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Folate Assay Development Report

Theranos, Inc.

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1 ASSAY INFORMATION[TC "ASSAY INFORMATION" \F C \L "2"]

1.1 [TC "Assay Specifications" \f C \l "3"]Analyte information

Folate (also known as Folic acid, Vitamin B9) is involved in many biological functions. It usually refers to a group of folic acid metabolite compounds. The major biologically active form of Folate in the body is 5-methyl Tetrahydrofolic Acid (5mTHF).

Folate plays an important role in making red blood cells, white blood cells, platelets, and for normal growth. It also is critical for the normal development of fetus. Folate deficiency can result in anemia and severe folate deficiency shows the symptoms such as fatigue and weakness, headaches and difficulty concentrating. The most common cause of folate deficiency is low intake from food.

Normal range of folate in plasma is often reported as 4-20ng/ml in adults. Children have a slightly higher level. Individuals with anemia and certain type of cancers have decreased folate level. Elevated folate level has been seen for people who take vitamin supplements.

1.2 Assay specifications

This assay determines the concentration of Folate (combination of Folic acid and its major active form 5mTHF) in human serum and plasma. The assay has a quantification range of 1 ng/mL to 64 ng/mL.

1.3 Reference assay[TC "Reference Assays and Standards" \f C \l "3"]

The following assay was used as reference method:

SIEMENS Immulite Folic acid, Catalog number: 10380911

1.4 Materials and methods[TC "Materials and Methods" \f C \l "1"]

A competitive immunoassay using folate binding protein was developed for the determination of folate in serum and plasma.

Folate Binding Protein (FBP) has high affinity of binding folate in body. In this assay, FBP was used as capture agent for folate determination. In order to disassociate folate from endogenous FBP, serum/plasma samples were treated with "Reductant" to release folate from FBP. By further treatment with high pH "Extractant", endogenous FBP was deactivated. Treated samples were neutralized to lower pH and then mixed with capture agent of Biotin labeled FBP. The mixture was then incubated with HSA-FA coated tips. After incubation, the tips were washed with wash buffer and incubated with Streptavidin-alkaline phosphatase conjugate. After the second incubation, tips were washed with wash buffer again and incubated with substrate buffer. The chemiluminescence results were measured and reported as Relative Light Units (RLU).

Table [SEQ Table * ARABIC]: Folate assay materials in final assay procedure

Name	Supplier	Catalog number
Folic Acid	RTC	PHR-1035
5-methyl Tetrahydrofolic Acid (5mTHF)	Sigma	M0132
Folate depleted serum	SunnyLab	SF238-7
Human serum albumin - folic acid conjugate	In house	Lot #: FA-HSA_001_060712
Folate binding protein	GenWay	11-511-248777
Folate binding protein – Biotin conjugate	In house	Lot #: OK-0341-164-A/B
Carbonate-bicarbonate coating buffer	Sigma	C3041
Tris buffer	Sigma	T6664
Human serum albumin	Meridian Life Science	H8P01-767
Sucrose	Sigma	S5016
1N NaOH solution	BDH	BDH 3221-1
1N HCl solution	BDH	BDH 3202-1
1M DTT solution	Sigma	646563
Sodium Phosphate	Sigma	S7907
Mannitol	MP Biomedicals	205988
NaCl	EMD	7760
0.5M EDTA solution	Calbiochem	4055
Borate buffer, pH9.0	Sigma	33648
StabilZyme AP stabilizer	Surmodics	SA01-1000
5% Sodium Azide solution	Teknova	S0208
Wash buffer (20x concentrate)	Enzo Life Science	80-1351
Streptavdin AP conjugate	Calbiochem	189732
AP substrate buffer	In house	Lot #3

2 ASSAY DEVELOPMENT [TC "ASSAY OPTIMIZATION" \F C \L "2"]

2.1 Initial antibody and folate binding protein screening on MTP

During initial assay development, 19 anti-folate antibodies and 7 folate binding proteins (FBP) from commercial sources were screened for binding to Folic acid-alkaline phosphatase (FA-AP) conjugate provided from in-house chemistry group on multi-titer plate (MTP).

Materials:

Table [SEQ Table * ARABIC]: Anti-folate antibodies and folate binding proteins screened

	Name	Supplier	Cat#	Lot#	Notes
Anti-FA antibody					
1	mouse anti-FA McAb	GenWay	20-783-310673	290410	clone FA2
2	mouse anti-Folate McAb	GenWay	20-251-400747	8779	clone M741809
3	mouse anti-FA McAb	GenWay	20-251-400709	2938	clone M608298
4	mouse anti-Folate McAb	MyBioSource	MBS532366	1622	clone 3310780
5	mouse anti-FA McAb	Millipore	CBL65	LV1827668	clone 8/33
6	mouse anti-Floate McAb	GenWay	20-511-242235	6A01812	B762F
7	mouse anti-FA McAb	GenWay	20-322-392263	12/01-FA3-A1	mixed clones
8	mouse anti-FA McAb	LifeSpan Biosciences	LS-C66254	24975	
9	mouse anti-FA McAb	LifeSpan Biosciences	LS-C66261	32382	
10	mouse anti-FA McAb	US Biological	F5800-14	L12012002	1.B.776
11	mouse anti-Folate McAb	GenWay	20-511-242193	5A01912	clone 8/33
12	mouse anti-Folate McAb	GenWay	20-511-242281	2B03212	clone B764F
13	mouse anti-Folate McAb	GenWay	20-511-242282	7B04612	clone B763F
14	mouse anti-FA McAb	LifeSpan Biosciences	LS-C66258	33053	
15	mouse anti-FA McAb	LifeSpan Biosciences	LS-C129137	33647	
16	mouse anti-FA McAb	LifeSpan Biosciences	LS-C129139	33648	
17	mouse anti-FA McAb	US Biological	F5800-10A	L12020167	
18	mouse anti-FA McAb	US Biological	F5800-12A	L12022376	
19	mouse anti-FA McAb	US Biological	F5800-12C	L12031620	
Folate Binding Protein					
1	FABP-1	R&D Systems	5646-FR	RTI0111081	recombinant
2	FABP-2	R&D Systems	5697-FR	RZI0211071	recombinant
3	FABP-3	R&D Systems	5319-FR	RBK0110121	recombinant
4	FOLR-1	Creative BioMart	FOLR1-3889H	392167	recombinant
5	FOLR-2	Creative BioMart	FOLR2-2244H	289196	recombinant
6	FBP	GenWay	11-511-248777	3K31008	Purified native protein

Table [SEQ Table * ARABIC]: Other materials used in initial screen

	Name	Supplier	Cat#/Lot#
1	FA-AP conjugate	In house	FA-AP_001_011912
2	Biotin-FBP	In house	OK-ii-2B/OK-ii-4B
3	UltraAvidin	Leinco	A110
4	Biotin labeling kit	Dojindo	LK10

Methods:

Antibodies and FBPs were labeled with Biotin using Dojindo Biotin Labeling kit. The MTP was first coated with UltraAvidin (UA) at 20 ug/ml in coating buffer and then coated with Biotin labeled antibody or Biotin labeled FBP at 10 ug/ml. FA-AP conjugate was diluted in a series dilution in Low Human serum albumin (HSA) buffer and incubated with coated antibodies or FBPs. Finally, AP substrate was added to each well and Relative Luminescence Unit (RLU) was measured by a plate reader. Modulations for each antibody and FBP were calculated using RLU of each conjugate concentration level divided by the RLU of background (buffer blank, no conjugate).

Results:

Six antibodies and six FBPs showed good modulations and were selected to move forward to Theranos readers for further screening.

Table [SEQ Table * ARABIC]: Results of initial screen on MTP

Antibody	# 1	# 2	# 3	# 4	# 5	# 6	# 7	# 8	# 9	# 10	# 11	# 12	# 13	# 14	# 15	# 16	# 17	# 18	# 19
Modulation																			
FBP	# 1	# 2	# 3	# 4	# 5	# 6													
Modulation																			

	Good modulations (>50 fold)
	Poor or no modulations

2.2 Antibody and FBP screening on readers

2.2.1 Antibody and FBP screening with FA-AP conjugate

From MTP screening, antibody #1, 4, 6, 7, 8, 9 and six FBPs (#1-#6) were chosen to screen on readers.

Methods:
Three tip coating formats were used for screening:

Format-1:
Reaction tips were coated with UA at 20ug/ml and then Biotin labeled antibody or FBP at various concentrations. Coated tips were incubated with series diluted FA-AP conjugate for 10min on readers. Tips were then washed and incubated with AP substrate buffer for 10min. RLU was measured for each tip.

Format-2:
Reaction tips were coated with UA and 20ug/ml and Biotin labeled goat and mouse IgG antibody. Anti-FA antibodies were incubated in solution with coated tips in cartridge.

Format-3;
Reaction tips were coated with non-labeled antibody or FBP directly.

Coated tips were incubated with FA-AP conjugate at series dilution. Modulations were calculated for each antibody and FBP.

Results:
Format-1 showed the best modulations for most antibodies and FBPs. Antibodies and FBPs which showed good modulations proceeded to further screen with folate competition.

2.2.2 Antibody and FBP screening with Folate competition to conjugate

Methods:
After antibody and FBP screening with FA-AP conjugate, a fixed FA-AP concentration was chosen for folate competition screening. Folic Acid and 5mTHF solution were prepared at 100ng/ml, 10ng/ml and 1ng/ml in Low HSA buffer. Reaction tips were coated with UA 20ug/ml and Biotin labeled anti-FA antibodies or FBPs. Anti-FA antibodies and FBPs were screened at 10ug/ml, 5ug/ml, and 1ug/ml coating concentration.

Both the capture surfaces were incubated with FA or 5mTHF in buffer for 10min. After washing, tips were incubated with FA-AP for 10min. After the third incubation with AP substrate buffer, tips were measured for RLU value. Modulation was calculated as "percentage of binding" to buffer blank (no FA or 5mTHF in buffer) to show the competition between free FA or 5mTHF and FA-AP conjugate.

Results:
Among six antibodies and six FBPs where were screened with folate competition, FBP showed dose dependent signal decreasing when being incubated folate first. FBP#6 showed the best modulation and was chosen for further evaluation.

Table [SEQ Table * ARABIC]: Results of antibody and FBP screening with folate competition

		Ab#1			Ab#4			Ab#6		
	Sample Conc. (ng/ml)	Mean RLU	%CV	Modulation (%Binding)	Mean RLU	%CV	Modulation (%Binding)	Mean RLU	%CV	Modulation (%Binding)
FA	100	55253	5	95	45855	10	102	43871	6	96
FA	10	50499	5	87	45970	11	102	42138	9	92
FA	1	53740	3	93	49947	6	111	44586	2	98
5mTHF	100	54587	4	94	49108	13	109	44628	4	98
5mTHF	10	47716	7	82	51181	3	114	39758	8	87
5mTHF	1	50960	9	88	51304	10	114	41805	12	91
buffer	0	57929	8	100	44990	6	100	45722	6	100
		Ab#7			Ab#8			Ab#9		
	Sample Conc. (ng/ml)	Mean RLU	%CV	Modulation (%Binding)	Mean RLU	%CV	Modulation (%Binding)	Mean RLU	%CV	Modulation (%Binding)
FA	100	49619	5	97	20935	12	103	22608	12	95
FA	10	46565	6	91	22147	6	109	26379	8	111
FA	1	47589	1	93	24295	3	120	26796	9	113
5mTHF	100	51257	3	100	23383	10	118	27189	6	115
5mTHF	10	44101	8	86	24403	10	120	25408	13	107
5mTHF	1	46151	11	90	24897	8	123	27404	4	115
buffer	0	51227	9	100	20258	13	100	23735	4	100
		FBP#1			FBP#2			FBP#3		
	Sample Conc. (ng/ml)	Mean RLU	%CV	Modulation (%Binding)	Mean RLU	%CV	Modulation (%Binding)	Mean RLU	%CV	Modulation (%Binding)
FA	100	2337	11	73	21160	6	72	4203	13	63
FA	10	2577	7	81	23611	10	80	4274	20	64
FA	1	3196	8	100	26025	6	88	5464	9	82
5mTHF	100	3316	7	104	26533	7	90	5375	7	81
5mTHF	10	3341	13	105	24773	9	84	5782	19	87
5mTHF	1	3405	14	107	27996	10	95	7019	9	105
buffer	0	3194	12	100	29433	15	100	6669	5	100
		FBP#4			FBP#5			FBP#6		
	Sample Conc. (ng/ml)	Mean RLU	%CV	Modulation (%Binding)	Mean RLU	%CV	Modulation (%Binding)	Mean RLU	%CV	Modulation (%Binding)
FA	100	9893	9	86	36072	7	79	20382	9	42
FA	10	12093	9	105	44319	7	97	49358	20	102
FA	1	11194	4	97	42263	8	92	45103	7	93
5mTHF	100	10448	12	90	40198	13	88	28300	15	58
5mTHF	10	11358	16	98	43893	11	96	48380	12	100
5mTHF	1	11769	10	102	46404	13	101	50571	11	104

buffer	0	11571	11	100	45784	8	100	48410	12	100

2.2.3 FBP screening with different assay format and with more conjugates

Although FBP#6 showed highest modulation among all antibodies and FBPs in initial screening, the modulation of folate competition with conjugate was not promising enough for assay development. FBP#6 was further evaluated with different assay format and with more conjugates for better condition for folate competition to conjugate.

