	1399173	1064885	1090120	1218996	166726	14	17876
20	170784	189911	118597	106810	146526	40117	27	2019