Table [SEQ Table * ARABIC]: Other materials used in FBP screening

	Name	Supplier	Cat#/Lot#
1	Pteroic Acid-AP	In house	OK-I-196-E
2	FBP-BNP-Biotin	In house	OK-ii-19-f2

Methods:
Two forms of Biotin-FBP conjugates obtained from in-house chemistry group were tested at this stage. FBPs were evaluated with direct coating on tips or coating on UA tips at 5ug/ml. First each format was performed with FA-AP or PA-AP conjugate titration to get the optimal conjugate concentration. The next step was to use each selected optimal concentration to test folate competition with conjugate.

A set of spiked samples from 0.5ng/ml to 64ng/ml of FA ot 5mTHF in depleted serum was also used to evaluate the binding condition of Folic acid and 5mTHF with the best assay format so far because native FBP was reported to have different affinity to Folic acid and 5mTHF. Our goal was to find the optimal condition to measure both Folic acid and 5mTHF.

At this stage, two Edison protocols were used: sandwich format (stepwise incubation) and co-incubation format.

Results:
From many experiments conducted, the following observation was obtained:

- Folic acid and 5mTHF showed different binding affinity to FBP at current condition
- FBP direct coating showed more sensitivity to folate competition than coating on UA tips
- Biotin-FBP was more sensitive than Biotin-BNP-FBP
- FA-AP conjugate was more sensitive than PA-AP conjugate

At this stage the major issue was identified as finding the optimal condition for Folic acid and 5mTHF binding to FBP.

Table [SEQ Table * ARABIC]: Folic acid and 5mTHF competition curve with best assay condition of FBP screening

Experiment:	FA/5mTHF competition curve
Edison Protocol:	Generic2_10x_coincubation
Tips:	FBP direct coating 2ug/ml
Calibrator:	FA or 5mTHF in serum, 2/7/2012
Sample dilution:	1:10

Sample (in depleted serum)	Conc. (ng/ml)	Mean RLU	%CV	Modulation (%Binding)		Sample (in depleted serum)	Conc. (ng/ml)	Mean RLU	%CV	Modulation (%Binding)
FA	64	874	9	3		5mTHF	64	17317	5	47
FA	32	1323	13	4		5mTHF	32	29994	11	81
FA	16	2492	8	7		5mTHF	16	30921	8	84
FA	8	4441	6	13		5mTHF	8	33441	11	91
FA	4	7339	16	21		5mTHF	4	32519	20	88
FA	2	13533	6	39		5mTHF	2	33438	12	91
FA	1	23761	10	69		5mTHF	1	36416	5	99
FA	0.5	27789	23	81		5mTHF	0.5	36631	6	99
depleted serum	0	34274	17	100		depleted serum	0	36822	13	100

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Figure [SEQ Figure * ARABIC]: Folate competition curve of FBP initial screen

2.3 Serum sample treatment method development

2.3.1 pH effect evaluation in buffer

Methods:
According to literatures, FBP binding affinity to FA and 5mTHF is pH sensitive. To evaluate pH effect on FBP binding, FA and 5mTHF were prepared as three-point calibrators in assay buffer. A series of sample diluent were prepared at different pH range from 7.4 to 9.7 to dilute FA/5mTHF samples at 1:10 dilution. Effect of Tween 20 in diluent was also compared. Percentage of binding comparing to buffer was calculated for each calibrator point. Sandwich assay format and co-incubation format were both evaluated as well.

Results:
A sample diluent pH of 9.0 to 9.3 demonstrated the most uniformed binding affinity of FA and 5mTHF to FBP compared to other pH conditions. Adding 0.1% Tween 20 to diluent seemed to improve the binding equivalency between FA and 5mTHF but the effect was not significant.

Table [SEQ Table * ARABIC]: pH and detergent effect on FBP binding with analyte in assay buffer

		pH7.4 w/ T20		pH8.0 w/ T20		pH9.6 w/ T20		pH9.0 no T20	
Sample	Conc. (ng/ml)	Mean RLU	Modulation (%Binding)	Mean RLU	Modulation (%Binding)	Mean RLU	Modulation (%Binding)	Mean RLU	Modulation (%Binding)
FA	100	707	2	678	3	579	13	2203	4
FA	10	3159	7	3705	16	1954	43	14892	29
FA	1	24797	59	19086	83	4183	91	46571	91
5mTHF	100	3367	8	2292	10	645	14	6892	13
5mTHF	10	7557	18	7228	31	1418	31	15757	31
5mTHF	1	37056	88	22724	98	3737	81	54813	107
buffer	0	42293	100	23119	100	4592	100	51351	100
		pH9.0 w/ T20		pH9.3 no T20		pH9.55 no T20		pH9.7 no T20	
Sample	Conc. (ng/ml)	Mean RLU	Modulation (%Binding)	Mean RLU	Modulation (%Binding)	Mean RLU	Modulation (%Binding)	Mean RLU	Modulation (%Binding)
FA	100	2137	6	1403	3	1274	5	1167	8
FA	10	10393	30	10176	24	8200	35	6152	40
FA	1	33019	97	32011	75	21234	90	11438	74
5mTHF	100	5251	15	3753	9	2735	12	1734	11
5mTHF	10	12198	36	9199	22	6645	28	4101	27
5mTHF	1	32954	96	34820	82	23495	100	9494	62
buffer	0	34204	100	42478	100	23514	100	15433	100

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Figure [SEQ Figure * ARABIC]: pH and detergent effect on FBP binding with analyte in assay buffer

2.3.2 Use of protein reducing agent for serum sample treatment

Serum folate is found bound to endogenous FBP as a major form. Folate needs to be released from binding protein for accurate measurement as free form. In order to disassociate folate binding to endogenous FBP in serum, a few protein reducing agents were used to "treat" serum samples before mixing with "reagent FBP".

Table [SEQ Table * ARABIC]: Other materials used in sample treatment method development

	Name	Supplier	Cat#/Lot#
1	β-mercaptoethanol	Sigma	M7522
2	Tris (2-carboxyethyl) phosphine (0.5M solution)	Sigma	646547
3	Urea	Sigma	U5378
4	Guanidine hydrochloride	Sigma	G3272
5	Clinical serum samples	Bioreclamation	BRH473794-BRH473813

Methods:
Protein reducing agents β-mercaptoethanol (BME), Dithiothreitol (DTT), and Tris (2-carboxyethyl) phosphine (TCEP) were added into phosphate buffer at different concentrations to make "sample treatment buffers" at pH 9.0-9.5. Folate was spiked in depleted serum at concentrations of 0.5 to 32 ng/ml. Spiked serum samples were mixed with different sample treatment buffers at 1:10 dilution and the proceed to further assay steps.

Assay formats included (1) sandwich format where FBP coated tips first react with treated samples and then incubate with FA-AP conjugate, or (2) co-incubation format where treated samples mix with FA-AP conjugate and FBP coated tips react with the mixture in one incubation step.

A few clinical serum samples were also used to check sample treatment effect with protein reducing agents.

Results:
Without adding protein reducing agents into sample treatment buffer, folic acid and 5mTHF spiked samples showed very different binding competition curve. After several attempts of using BME, DTT, and TCEP in treatment buffer, certain conditions showed that Folic acid and 5mTHF spiked serum samples had similar binding to "reagent FBP" which was indicated by similar modulations at same concentration levels. BME, DTT and TCEP didn't show significant difference on treatment. However, when clinical samples were used to evaluate sample treatment effect, no modulation was seen with samples having different folic acid concentrations.

Table [SEQ Table * ARABIC]: Example of sample treatment results

	FA spiked in depleted serum	5mTHF spiked in depleted serum	Clinical samples from Bioreclamation

Nominal Conc (ng/ml)	Mean RLU	% Binding	Mean RLU	% Binding	Conc by SIEMENS	Mean RLU	% Binding
32	6693	30	7987	36	2.96	25382	113
8	10652	48	15448	69	13.5	26767	119
2	18458	82	19174	86	23.8	25248	113
1	20606	92	21277	95			
0.5	20699	92	21143	94			
0	22410	100	22410	100			

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Figure [SEQ Figure * ARABIC]: Example of sample treatment results

2.3.3 Development of full procedure for sample treatment method

Using protein reducing agents for sample treatment increased folate binding in spiked samples with initial assay format. However, preliminary sample treatment method didn't work for clinical samples. Further development of serum sample treatment was conducted by evaluating more protein denaturing reagents, testing various treatment procedures and using different assay formats.

Methods:

Besides BME, DTT and TCEP, more reagents such as Urea and Guanidine were also used for protein denaturing at various concentrations. Sample treatment protocols were evaluated with several different procedures. Sample treatment was also conducted at room temperature and 37C for comparison. Different assay formats were also included. Several clinical samples were used to indicate treatment results. Table 11 showed the summary of some experiments for comparison.

Results:

Many different combinations of treatment reagents, reagent concentrations and treatment protocols were evaluated. Some conditions showed good dose-dependent folate competition with spiked samples but did not show good correlation with clinical samples. Some conditions worked better with clinical samples. With the consideration of experiment results and literature reference, sample treatment effect might be blocked by un-optimized assay procedure. Based on the results from different sample treatment methods, a preliminary procedure was chosen to evaluate different assay formats.

A preliminary procedure of treatment was temporarily fixed as

(1) sample mixing with "reductant" which contains protein reducing agent to disassociate folate with endogenous FBP
(2) then mixing with "extractant" which has high pH to denature endogenous FBP

(3) final mixing with "neutralizer" to bring pH around 9.0 for best binding condition for released folate in serum to "reagent FBP"

Table [SEQ Table * ARABIC]: Summary of sample treatment development effort

1. Assay format: FBP direct coating, sandwich format unless indicated for co-incubation							
		RT					
Sample	Conc.(ng/ml)	1mM BME sample/conj coincubation %Binding	50mM BME sample/conj coincuabtion %Binding	50mM BME %Binding	40mM DTT %Binding	40mM DTT overnight %Binding	40mM DTT neatralizing to ~pH9 %Binding
F5	2.96		reading too low	54	73	46	113
F6	13.5			168	98	114	119
F18	23.8			171	66	212	113
FA	4	64		111	136	97	82
FA	32	9		47	77	70	30
5mTHF	4	102		22	162	188	86
5mTHF	32	59		38	69	58	36
blank serum	0	100		100	100	100	100

		37C				
Sample	Conc. (ng/ml)	100mM DTT %Binding	250mM DTT %Binding	500mM DTT %Binding	40mM TCEP %Binding	125mM TCEP %Binding
F5	2.96	99	109	80	99	95
F6	13.5	93	96	98	82	83
F18	23.8	83	108	107	78	80
FA	4	48	67	50	43	42
FA	32	29	39	36	20	26
5mTHF	4	88	88	98	79	68
5mTHF	32	34	62	65	37	51
blank serum	0	100	100	100	100	100

		37C			
		125mM TCEP 4M Urea	125mM TCEP 8M Urea	125mM TCEP 3M Gnd	Proteinase K 40ug/ml 1:1 mix w sample

Sample	Conc. (ng/ml)	%Binding	%Binding	%Binding	%Binding
F5	2.96	78	88	82	78
F6	13.5	69	83	79	79
F18	23.8	72	74	81	76
FA	4	33	52	40	41
FA	32	21	21	23	17
5mTHF	4	62	65	67	88
5mTHF	32	32	36	32	102
blank serum	0	100	100	100	100

2. B-FBP coating						
		RT				
Sample	Conc.(ng/ml)	40mM DTT overnight	100mM DTT	100mM DTT reversed ratio (equivalent to 200mM DTT, sample diluted 1:20)	40mM TCEP	40mM TCEP reversed ratio (equivalent to 80mM TCEP, sample diluted 1:20)
		%Binding	%Binding	%Binding	%Binding	%Binding
F5	2.96	124	103	109	100	101
F6	13.5	152	87	91	90	92
F18	23.8	155	85	94	83	98
FA	4	(2)116	97	105	89	99
FA	32	85	77	87	80	85
5mTHF	4	(2)154	102	104	98	104
5mTHF	32	91	94	103	95	95
blank serum	0	100	100	100	100	100

		3. UA coating, B-FBP in solution		
		37C		
		500mM DTT	125mM TCEP 4M Urea	10mM TCEP sample/BFBP/Conj coincubation

Sample	Conc.(ng/ml)	%Binding	%Binding	%Binding
F5	2.96	114	92	53
F6	13.5	114	88	85
F18	23.8	105	93	81
FA	4	96	91	76
FA	32	91	78	92
5mTHF	4	107	97	83
5mTHF	32	91	93	100
blank serum	0	100	100	100

2.4 Assay format evaluation with preliminary sample treatment method

With the preliminary sample treatment method, multiple assay formats were evaluated for better modulation differentiation. As the focus of this stage was to find the best condition for clinical samples, several clinical samples from Bioreclamation with known concentration of folate were chosen to use as a group of calibrators (indicators) to compare the results among experiments.

2.4.1 Assay format comparison and selection

First attempt was to use the same sample treatment method to try on many assay formats and conditions.

Methods:
Serum sample treatment procedure was described in section 2.3.3 was kept the same to compare different assay format and procedure. 40mM DTT was used for treatment and all samples were diluted 1:24 before being loaded to cartridge. FBP from different vendors were also used for comparison.

Table [SEQ Table * ARABIC]: Other materials used for assay format evaluation

	Name	Supplier	Cat#/Lot#
1	FBP	Sigma	FA-AP_001_011912
2	FBP	Fitzgerald	30C-CP8104
3	BSA-FA	US Biological	F5800-11A
4	Goat anti-FBP	GenWay	18-732-292005
5	FBP-AP	In house	OK-II-15-AF3

Table [SEQ Table * ARABIC]: Major assay formats evaluated

	Reaction Tip Coating	Capture agent	Tracer	Detection agent	Incubation Steps

1	FBP direct	FBP	FA-AP conj.	FA-AP conj.	Sample and conjugate co-incubation
2	FBP direct	FBP	Biotin-FA	Streptavidin-AP	Sandwich format: Sample and tracer premix, AP conj in separate step
3	FBP direct	FBP	Biotin-FA	Streptavidin-AP	Sample, tracer and AP conj co-incubation
4	UA	Biotin-FBP in solution	FA-AP conj	FA-AP conj.	Sandwich format: Sample and tracer premix, AP conj in separate step
5	UA	Biotin-FA in solution	Biotin-FA	FBP-AP conj	Sample and tracer and AP conj. co-incubation
6	UA	Biotin-FA in solution	Biotin-FA	FBP-AP conj	Sandwich format: Biotin-FA 1st step, sample and AP conj premix in 2nd step
7	UA and Biotin-FA	FBP	Biotin-FA	FBP-AP conj.	Co-incubation
7	Goat anti-FBP coating	FBP in solution	FA-AP	FA-AP conj.	Co-incubation or sandwich format
8	Goat anti-FBP coating	FBP in solution	Biotin-FA	Streptavidin-AP	Sandwich format
9	BSA-FA direct	Biotin-FBP	BSA-FA	Streptavidin-AP	Sandwich format
10	HSA-FA direct	Biotin-FBP	HSA-FA	Streptavidin-AP	Sandwich format

Results:
Among all the assay conditions evaluated, two formats looked promising which showed better modulations:

(1) Tips coated with UA only, Biotin-FA was put in solution and incubated with coated tip first. Treated sample was mixed with FBP-AP conjugate and reacted with tips in second incubation.

(2) HAS-FA direct coating on tips. Treated sample was mixed with Biotin-FBP and incubated with coated tips in first incubation. Streptavidin-AP conjugate was incubated in second incubation step.

Above two formats were selected as tentative format for further evaluation.

Table [SEQ Table * ARABIC]: Results summary of most assay format/conditions

Assay format category	**FBP direct coating**	**FBP direct coating**

Method Descriptions		Sample and Conj. Co-incubation Sigma FBP	Sample and Conj. Co-incubation Fitzgerald FBP	Sample and Conj. Co-incubation GenWay FBP	sample/conj co-in, use blood tip to mix to shorten the time of "conj w DTT" Fitzgerald FBP	sample/conj co-in, use blood tip to mix to shorten the time of "conj w DTT" GenWay FBP
Clinical Sample	**Conc. (ng/ml) by SIEMENS**	**%Binding**	**%Binding**	**%Binding**	**%Binding**	**%Binding**
F2	**2.18**	99	66	81	80	75
F13	**9.19**	86	67	76	60	67
F20	**45.2**	93	57	69	44	36
blank serum	**0**	100	100	100	100	100
Modulation to highest sample						
blk/F20		***1.1***	***1.8***	***1.5***	***2.3***	***2.8***
F2/F20		***1.0***	***0.7***	***0.8***	***0.8***	***0.7***
F13/F20		***0.9***	***0.7***	***0.8***	***0.6***	***0.7***
F20/F20		***0.9***	***0.6***	***0.7***	***0.4***	***0.4***

Assay format category		UA tip		UA tip		UA+B-DNP-FBP coating
Method Descriptions		B-FBP or B-DNP-FBP in sln 1st, sample/conj co-in 2nd (2-step incubation)	B-FBP or B-DNP-FBP in sln 1st, sample/conj co-in 2nd (2-step incubation)	Biotin-FA in sln, sample/conj co-in 2-step incubation	Biotin-FA/sample/conj all co-incubation	sample/conj co-in
Clinical Sample	**Conc. (ng/ml) by SIEMENS**	**%Binding**	**%Binding**	**%Binding**	**%Binding**	**%Binding**
F2	**2.18**	94	91	89	122	114
F13	**9.19**	79	79	71	101	103

F20	**45.2**	43	38	28	110	71
blank	**0**	100	100	100	100	100
Modulation to highest sample						
blk/F20		***2.3***	***2.6***	***3.6***	***0.9***	***1.4***
F2/F20		***0.9***	***0.9***	***3.2***	***1.1***	***1.6***
F13/F20		***0.8***	***0.8***	***2.5***	***0.9***	***1.5***
F20/F20		***0.4***	***0.4***	***1.0***	***1.0***	***1.0***

Assay format category		**Goat-anti-FBP coating**			**Goat-anti-FBP coating**		
Method Descriptions		FBP 1st, sample/conj 2nd, Sigma FBP	FBP 1st, sample/conj 2nd, Fitzgerald	FBP 1st, sample/conj 2nd, GenWay	FBP/sample 1st then conj 2nd, Sigma	FBP/sample 1st then conj 2nd, Fitzgerald	FBP/sample 1st then conj 2nd, GenWay
Clinical Sample	**Conc. (ng/ml) by SIEMENS**	**%Binding**	**%Binding**	**%Binding**	**%Binding**	**%Binding**	**%Binding**
F2	**2.18**	99	66	81	135	68	79
F13	**9.19**	86	67	76	128	64	72
F20	**45.2**	93	57	69	135	57	71
blank	**0**	100	100	100	100	100	100
Modulation to highest sample							
blk/F20		***1.1***	***1.8***	***1.5***	***0.7***	***1.7***	***1.4***
F2/F20		***1.1***	***1.2***	***1.2***	***1.0***	***1.2***	***1.1***
F13/F20		***0.9***	***1.2***	***1.1***	***1.0***	***1.1***	***1.0***
F20/F20		***1.0***	***1.0***	***1.0***	***1.0***	***1.0***	***1.0***

Assay format category		**Goat-anti-FBP coating**			
Method Descriptions		FBP/sample then conj all co-incubation, Fitzgerald	FBP/sample then conj all co-incubation, GenWay	FBP/sample then conj all co-incubation, FOLR1CB	FBP/sample then conj all co-incubation, FOLR2CB
Clinical Sample	**Conc. (ng/ml) by SIEMENS**	**%Binding**	**%Binding**	**%Binding**	**%Binding**
F2	**2.18**	92	115	84	120
F13	**9.19**	95	117	88	111

F20	**45.2**	77	75	109	131
blank	**0**	100	100	100	100
Modulation to highest sample					
blk/F20		***1.3***	***1.3***	***0.9***	***0.8***
F2/F20		***1.2***	***1.5***	***0.8***	***0.9***
F13/F20		***1.2***	***1.6***	***0.8***	***0.9***
F20/F20		***1.0***	***1.0***	***1.0***	***1.0***

Assay format category		**BSA-FA direct coating**	**HSA-FA direct coating**	
Method Descriptions		BSA-FA direct coating	HSA-FA direct coating	HSA-FA direct coating, 2-step format, sample mixed with Biotin-FBP, UA-AP in 2nd incu
Clinical Sample	**Conc. (ng/ml) by SIEMENS**	**%Binding**	**%Binding**	**%Binding**
F2	**2.18**	117	114	74
F13	**9.19**	110	93	49
F20	**45.2**	76	35	12
blank	**0**	100	100	100
Modulation to highest sample				
blk/F20		***1.3***	***2.9***	***8.3***
F2/F20		***1.5***	***3.3***	***6.1***
F13/F20		***1.4***	***2.7***	***4.1***
F20/F20		***1.0***	***1.0***	***1.0***

Assay format category	**FBP direct coating**						
Method Descriptions	FBP direct tip, Biotin-FA/sample premix, UA-AP: Biotin-FA 1ng/ml, UA-AP 10ng/ml	FBP direct tip, Biotin-FA/sample premix, UP-AP: Biotin-FA 5ng/ml, UP-AP 5ng/ml	FBP direct, fix-1, Biotin-FA treated w sample	FBP direct, fix-2, Biotin-FA treated w sample	Altogether format: FBP coating, Biotin-FA/sample/UA-AP co-incu	Altogether format: UA coating, Biotin-FBP/sample/UA-AP altogether	FBP direct coating 0.1ug/ml, 2-step format

Clinical Sample	Conc. (ng/ml) by SIEMENS	%Binding	%Binding	%Binding	%Binding	%Binding	%Binding	%Binding
F2	**2.18**	100	98	84	83	95	96	83
F13	**9.19**	82	90	93	88	81	105	89
F20	**45.2**	44	64	46	43	86	44	56
blank	**0**	100	100	100	100	100	100	100
Modulation to highest sample								
blk/F20		***2.3***	***1.6***	***2.2***	***2.3***	***1.2***	***2.3***	***1.8***
F2/F20		***2.3***	***1.5***	***1.8***	***1.9***	***1.1***	***2.2***	***1.5***
F13/F20		***1.8***	***1.4***	***2.0***	***2.0***	***0.9***	***2.4***	***1.6***
F20/F20		***1.0***	***1.0***	***1.0***	***1.0***	***1.0***	***1.0***	***1.0***

2.4.2 Further test of UA coating format

Form assay format/condition comparison, tentative format 1 which used UA coated tips was first optimized. More detailed assay conditions were further verified with different reagent buffers, reagent concentrations etc.

Assay format was kept as using UA coated tips, Biotin-FA is first incubation, and treated sample and FBP-AP conjugate incubate together in second incubation. Concentration Biotin-FA and FBP-AP were evaluated in different combinations. Sample treatment procedure was kept the same as before but different DTT concentrations and different buffer were tested.

This format gave good modulation after optimization. However, only Biotin-FA diluted in "WS buffer" showed better results. Selection or formulation of buffer for Biotin-FA would be needed for the format.

Table [SEQ Table * ARABIC]: Optimization of tentative format I

Assay format	**to optimize "new pair": UA tip, Biotin-FA in sln 1st incu, treated sample/FBP-AP co-in 2nd step**				
Method description	Biotin-FA 2ug/ml, FBP-AP 10ng/ml	Biotin-FA 5ug/ml, FBP-AP 5ng/ml, Biotin-FA in WS buffer	40mM DTT use Biostab to neutralize	40mM DTT, Boric buffer, Biotin-FA in WS buffer	10mM DTT, Boric buf to neutralize, Biotin-FA in WS buffer

Sample	Conc. (ng/ml) by SIEMENS	%Binding	%Binding	%Binding	%Binding	%Binding
F2	**2.18**	94	79	146	107	86
F13	**9.19**	95	46	44	66	58
F20	**45.2**	23	14	45	18	15
blank	**0**	100	100	100	100	100
Modulation						
blk/F20		*4.3*	*7.3*	*2.2*	*5.6*	*6.5*
F2/F20		*4.1*	*5.7*	*3.3*	*6.0*	*5.6*
F13/F20		*4.1*	*3.4*	*1.0*	*3.7*	*3.7*
F20/F20		*1.0*	*1.0*	*1.0*	*1.0*	*1.0*

2.4.3 Further test of HSA-FA coating format

To optimize tentative format II, HAS-FA coating concentration was chosen at 2ug/ml, Biotin-FBP and SA-AP concentration changed in different combinations. Sample treatment was kept at using 40mM DTT and 1:24 dilution.

Most conditions in this format gave promising modulation with "3-point" clinical samples. This format was chosen as final format for further development.

Table [SEQ Table * ARABIC]: Optimization of tentative format II

Assay format		**HAS-FA coated tips, Biotin-FBP and treated sample in first incubation, Streptavidin AP in second incubation**						
Method description		Biotin-FBP 50 ng/ml, SA-AP 50 ng/ml	Biotin-FBP 25 ng/ml, SA-AP 25 ng/ml	Biotin-FBP 12.5 ng/ml, SA-AP 25 ng/ml	Biotin-FBP 25 ng/ml, SA-AP 12.5 ng/ml	B-FBP 25 ng/ml, SA-AP 12.5 ng/ml (repeat)	B-DNP-FBP 25 ng/ml, SA-AP 12.5 ng/ml	B-FBP 10 ng/ml, SA-AP 5 ng/ml
Sample	**Conc. (ng/ml) by**	**% Binding**	**% Binding**	**% Binding**	**% Binding**	**% Binding**	**% Binding**	**% Binding**

	SIEMENS							
F2	**2.18**	74	84	68	77	79	79	82
F13	**9.19**	49	67	40	45	47	55	52
F20	**45.2**	12	15	16	12	11	30	20
blank	**0**	100	100	100	100	100	100	100
Modulation								
blk/F20		*8.3*	*6.7*	*6.3*	*8.3*	*9.1*	*3.3*	*4.9*
F2/F20		*6.2*	*5.6*	*4.3*	*6.4*	*7.2*	*2.6*	*4.0*
F13/F20		*4.1*	*4.5*	*2.5*	*3.8*	*4.3*	*1.8*	*2.5*
F20/F20		*1.0*	*1.0*	*1.0*	*1.0*	*1.0*	*1.0*	*1.0*

2.5 Edison protocol development with final assay format

2.5.1 Sample treatment condition optimization

Until this stage of assay development, all sample treatment was done "off line" manually and treated samples were loaded into cartridge for Edison run although all incubations were done on Edison. Several Edison protocols were used for early stage development without sample treatment procedure in consideration. In order to transfer sample treatment into Edison protocol, treatment protocol needed to be finalized for creating Edison protocol.

"3-point" clinical samples were still in use as indicators for comparison of sample treatment results. Major reagents in preliminary treatment method were further optimized.

First DTT concentration used in treatment were tested from 10mM to 80mM. Because DTT might also have denaturing effect on other proteins in the system, the effort was to balance between the disassociation of folate with endogenous FBP and the denaturing effect to other "reagent FBP" and capture protein.

Table [SEQ Table * ARABIC]: Comparison of DTT concentration

DTT conc.		**10mM**			**20mM**			**40mM**		
Sample	**Conc.(ng/ml) by SIEMENS**	**Mean RLU**	**%CV**	**%Binding**	**Mean RLU**	**%CV**	**%Binding**	**Mean RLU**	**%CV**	**%Binding**
F4	**3.87**	49868	20	54	47304	16	53	51859	15	68
F10	**6.73**	39579	13	43	34792	11	39	33131	17	43
F17	**24.1**	22070	13	24	18323	20	21	15453	20	20
blank serum	**0**	91570	13	100	88650	19	100	76421	21	100
DTT conc.		**60mM**			**80mM**					

Sample	Conc.(ng/ml) by SIEMENS	Mean RLU	%CV	%Binding	Mean RLU	%CV	%Binding
F4	**3.87**	29480	22	55	27803	27	67
F10	**6.73**	25182	16	47	21627	25	52
F17	**24.1**	9317	21	17	8628	20	21
blank serum	**0**	53654	16	100	41585	17	100

Different lots of biotin-FBP was also tested to compare and to confirm the effect on assay result. Formulation of Biotin-FBP buffer and neutralizer were also done by preliminary stability test.

Table [SEQ Table * ARABIC]: Comparison of Biotin-FBP materials

		B-FBP Lot OK-ii-4B		B-DNP-FBP Lot OK-ii-19-f2		B-FBP Lot Ok-ii-2B		B-FBP new method Lot Ok-ii-121	
Sample	Conc. (ng/ml) by SIEMENS	Mean RLU	%Binding	Mean RLU	%Binding	Mean RLU	%Binding	Mean RLU	%Binding
F3	**2.35**	121040	59	105954	97	113077	67	70948	75
F10	**6.73**	72751	36	61293	56	77567	46	58941	63
F17	**24.1**	37703	18	38584	35	30298	18	36502	39
blank serum	**0**	203842	100	108698	100	167882	100	94230	100

Table [SEQ Table * ARABIC]: Preliminary stability test for Biotin-FBP formulation

	B-FBP working solution	Freshly prepared			2-week old with formulation			2-week old in buffer w/o HSA and sucrose		
Sample	Conc. (ng/ml) by SIEMENS	Mean RLU	%CV	% Binding	Mean RLU	%CV	% Binding	Mean RLU	%CV	% Binding
F4	**3.87**	86487	26	55	95108	15	56	1778	14	70
F10	**6.73**	65978	7	42	77940	10	45	1656	17	65
F17	**24.1**	31354	14	20	35308	20	21	1296	16	51
blank	**0**	158266	16	100	171345	9	100	2553	8	100

2.5.2 Development of sample treatment protocol for Edison

To transfer sample treatment from manual procedure to Edison protocol, some changes were made to be accommodated to Edison cartridge's volume and dilution factors.

All changes made were first verified by manual process and tested at room temperature vs. 37C for verification of optimal results on Edison reader. The final version for "on board" treatment in Edison reader was as the followings:

(1) Sample was mixed with DTT "reductant" to disassociate folate from endogenous FBP
(2) NaOH "extractant" was added to reach pH for deactivation of endogenous FBP
(3) HCl was used as neutralizer to decrease pH of sample mixture
(4) Boric buffer pH9.0 was the sample diluent to further bring pH 9.0 of sample mixture
(5) Biotin-FBP solution was finally added into sample mixture, final sample dilution 1:24
(6) Proceed to reaction with coated reaction tips

A new Edison protocol was created to conduct step by step sample treatment in cartridge. After sample treatment was done, the sample mixture was incubated with HSA-FA coated tips. After washing, the tips were incubated with Streptavidin-AP conjugate and then incubated with AP substrate buffer. RLU was measured after incubation was done.

[SHAPE * MERGEFORMAT]

Figure [SEQ Figure * ARABIC]: Protocol "Folate_treatment_1_24x_1" cartridge layout

Reagent Legend and Position:

Symbol	Location	Description	Volume (µl)
Sub	A1-A4	Substrate	80
WB	B1-B4	Wash Buffer	400
A	F5	Reductant solution	80
B	A7	Extractant solution	80
C	A8	Neutralizer	80
D	F3	Sample Diluent	400
E	F4	FBP solution	30
Conj	D1-D4	AP conjugate	30
BT1, BT2	E8, E7	Blood Tip	2 tips
X	--	Sealed Empty PCR Tube	
S	--	Unsealed Empty PCR Tube	
#1-8		Assay Coated Test Tip Location	

2.5.3 Optimization of assay conditions with Edison protocol

After the sample treatment procedure was fixed with Edison protocol, further optimization was done to optimize reagent conditions. Folic acid and 5mTHF spiked serum samples were used as calibrators for optimization.

Lower tip coating concentration was first tested to increase sensitivity and reduce material consuming. The optimal coating was chosen at 1ug/ml of HSA-FA in coating buffer.

Table [SEQ Table * ARABIC]: Coating concentration optimization

		coating at 2ug/ml			coating at 1ug/ml		
Calibrator	Nominal Conc. (ng/ml)	Mean RLU	%CV	%Binding	Mean RLU	%CV	%Binding
FA	64	7116	12	6	4456	12	6
FA	16	18542	18	16	9039	27	12
FA	4	38092	13	32	29482	22	38
FA	1	72521	15	61	50567	7	65
5m	64	7042	6	6	4982	20	6
5m	16	42393	11	36	27502	32	35
5m	4	84247	24	71	45626	28	59
5m	1	105674	19	89	73364	17	94
blk	0	118698	8	100	77771	23	100

Final pH titration was also done to make sure the sample treatment resulted in optimal folate binding condition on board. Samples spiked with FA and 5mTHF respectively were tested using on board treatment. Because the pH of the sample mixture was not able to be measured after treatment on board, the pH optimization was conducted by adjusting the ratio of NaOH and HCl amount. The objective was to bring the optimal binding affinity for FA and 5mTHF as close as possible. Condition-3 which used 0.5N NaOH and 0.5N HCl showed the best results among three conditions.

Table [SEQ Table * ARABIC]: Results of pH titration

		pH condition 1			pH condition 2			pH condition 3		
Sample	Conc. (ng/ml)	Mean RLU	%CV	% Binding	Mean RLU	%CV	% Binding	Mean RLU	%CV	% Binding
FA	32	3427	19	70	4333	15	13	7964	18	10
FA	8	4349	20	89	9100	19	27	21880	16	29
FA	2	4279	13	88	13542	20	40	46738	23	61
FA	0.5	4517	15	93	25463	36	76	75200	24	99
5m	32	3543	13	73	4692	32	14	8349	7	11
5m	8	3593	25	74	12271	30	37	29921	19	39
5m	2	4541	22	93	24255	13	72	60428	24	79
5m	0.5	4407	19	91	30605	14	91	78038	24	102
blk	0	4865	13	100	33499	12	100	76317	13	100

[SHAPE * MERGEFORMAT]

Figure [SEQ Figure * ARABIC]: Optimal pH condition for sample treatment and binding affinity

2.6 Calibration of folate assay with final protocol on Edison

2.6.1 Calibrator selection

Once the optimal assay condition for each reagent was finalized, calibration was first done by comparison of each calibrator sets with clinical samples. Although FA and 5mTHF binding affinity looked similar at optimal assay condition at this stage, other possible calibrators were also tested with the consideration of potential FA and 5mTHF affinity discrepancy in real sample. FA alone or 5mTHF alone in calibration curve was tested for comparison. With the hope of minimize the affinity difference between FA and 5mTHF, Pteroic Acid was tested as calibration analyte in depleted serum because FA and 5mTHF shared core structure of Pteroic Acid. A set of calibrators was also prepared with FA and 5mTHF in 1:3 ratio of amount to mimic the ratio of two major folate forms in body.

Table [SEQ Table * ARABIC]: Calibrator selection

	FA alone			5mTHF alone			PA alone			FA:5mTHF 1:3		
Conc. (ng/ml)	Mean RLU	%CV	% Binding	Mean RLU	%CV	% Binding	Mean RLU	%CV	% Binding	Mean RLU	%CV	% Binding
64	6445	38	8	5110	9.4	6	31073	28	54	4186	9	6
32	8906	19	11	8861	8.7	11	32664	6	56	8196	20	11
16	15812	25	19	18696	5.5	23	42059	9	73	14502	21	20

8	23351	16	28	34665	5.5	43	47354	22	82	24930	7	34
4	38683	36	46	56371	31.9	69	61353	18	106	41529	3	57
2	55301	20	65	62624	10.9	77	57048	7	98	54923	13	75
1	68824	29	81	71764	15.1	88	61741	29	107	64643	12	89
0.5	75181	20	89	75201	19.3	93	48408	0	84	69653	25	95
0	84516	16	100	81207	16.5	100	57928	16	100	72952	3	100

[SHAPE * MERGEFORMAT]

Figure [SEQ Figure * ARABIC]: Calibrator selection

PA alone calibrators didn't give calculable calibration curve. FA, or 5mTHF, or Folate (FA: 5mTHF=1:3) calibration curve were each calculated to evaluate back calculation accuracy for each calibration point.

Table [SEQ Table * ARABIC]: Back calculation accuracy of each calibration curve

	FA calibration curve				5mTHF calibration curve				Folate (FA:5mTHF 1:3) calibration curve			
Calibrator Conc. (ng/ml)	Mean RLU	%CV	Back Cal conc.	% Accuracy	Mean RLU	%CV	Back Cal conc.	% Accuracy	Mean RLU	%CV	Back Cal conc.	% Accuracy
64	6445	38	66.17	103.4	5110	9	66.00	103.1	4186	22	67.45	105.4
32	8906	19	32.30	100.9	8861	9	25.14	78.6	8196	16	27.34	85.5
16	15812	25	14.48	90.5	18696	6	17.12	107.0	14502	28	17.21	107.6
8	23351	16	9.32	116.5	34665	6	9.44	118.0	24930	29	9.71	121.4
4	38683	36	4.32	107.9	56371	32	2.54	63.6	41529	17	3.66	91.6
2	55301	20	1.81	90.3	62624	11	1.65	82.5	54923	11	1.61	80.7
1	68824	29	0.87	86.6	71764	15	0.86	85.8	64643	10	0.89	88.9
0.5	75181	20	0.61	122.2	75201	19	0.67	133.6	69653	22	0.65	131.0

[SHAPE * MERGEFORMAT]

Figure [SEQ Figure * ARABIC]: Curve regression analysis of each calibrator set

2.6.2 Training set

To confirm the performance of new protocol, and to select the calibration analyte to best represent clinical samples, a set of 20 health donor serum samples was run using new Edison protocol on readers and calculated from each calibration curves at 1-64ng/ml quantification range. Correlation was calculated by comparing the results with folate concentration measured by SIEMEN Immulite folic acid method.

Table [SEQ Table * ARABIC]: Training set of 20 health donor serum samples from Bioreclamation

Serum Sample from Biorec	Conc by SIEMENS (ng/ml)	Mean RLU	%CV	Cal. Conc from FA curve (ng/ml)	Cal. Conc from 5mTHF curve (ng/ml)	Cal. Conc from Folate curve (ng/ml)
F1	**3.42**	41729	4	3.55	6.53	3.48
F2	**2.18**	45970	6	2.92	5.24	2.79
F3	**2.35**	49188	13	2.52	4.41	2.36
F4	**3.87**	33698	22	5.21	9.67	5.35
F5	**2.96**	43132	20	3.33	6.08	3.23
F6	**13.5**	14368	21	16.7	20.6	16.7
F7	**7.19**	24024	32	8.59	14.5	9.19
F8	**3.77**	35025	7	4.88	9.09	4.97
F9	**4.25**	30288	10	6.17	11.3	6.45
F10	**6.73**	32172	9	5.61	10.4	5.81
F11	**5.51**	25020	6	8.13	14.0	8.67
F12	**6.18**	30930	27	5.97	11.0	6.22
F13	**9.19**	21532	5	9.93	16.0	10.6
F14	**7.60**	25161	6	8.07	13.9	8.60
F15	**9.64**	27975	10	6.94	12.4	7.34
F16	**52.2**	6522	14	62.4	42.8	39.5
F17	**24.1**	9506	15	30.4	27.4	24.9
F18	**23.8**	8634	3	35.8	29.9	27.3
F19	**11.0**	26676	25	7.43	13.1	7.89
F20	**45.2**	5617	9	64.0	55.6	43.4

[SHAPE * MERGEFORMAT]

Figure [SEQ Figure * ARABIC]: Calibration curve at 1-64ng/ml quantification range for training set calculation

[SHAPE * MERGEFORMAT]
9-1: Clinical correlation from Theranos FA curve

[SHAPE * MERGEFORMAT]
9-2: Clinical correlation from Theranos 5mTHF curve

[SHAPE * MERGEFORMAT]
9-3: Clinical correlation from Theranos Folate curve

Figure [SEQ Figure * ARABIC]: Clinical correlation between Theranos result and SIEMENS result

Folate concentration calculated from all three calibration curves showed reasonable recovery and clinical correlation. From the consideration of calibration curve accuracy and clinical correlation, Folate calibrators with Folic Acid and 5mTHF at 1:3 ratio of amount was chosen as final calibrators.

2.6.3 Calibration of folate calibrators with SIEMENS method

Folate calibrators with Folic Acid and 5mTHF at 1:3 ratio of amount were then analyzed by SIEMENS Immunlite Folic Acid assay method.

Table [SEQ Table * ARABIC]: Calibration of Folate calibrator with SIEMENS method

Calibrators	Nominal conc. (ng/ml)	Measured by SIEMENS (ng/ml)
1	64	77.8
2	32	36.8
3	16	21.1
4	8	11.1
5	4	4.86
6	2	2.66
7	1	1.4
8	0.5	<1
9	depleted serum	<1

[SHAPE * MERGEFORMAT]

Figure [SEQ Figure * ARABIC]: Calibration of Folate calibrator with SIEMENS method

2.7 Final optimization of assay protocol

2.7.1 Incubation time comparison

After sample treatment procedure was fixed in Edison Folate protocol, other parameters were verified to fine tune the protocol to final version. Reaction times of treated sample incubation with tips, AP conjugate incubation with tips, and substrate incubation were evaluated at 10-10-10, 5-5-5, and 2-2-1 minutes.

Table [SEQ Table * ARABIC]: Comparison of three Edison protocols

		Protocol-1: "10_10_10", 3xPSW			Protocol-2: "5_5_5", 1xPSW			Protocol-3: "2_2_1", 1xPSW		
		"Folate_treatment_1_24x_1"			"Folate_treatment_1_24x_2"			"Folate_treatment_1_24x_3"		
Sample	Conc. (ng/ml)	Mean RLU	%CV	%Binding	Mean RLU	%CV	%Binding	Mean RLU	%CV	%Binding
Folate	64	6445	37.7	8	3286	5.5	6	549	13.5	14
Folate	32	8906	19.4	11	4439	27.4	8	511	11.2	13
Folate	16	15812	24.7	19	8287	4.6	15	766	5.3	19
Folate	8	23351	16.5	28	14176	11.5	25	1106	5.5	27
Folate	4	38683	35.5	46	21270	22.9	38	1873	16.1	46
Folate	2	55301	19.5	65	30644	20.7	55	2422	15.4	60
Folate	1	68824	28.7	81	38398	3.6	69	3420	8.8	85
Folate	0.5	75181	19.6	89	50634	25.7	91	4316	4.5	107
Folate	0	84516	15.7	100	55665	18.4	100	4031	17.2	100

[SHAPE * MERGEFORMAT]

Figure [SEQ Figure * ARABIC]: Comparison of three Edison protocols

Both incubation time "10_10_10" and "5_5_5" gave good signal and modulation. RLU signal of protocol incubation 2_2_1 was too low to have enough resolution for measurement. In order to shorten whole protocol running time, "5_5_5" incubation time was initially preferred.

2.7.2 Trouble shooting of signal losing of "5-5-5"

With "5_5_5" incubation time being selected, final optimization plan was to determine conditions of other reagents which were not verified during early development. However, when tracking reagent effect, RLU signal was shown decreasing over time. Trouble shooting was conducted to find out which reagent(s) caused signal decreasing. During this trouble shooting

experiments, more lots of reagents were tested, and more condition comparison was also done. Table 27 summarized the major factors of trouble shooting experiments.

Table [SEQ Table * ARABIC]: Trouble shooting result summary

Reagent Trouble Shooting						
Protocol	**"Folate_treatment_1_24x_2" ("5-5-5")**					
Variables	Tips	DTT	DTT buffer	NaOH	HCl	Neutralizer
experiments details	compared coating on different dates, no coating conc change	compared working solution prepared on different dates, and with DTT buffer prepared on different dates	freshly prepared lot vs existing lot in use	compared working solution prepared on different dates, and from different lots of stock solutions	compared working solution prepared on different dates, and from different lots of stock solutions	compared two lots
Observation	no significant impact	no significant impact	no significant impact	problem with stock solution	no significant impact	no significant impact

Reagent Trouble Shooting					
Protocol	**"Folate_treatment_1_24x_2" ("5-5-5")**				
Variables	Biotin-FBP	B-FBP buffer	SA-AP	Substrate	Wash buf
experiments details	two lots of Biotin-FBP, compared working solution prepared on different dates, and with buffer prepared on different dates	freshly prepared lot vs existing lot in use	compared two lots of SA-AP conjugate stock and two lots of conjugate buffer	compared two lots	compared two lots
Observation	no significant impact	no significant impact	no significant impact	no significant impact	no significant impact

While trouble shooting was conducted for signal decreasing, most reagents were tested for lot-to-lot variation and preparation variation. Tip coating, DTT solution, Biotin-FBP solution, SA-AP conjugate and substrate showed minimum variation between two lots of materials and between different preparations of working solution. New NaOH 1N stock solution gave signal back to original RLU level.

2.7.3 Re-test training set of clinical samples to confirm "5_5_5" protocol

Using new batch of each reagents, a calibration curve from protocol "Folate_treatment_1_24x_2" ("5_5_5" incubation) was generated and "training set" of 20 clinical samples were re-tested using the same protocol.

Table [SEQ Table * ARABIC]: Calibration curve from protocol "5_5_5"

Calibrator	Conc. (ng/ml)	Mean RLU	%CV	%Binding
1	64	3525	22	3
2	32	6309	12	5
3	16	16842	27	14
4	8	28942	25	25
5	4	43451	9	37
6	2	69614	12	59
7	1	99050	7	84
8	0.5	108494	2	92
9	0	117568	12	100

[SHAPE * MERGEFORMAT]

Figure [SEQ Figure * ARABIC]: Calibration curve from protocol "5_5_5"

Table [SEQ Table * ARABIC]: Training set of clinical samples from protocol "5_5_5"

Sample	Conc by SIEMENS	Mean RLU	%CV	Calc conc. (ng/ml)
F1	3.42	51464	27	3.40
F2	2.18	70083	12	1.84
F3	2.35	61693	12	2.41
F4	3.87	47230	13	3.95
F5	2.96	39551	19	5.26
F6	13.5	23459	22	10.3
F7	7.19	39244	14	5.32
F8	3.77	47731	18	3.88
F9	4.25	36609	15	5.89
F10	6.73	50573	21	3.51
F11	5.51	31254	10	7.31
F12	6.18	32680	22	6.89
F13	9.19	28539	21	8.19

F14	**7.60**	34474	16	6.41
F15	**9.64**	42192	12	4.76
F16	**44.6**	11456	20	20.1
F17	**24.1**	15026	15	15.9
F18	**23.8**	18316	10	13.2
F19	**11.0**	34257	24	6.47
F20	**45.2**	10355	34	21.8

[SHAPE * MERGEFORMAT]

Figure [SEQ Figure * ARABIC]: Correlation of clinical samples from protocol "5_5_5"

Although 20 clinical samples showed good correlation between concentration measured using protocol "5_5_5"and concentration measured by SIEMENS method, overall recovery was much lower than result from protocol "10_10_10". Because this protocol required samples treatment, different matrix might require different response time. Clinical samples seemed to need longer incubation time to reach the same reaction response as to calibrators which were prepared in FA depleted serum. The final decision was to use protocol "10_10_10" with longer incubation time for better clinical correlation.

2.7.4 Finalization of "10_10_10" protocol

In order to finalize protocol "10_10_10", a few more parameters which were not optimized during early development stage were modified and compared to finalize the best conditions.

2.7.4.1 Confirm protocol "10_10_10" with WHO standard

First, to confirm protocol "10_10_10", WHO Standard was used to verify the assay performance besides training set of clinical samples. WHO standard was reconstituted following the instruction and was also diluted 1:2 and 1:4 with depleted serum. A folate calibration curve was created using freshly prepared reagents after reagent trouble shooting using new batches of materials. WHO standard and diluted samples were measured using "10_10_10" protocol. The performance of "10_10_10" was confirmed by acceptable recovery of WHO standard and correlation from diluted samples.

Table [SEQ Table * ARABIC]: Calibration curve from protocol "10_10_10"

Calibrator	Conc. (ng/ml)	Mean RLU	%CV	%Binding	Back cal conc (ng/ml)	%Accuracy
Folate	**64**	9123	29	5	67.2	105
Folate	**32**	20910	18	11	25.8	81
Folate	**16**	28879	36	15	18.9	118
Folate	**8**	53253	15	27	9.3	116
Folate	**4**	88163	23	45	3.9	98

Folate	**2**	124428	22	64	1.7	86
Folate	**1**	151283	12	78	1.0	97
Folate	**0.5**	176232	20	90	0.6	116
Folate	**0**	194823	18	100		

[SHAPE * MERGEFORMAT]

Figure [SEQ Figure * ARABIC]: Calibration curve of protocol "10_10_10"

Table [SEQ Table * ARABIC]: WHO standard measured by protocol "10_10_10"

Sample	Conc. (ng/ml)	Mean RLU	%CV	Back cal conc (ng/ml)	%Accuracy
WHO	**5.33**	84459	15	4.3	80
WHO 1:2	**2.67**	122742	11	1.8	67
WHO 1:4	**1.34**	142278	7	1.2	87

[SHAPE * MERGEFORMAT]

Figure [SEQ Figure * ARABIC]: Correlation of WHO standard and diluted samples

2.7.4.2 Compare coating buffers

HSA-FA was originally prepared in carbonate-bicarbonate coating buffer. PBS was used to compare the coating buffer effect. Although PBS showed lower back ground signal, it gave higher %CV and flatter curve across the range. Carb-Bicarb buffer was continued to use for coating.

Table [SEQ Table * ARABIC]: Comparison of coating buffer

Coating buffer		Carb-Bicarb buffer			PBS		
Calibrator	Conc. (ng/ml)	Mean RLU	%CV	%Binding	Mean RLU	%CV	%Binding
Folate	**64**	9123	29	5	4803	20	3
Folate	**32**	20910	18	11	9700	34	7
Folate	**16**	28879	36	15	19971	41	14
Folate	**8**	53253	15	27	32122	33	22
Folate	**4**	88163	23	45	47952	18	33

Folate	**2**	124428	22	64	63478	3	44
Folate	**1**	151283	12	78	88646	10	62
Folate	**0.5**	176232	20	90	97262	7	68
Folate	**0**	194823	18	100	143459	14	100

[SHAPE * MERGEFORMAT]

Figure [SEQ Figure * ARABIC]: Comparison of coating buffer

2.7.4.3 Use of in house substrate

With in-house substrate buffer ready to use, test was done to compare KPL AP substrate and Theranos in–house substrate and then further optimization was transferred to in-house substrate.

Table [SEQ Table * ARABIC]: Transition from KPL substrate to in-house substrate

Calibrator	Conc . (ng/ml)	Mean RLU	%CV	%Binding	Substrate comparison
Folate	**64**	6978	4	5	KPL PhasphoGLO substrate
Folate	**0.5**	124548	13	82	
Folate	**0**	151884	14	100	
Folate	**64**	11487	7	5	in-house substrate Lot-2
Folate	**0.5**	216152	3	95	
Folate	**0**	228315	12	100	

2.7.4.4 Selection of AP conjugate stabilizer

Using in-house AP substrate, three AP conjugate stabilizers were tested at original SA-AP concentration of 12.5ng/ml. In-house AP buffer gave the highest signal but showing higher background and less sensitivity. Sigma BioStab AP Stabilizer and Surmodics StabilZyme AP Stabilizer showed similar results. StabilZyme had lower background signal and was selected as final AP buffer.

Table [SEQ Table * ARABIC]: Comparison of AP Stabilizers

Stabilizer		BioStab			In-house AP buffer			StabilZyme		
Calibrator	Conc. (ng/ml)	Mean RLU	%CV	% Binding	Mean RLU	%CV	% Binding	Mean RLU	%CV	% Binding
Folate	**64**	10025	21	4	13993	11	4	7274	22	3
Folate	**32**	21036	15	8	33080	15	9	12707	6	5
Folate	**16**	37418	11	14	59616	13	17	20486	14	8

Folate	**8**	68847	27	27	133091	38	38	50003	8	20
Folate	**4**	125017	7	48	188352	9	53	96497	21	38
Folate	**2**	154677	4	60	253159	26	72	135518	10	53
Folate	**1**	189333	2	73	356356	18	101	194175	22	76
Folate	**0.5**	234589	10	91	366851	9	104	242793	10	95
Folate	**0**	258975	11	100	353901	12	100	255268	14	100

[SHAPE * MERGEFORMAT]

Figure [SEQ Figure * ARABIC]: Comparison of AP stabilizer

2.7.4.5 Titration of AP conjugate concentration

Streptavidin-AP conjugate concentration was also further titrated in StabilZyme. The concentrations were tested at 12.5ng/ml, 8ng/ml and 5ng/ml. All three concentrations gave similar results. With consideration of choosing RLU signal modulation and background, and keep the similar ratio with Biotin-FBP amount, 8ng/ml of SA-AP was used as final concentration.

Table [SEQ Table * ARABIC]: Titration of AP concentration

AP concentration		SA-AP 12.5ng/ml			SA-AP 8ng/ml			SA-AP 5ng/ml		
Calibrator	Conc. (ng/ml)	Mean RLU	%CV	% Binding	Mean RLU	%CV	% Binding	Mean RLU	%CV	% Binding
Folate	**64**	10025	21	4	4114	18	3	2938	14	4
Folate	**32**	21036	15	8	8788	8	7	4924	11	6
Folate	**16**	37418	11	14	17895	23	14	8240	15	10
Folate	**8**	68847	27	27	37362	4	29	16407	15	21
Folate	**4**	125017	7	48	54191	5	42	34337	10	43
Folate	**2**	154677	4	60	84805	19	65	45616	7	58
Folate	**1**	189333	2	73	114476	22	88	61987	19	78
Folate	**0.5**	234589	10	91	125461	15	97	70986	20	90
Folate	**0**	258975	11	100	129824	11	100	79124	19	100

[SHAPE * MERGEFORMAT]

Figure [SEQ Figure * ARABIC]: Titration of AP concentration

2.7.4.6 Final calibration (Dexter data)

Calibration curve was generated from final assay conditions and analyzed by Dexter.
[SHAPE * MERGEFORMAT]

Figure [SEQ Figure * ARABIC]: Calibration curve from Dexter analysis

Table [SEQ Table * ARABIC]: Calibration curve parameter from Dexter analysis

Model Type	4PL
Model Equation	RLU = b1+(b2-b1)/(1+(Conc/b3)^b4
Calibration Equation	conc=3.227*(((135843.958)/(RLU-335.348))-1)^(1/1.186)
b1	335.348
b2	135843.958
b3	3.227
b4	1.186
LLOQ	0.5 ng/ml
ULOQ	64 ng/ml
LLOQ accuracy	75%
LLOQ precision	35%
ULOQ accuracy	105%
ULOQ precision	14%

Table [SEQ Table * ARABIC]: Calibration curve back calculation result from Dexter analysis

Calibrator	Conc. (ng/ml)	Mean RLU	%CV	%Binding	Back Cal (ng/ml)	% Accuracy
Folate	**64**	4114	18	3	64.5	101
Folate	**32**	8788	8	7	31.7	99
Folate	**16**	17895	23	14	16.1	100
Folate	**8**	37362	4	29	7.36	92
Folate	**4**	54191	5	42	4.58	115
Folate	**2**	84805	19	65	2.11	106
Folate	**1**	114476	22	88	0.79	79
Folate	**0.5**	125461	15	97	0.40	79
Folate	**0**	129824	11	100		

2.8 Analysis of clinical samples

Clinical samples were analyzed using the final assay protocol and folate concentrations were calculated from calibration curve. Folate concentration results from Theranos method were compared with results from SIEMENS Immulite Folic Acid assay.

2.8.1 Cross reactivity and interference

Methotrexate, Aminopterin, and Folinic Acid are therapeutic drugs which have similar structure of folic acid and literatures reported they might interfere with Folate in assays. Three compounds were spiked into folic acid depleted serum and analyzed by both Theranos method and SIEMENS method to compare the analysis results.

First batch of analysis showed certain cross reactivity levels with three compounds. To rule out any possible preparation issue, a second batch of sample was prepared and analyzed by both Theranos method and SIEMENS methods. Both Aminopterin and Folinic acid showed consistent results from two batches of preparation. The second batch of Methotrexate samples showed no cross reactivity comparing to relatively high cross reactivity in the first preparation. Results from Theranos method tracked with SIEMENS results well. It indicated the cross reactivity of Methotrexate in first preparation might be caused by preparation error. To further confirm the interference effect, 10 clinical samples from patients who were receiving Methotrexate were ordered. Clinical samples were analyzed by both Theranos and SIEMENS methods for folate concentration. The accuracy of Theranos results comparing to SIEMENS results was acceptable. This result showed the interference of Methotrexate presenting to folate assay was minimum.

Table [SEQ Table * ARABIC]: Cross reactivity check using spiked samples

Sample batch-1					
Compound spiked	**Spiking Conc. (ng/ml)**	**MEAN RLU**	**%CV**	**Folate conc from Theranos (ng/ml)**	**Folate conc from SIEMENS (ng/ml)**
Methotrexate	200	18080	20	15.9	10.7
Methotrexate	20	98636	13	1.4	1.02
Methotrexate	2	165067	18	<1	< 1.00
Aminopterin	200	13688	37	20.9	11.1
Aminopterin	20	107504	9	1.1	1.14
Aminopterin	2	130198	17	<1	< 1.00
Folinic Acid	200	44713	12	5.9	4.14

Folinic Acid	20	97309	12	1.5	< 1.00
Folinic Acid	2	71090	24	3.0	4.62

Sample batch-2					
Compound spiked	**Spiking Conc. (ng/ml)**	**MEAN RLU**	**%CV**	**Folate conc from Theranos (ng/ml)**	**Folate conc from SIEMENS (ng/ml)**
Methotrexate	200	127440	8	<1	< 1.00
Methotrexate	20	153416	6	<1	< 1.00
Methotrexate	2	119329	16	<1	< 1.00
Aminopterin	200	83473	42	2.2	< 1.00
Aminopterin	20	94770	37	1.6	< 1.00
Aminopterin	2	121837	13	<1	< 1.00
Folinic Acid	200	66241	5	3.4	2.07
Folinic Acid	20	127822	26	<1	< 1.00
Folinic Acid	2	130791	8	<1	< 1.00

Table [SEQ Table * ARABIC]: Folate assay results of patients having Methotrexate medication

Sample #	**Bioreclamation Lot#**	**UID #**	**Medication information**	**Folate measured by SIEMENS (ng/ml)**	**Folate measured by Theranos (ng/ml)**	**%Accuracy (Theranos/SIEMENS)**
1	614356	26435	Actonel; Methotrexate	8.39	7.45	89
2	614357	25256	Methotrexate; Fosamax	12.6	11	87
3	614358	71705	Synthroid 5mg,Prednisone 5mg,Methotrexate 2.5mg,Leucovorin	18.6	13.66	73

4	614359	60863	Nexium, Prednisone, Methotrexate, B12, Glucovance	OORH*	OORH*	
5	614360	94454	Symbicort, Remicade, Methotrexate, Nasonex	OORH*	OORH*	
6	614361	89401	ASA, Metoprolol, Nasonex, Remicade, Zyrtec, Methotrexate, Aciphex, Crestor	12.4	14.06	113
7	614362	87856	Enbrel, Methotrexate, Loucovorin, Levothyroxine, Propanolol, ASA	11.9	9.16	77
8	614363	37648	Methotrexate; Sulfazine; Vitamin D; Potassium; Vitamin B12; ASA etc.	13.8	11.76	85
9	614364	96323	Arimidex, Cozaar, Calcium, Vitamin D,Methotrexate 15mg	9.9	11.69	118
10	614365	38105	Nexium; Rituxan; Methotrexate; Naprosyn	9.23	10.05	109

**OORH: concentration of folate was higher than upper limit of quantification range*

2.8.2 Matrix effect

Folate was spiked into Lipemic and Icteric plasma at three different levels and spiked samples were analyzed by Theranos method. Recovery was calculated as measured value vs. nominal value. Most of spiked samples had recovery within 80-120% range in both matrix. Lipemic and Icteric plasma showed no significant effect on folate measurement. Hemolyzed plasma was not evaluated because of high level of folate in red blood cell.

Table [SEQ Table * ARABIC]: Spike recovery in lipemic and icteric matrix

Lipemic samples:

Blank (ng/ml)	**Spiking conc. (ng/ml)**	**Nominal conc. (ng/ml)**	**Mean RLU**	**%CV**	**Measured conc. (ng/ml)**	**Recovery (%)**

11.5	32	43.5	4735	5	56.5	129.9
	8	19.5	14007	18	20.4	104.6
	2	13.5	21332	16	13.5	100.0

Icteric samples:

Blank (ng/ml)	Spiking conc. (ng/ml)	Nominal conc. (ng/ml)	Mean RLU	%CV	Measured conc. (ng/ml)	Recovery (%)
13.4	32	45.4	5514	2	49	107.9
	8	21.4	12767	18	22.3	104.2
	2	15.4	19011	29	15.1	98.1

2.8.3 Paired Samples from healthy donors

Samples of twenty healthy donors (10 male, 10 female) were collected in pairs of serum, EDTA plasma and Heparin plasma. All samples were analyzed by Theranos method and SIEMENS method. To evaluate the matrix differences, results from plasma were compared with results from serum. The difference between plasma vs. serum might be caused by different amount of red blood cells in plasma separation. The correlation between Theranos results and SIEMENS results was reasonable for all three matrix. It was indicated that Theranos method was compatible to analyze all serum, EDTA plasma and Heparin plasma.

Table [SEQ Table * ARABIC]: Folate concentration of paired samples from Stanford Blood Center healthy donors

		Serum		EDTA-plasam		Heparin-plasma	
Sample ID	Unit # (from Blood Center)	Theranos Result (ng/ml)	SIEMENS Result (ng/ml)	Theranos Result (ng/ml)	SIEMENS Result (ng/ml)	Theranos Result (ng/ml)	SIEMENS Result (ng/ml)
F1	W070512100991	14.1	17.6	15.2	18.1	16.0	23.6
F2	W070512100992	21.7	23.7	22.9	20.1	14.3	22.1
F3	W070512201079	47.5	42.9	43.7	42.1	46.6	49.5
F4	W070512201080	21.8	25.4	28.4	21.1	28.8	24.3
F5	W070512201083	32.1	40.3	31.1	34.7	44.4	46.0
F6	W070512101024	13.1	14.1	15.2	12.7	15.4	14.5
F7	W070512101028	37.7	39.1	33.6	27.6	23.3	24.0
F8	W070512101029	16.6	22.9	21.1	22.5	21.3	21.8
F9	W070512101131	20.5	23.2	15.3	19.6	18.7	23.6
F10	W070512101134	18.0	21.8	14.8	18.0	18.2	26.1
M1	W070512100987	17.6	22.9	13.0	16.0	17.5	20.6
M2	W070512100988	13.3	11.2	11.0	12.6	15.2	15.4

M3	**W070512100989**	10.4	11.7	11.3	10.2	12.1	15.5
M4	**W070512100990**	16.2	17.9	9.7	13.2	12.5	15.7
M5	**W070512100993**	15.6	19.9	11.5	14.0	16.4	19.1
M6	**W070512201081**	30.4	32.5	18.0	21.9	21.7	34.7
M7	**W070512201082**	42.9	42.7	33.9	38.3	49.6	51.2
M8	**W070512201084**	13.7	11.6	7.8	10.2	11.1	13.3
M9	**W070512201085**	22.4	28.3	17.7	19.2	14.3	23.7
M10	**W070512201086**	14.1	14.7	11.3	10.2	13.0	13.1

[SHAPE * MERGEFORMAT]

Figure [SEQ Figure * ARABIC]: Correlation of folate concentration in healthy donor serum samples measured by Theranos method and SIEMENS method

[SHAPE * MERGEFORMAT]

Figure [SEQ Figure * ARABIC]: Correlation of folate concentration in healthy donor EDTA plasma samples measured by Theranos method and SIEMENS method

[SHAPE * MERGEFORMAT]

Figure [SEQ Figure * ARABIC]: Correlation of folate concentration in healthy donor heparin plasma samples measured by Theranos method and SIEMENS method

[SHAPE * MERGEFORMAT]

Figure [SEQ Figure * ARABIC]: Theranos result comparison: EDTA plasma vs. serum

[SHAPE * MERGEFORMAT]

Figure [SEQ Figure * ARABIC]: Theranos result comparison: Heparin plasma vs. serum

2.8.4 Serum samples from pregnancy women donors

Ten serum samples from pregnant women were obtained from Bioreclamation and were analyzed by both Theranos method and SIEMENS methods. Reasonable correlation was seen with most samples except one possible outlier.

Table [SEQ Table * ARABIC]: Folate concentration of serum samples from pregnancy women

Samples ID	Bioreclamation Lot#	THERANOS result (ng/ml)	SIEMENS result (ng/ml)
S11	BRH468993	13.6	11.6
S12	BRH468994	10.7	8.1
S13	BRH468995	13.8	6.2

S14	BRH468996	9.7	6.7
S15	BRH468997	12.1	10.5
S16	BRH468998	16.4	12.3
S17	BRH468999	47.1	19.9
S18	BRH469000	33.0	29.2
S19	BRH469001	11.5	10.1
S20	BRH469002	7.1	6.1

[SHAPE * MERGEFORMAT]

Figure [SEQ Figure * ARABIC]: Correlation of folate concentration in pregnant women serum samples

[SHAPE * MERGEFORMAT]

Figure [SEQ Figure * ARABIC]: Correlation of folate concentration in pregnant women serum samples with one possible outlier removed

2.8.5 Serum samples from Anemia patient

Twenty serum samples from Anemia patients were obtained from Bioreclamation and were analyzed for folate concentration by both Theranos and SIEMENS methods. Folate results from two methods correlated well. Low level of folate was expected in Anemia patient samples. However, this batch of samples had folate in normal range. All the patients were taking medication for Anemia while serum samples were collected.

Table [SEQ Table * ARABIC]: Folate concentration of serum samples from Anemia patients

Sample ID	Bioreclamation Lot #	Theranos result (ng/ml)	SIEMENS result (ng/ml)
B1	BRH570442	6.8	9.5
B2	BRH570443	51.3	42.5
B3	BRH570444	16.8	17.6
B4	BRH570445	12.6	17.9
B5	BRH570446	17.6	17.8
B6	BRH570447	101.5*	89.0*
B7	BRH570448	51.0	30.5*
B8	BRH570449	18.7	15.1
B9	BRH570450	55.8	71.0*
B10	BRH570451	12.1	8.5

B11	BRH570452	18.9	23.4
B12	BRH570453	21.8	22.3
B13	BRH570454	8.2	6.3
B14	BRH570455	26.2	17.4
B15	BRH570456	17.9	10.1
B16	BRH570457	15.0	9.6
B17	BRH570458	15.3	8.6
B18	BRH570459	17.4	10.0
B19	BRH570460	27.8	26.2
B20	BRH570461	10.7	9.1

**Samples had folate concentration out of the calibration range of both assays. Results from re-analysis at 1:5 dilution*

[SHAPE * MERGEFORMAT]

Figure [SEQ Figure * ARABIC]: Correlation of folate concentration in Anemia patient serum samples

2.8.6 HAMA and RF positive samples

Six RF positive serum samples and six HAMA positive serum samples from ProMedDx were analyzed by both Theranos and SIEMENS methods. Because some samples showed very low concentration of folate, correlation was not calculated. The comparison of two methods was conducted by calculation accuracy of Theranos result vs. SIEMENS result.

Table [SEQ Table * ARABIC]: Folate concentration of RF positive serum samples

Sample ID	ProMedDx Barcode #	SIEMENS result (ng/ml)	Theranos result (ng/ml)	% Accuracy
R11	2047139	3.38	3.7	110.6
R15	2046911	4.83	4.8	100.4
R16	2047135	17.6	26.9	152.9
R17	2047094	6.61	6.4	96.9
R24	2046859	2.99	4.0	132.4
R25	2046938	7.55	8.6	113.8

Table [SEQ Table * ARABIC]: Folate concentration of HAMA positive serum samples

Sample ID	ProMedDx Barcode #	SIEMENS result (ng/ml)	Theranos result (ng/ml)	% Accuracy
H14	1291446	2.47	3.52	142.6
H15	1291482	6.01	4.86	80.8
H16	1291411	<1	1.18	

H17	1291421	2.25	2.50	111.2
H18	1291433	<1	1.14	
H19	1291448	2.17	2.60	119.7

2.8.7 Bio Rad controls

A few clinical quality control samples from Bio Rad were also analyzed to confirm assay performance. Folate results from Theranos method were tracking well with data provided by Bio Rad.

Table [SEQ Table * ARABIC]: Analysis of quality control samples

Sample ID	Bio Rad Cat#	Lot#	Bio Rad result by SIEMENS Immulite 2000	Theranos result
Bio Rad Level 1	Lyphocheck Immunoassay Plus control 370X	40250	2.55-3.83 ng/ml	2.3 ng/ml
Bio Rad Level 2	Lyphocheck Immunoassay Plus control 370X	40250	5.89-8.83 ng/ml	7.8 ng/ml
Bio Rad Level 3	Lyphocheck Immunoassay Plus control 370X	40250	11.8-17.8 ng/ml	12.4 ng/ml
Bio Rad Anemia control	Lyphocheck Anemia control 500X	43190	<1 ng/ml	0.7 ng/ml

2.9 Stability

2.9.1 Stability monitoring

Reagents stability monitoring was done with all reagents and coated tips stored at 4C for 12 weeks. Reagents evaluation and formulation development will be conducted to increase reagent stability.

Table [SEQ Table * ARABIC]: Reagent stability monitoring

		week 0				week 2			
Calibrator	Nominal conc (ng/ml)	RLU	%CV	Back calculation	%Accuracy	RLU	%CV	Back calculation	%Accuracy
1	32	8788	8.3	30.7	95.9	7794	9.2	35.5	110.9
2	8	37362	4.0	7.87	98.4	28565	13.1	9.9	124.3
3	2	84805	19.1	1.83	91.5	82755	6.0	2.2	111.4

4	blank	129824	11.1			119132	8.9		
		week 4				**week 8**			
Calibrator	**Nominal conc (ng/ml)**	**RLU**	**%CV**	**Back calculation**	**%Accuracy**	**RLU**	**%CV**	**Back calculation**	**%Accuracy**
1	32	7169	22.8	38.3	119.8	9126	14.4	30.6	95.7
2	8	32572	18.2	8.6	107.7	24371	11.7	11.8	147.1
3	2	78301	23.8	2.5	124.9	64818	9.5	3.5	175.1
4	blank	119420	8.1			106822	1.1		
		week 12							
Calibrator	**Nominal conc (ng/ml)**	**RLU**	**%CV**	**Back calculation**	**%Accuracy**				
1	32	6266	19.6	43.5	135.8				
2	8	18184	10.4	15.8	197.8				
3	2	41095	5.4	6.57	328.6				
4	blank	91397	16.9						

2.9.2 Reagent optimization for improving stability (experiments conducted by Darren Crandall)

In order to improve reagents stability, new concentration and formulations were tested. HSA-FA coating concentration was increased to 2.5ug/ml or 5ug/ml. Biotin-FBP working solution was prepared with or without 10% Glycerol for comparison at each coating concentration levels. A new round of 12 weeks stability monitoring was conducted for all four new conditions.

After 12 weeks, coating at 2.5ug/ml gave quite stability RLU signal. Adding glycerol to FBP solution didn't show significant difference.

Table [SEQ Table * ARABIC]: Stability monitoring with new reagent conditions

		Tip Coating Concentration: 2.5 ug/mL FBP Solution: 250ng/mL w/out Glycerol						
		Week 0			**Week 2**			
Calibrator	Nominal conc (ng/ml)	Mean RLU	%CV	S/B Modulation	Mean RLU	%CV	S/B Modulation	% Signal Change
1	32	**24886**	15.9%	9.9	**35715**	13.6%	8.6	43.5
2	8	**139604**	15.8%	1.8	**138080**	17.6%	2.2	-1.1
3	2	**200678**	5.2%	1.2	**223459**	23.2%	1.4	11.4
4	0	**245291**	7.0%		**307300**	30.3%		25.3
		Week 4			**Week 6**			

Calibrator	Nominal conc (ng/ml)	Mean RLU	%CV	S/B Modulation	% Signal Change	Mean RLU	%CV	S/B Modulation	% Signal Change
1	32	**15249**	29.7%	13.8	-38.7	**20613**	18.4%	10.4	-17.2
2	8	**78012**	13.9%	2.7	-44.1	**89518**	15.1%	2.4	-35.9
3	2	**138867**	36.9%	1.5	-30.8	**142969**	36.3%	1.5	-28.8
4	0	**210339**	25.3%		-14.2	**215304**	19.0%		-12.2
		Week 8				**Week 12**			
Calibrator	Nominal conc (ng/ml)	Mean RLU	%CV	S/B Modulation	% Signal Change	Mean RLU	%CV	S/B Modulation	% Signal Change
1	32	**27557**	14.8%	9.6	10.7	**26802**	19.0%	7.6	7.7
2	8	**126213**	14.8%	2.1	-9.6	**88117**	28.9%	2.3	-36.9
3	2	**149767**	16.1%	1.8	-25.4	**144693**	15.5%	1.4	-27.9
4	0	**264543**	20.9%		7.8	**204030**	40.9%		-16.8

		Tip Coating Concentration: 2.5 ug/mL FBP Solution: 250ng/mL w/ 10% Glycerol							
		Week 0				**Week 2**			
Calibrator	Nominal conc (ng/ml)	Mean RLU	%CV	S/B Modulation		Mean RLU	%CV	S/B Modulation	% Signal Change
1	32	**22765**	13.9%	13.2		**28555**	12.8%	14.6	25.4
2	8	**176979**	14.0%	1.7		**119750**	6.2%	3.5	-32.3
3	2	**289391**	12.2%	1.0		**272070**	13.9%	1.5	-6.0
4	0	**300908**	11.7%			**417697**	11.7%		38.8
		Week 4				**Week 6**			
Calibrator	Nominal conc (ng/ml)	Mean RLU	%CV	S/B Modulation	% Signal Change	Mean RLU	%CV	S/B Modulation	% Signal Change
1	32	**20831**	36.5%	11.1	-8.5	**22843**	39.5%	13.7	0.3
2	8	**104009**	17.0%	2.2	-41.2	**138076**	26.4%	2.3	-22.0
3	2	**155146**	23.4%	1.5	-46.4	**207638**	33.5%	1.5	-28.2
4	0	**231186**	11.8%		-23.2	**312962**	19.8%		4.0
		Week 8				**Week 12**			
Calibrator	Nominal conc (ng/ml)	Mean RLU	%CV	S/B Modulation	% Signal Change	Mean RLU	%CV	S/B Modulation	% Signal Change
1	32	**23631**	18.6%	12.7	3.8	**47706**	11.7%	9.1	109.6
2	8	**188471**	20.6%	1.6	6.5	**195312**	36.2%	2.2	10.4

3	2	**289637**	12.6%	1.0	0.1	**348787**	16.0%	1.2	20.5
4	0	**300544**	29.4%		-0.1	**433364**	10.4%		44.0

		TipCoating Concentration: 5 ug/mL							
		FBP Solution: 250ng/mL w/out Glycerol							
		Week 0				**Week 2**			
Calibrator	Nominal conc (ng/ml)	Mean RLU	%CV	S/B Modulation		Mean RLU	%CV	S/B Modulation	% Signal Change
1	32	**13840**	18.6%	10.6		**12148**	8.2%	13.2	-12.2
2	8	**58570**	17.4%	2.5		**42352**	18.2%	3.8	-27.7
3	2	**88007**	20.1%	1.7		**74963**	16.9%	2.1	-14.8
4	0	**146622**	15.1%			**160545**	14.8%		9.5
		Week 4				**Week 6**			
Calibrator	Nominal conc (ng/ml)	Mean RLU	%CV	S/B Modulation	% Signal Change	Mean RLU	%CV	S/B Modulation	% Signal Change
1	32	**24425**	59.7%	7.1	76.5	**21491**	0.3%	8.6	55.3
2	8	**35059**	17.8%	4.9	-40.1	**47300**	30.8%	3.9	-19.2
3	2	**75494**	2.0%	2.3	-14.2	**153896**	17.4%	1.2	74.9
4	0	**173358**			18.2	**183834**	32.6%		25.4
		Week 8				**Week 12**			
Calibrator	Nominal conc (ng/ml)	Mean RLU	%CV	S/B Modulation	% Signal Change	Mean RLU	%CV	S/B Modulation	% Signal Change
1	32	**23521**	19.8%	9.1	70.0	**25914**	23.7%	7.6	87.2
2	8	**86207**	14.6%	2.5	47.2	**93363**	19.3%	2.1	59.4
3	2	**131112**	33.9%	1.6	49.0	**94470**	30.4%	2.1	7.3
4	0	**213041**	13.1%		45.3	**197451**	14.5%		34.7

		Tip Coating Concentration: 5 ug/mL						
		FBP Solution: 250ng/mL w/ 10% Glycerol						
		Week 0				**Week 2**		
Calibrator	Nominal conc (ng/ml)	Mean RLU	%CV	S/B Modulation	Mean RLU	%CV	S/B Modulation	% Signal Change
1	32	**12297**	9.1%	19.5	**10875**	19.9%	22.7	-11.6
2	8	**86970**	61.0%	2.8	**62531**	15.2%	3.9	-28.1
3	2	**139098**	23.0%	1.7	**147394**	13.9%	1.7	6.0
4	0	**240293**	33.2%		**246485**	33.5%		2.6

		Week 4				Week 6			
Calibrator	Nominal conc (ng/ml)	Mean RLU	%CV	S/B Modulation	% Signal Change	Mean RLU	%CV	S/B Modulation	% Signal Change
1	32	**10294**	35.8%	22.8	-16.3	**21614**	62.8%	10.1	75.8
2	8	**83908**	48.0%	2.8	-3.5	**103066**	58.1%	2.1	18.5
3	2	**114932**	16.5%	2.0	-17.4	**160108**	47.8%	1.4	15.1
4	0	**234218**	44.2%		-2.5	**217625**	38.2%		-9.4
		Week 8				**Week 12**			
Calibrator	Nominal conc (ng/ml)	Mean RLU	%CV	S/B Modulation	% Signal Change	Mean RLU	%CV	S/B Modulation	% Signal Change
1	32	**21609**	8.9%	13.5	75.7	**48420**	9.5%	7.5	293.8
2	8	**163717**	20.7%	1.8	88.2	**185788**	27.7%	2.0	113.6
3	2	**220979**	18.5%	1.3	58.9	**273743**	20.8%	1.3	96.8
4	0	**291130**	3.7%		21.2	**364676**	18.1%		51.8

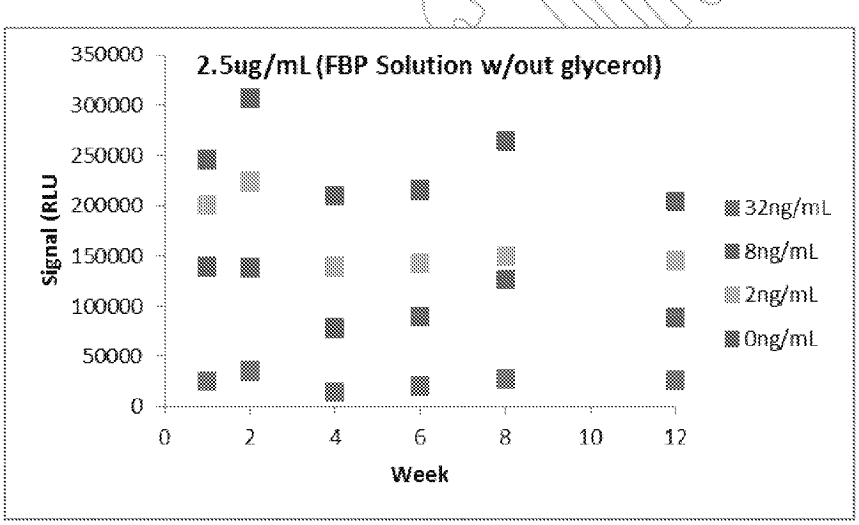

Figure [SEQ Figure * ARABIC]: 12-week's stability with coating at 2.5ug/ml

2.9.3 Calibration curve at new reagent condition

A calibration curve was generated at the new condition of 2.5ug/ml coating concentration and using Biotin-FBP original formulation after 12-week stability was done. All reagents used for calibration curve were prepared 12 weeks ago for Stability test. The calibration curve reflected the overall reagent stability for 12 weeks. Satisfied modulation and sensitivity were achieved.

Table [SEQ Table * ARABIC]: Calibration curve at new reagent condition

Calibrator	Nominal FA Conc. (ng/mL)	Mean RLU	%CV	Modulation	Back cal conc (ng/ml)	% Recovery
1	64.0	9378	0.10	25.05	63.91	99.86
2	32.0	24478	0.12	9.60	30.67	95.84
3	16.0	40967	0.17	5.73	17.76	110.99
4	8.0	70716	0.10	3.32	7.32	91.54
5	4.0	98068	0.10	2.40	3.60	89.94
6	2.0	114780	0.14	2.05	2.13	106.27
7	1.0	153904	0.10	1.53	1.01	100.78
8	0.5	188261	0.07	1.25	0.50	99.42
9	0.0	234902	0.06	1		

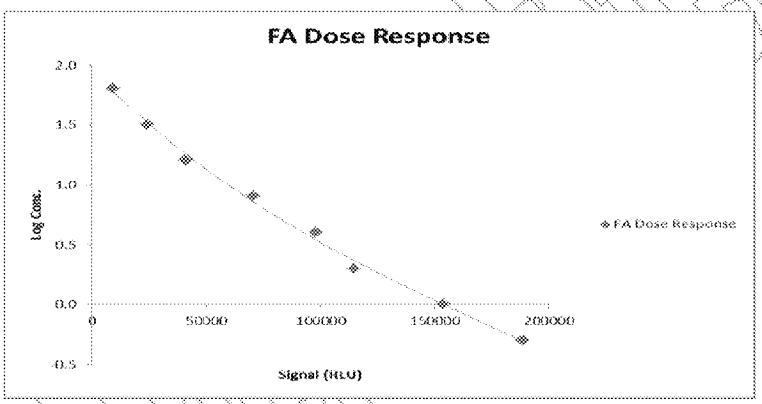

Figure [SEQ Figure * ARABIC]: Calibration curve at new reagent condition

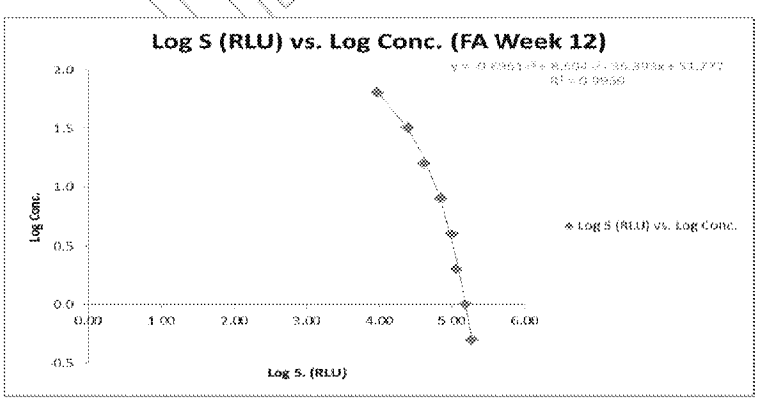

Figure [SEQ Figure * ARABIC]: Calculation of new calibration curve

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