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Flu B
Assay Development Report

Theranos, Inc.

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

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

This assay is designed to qualitatively determine the presence of Influenza B nucleoprotein antigens in a nasal swab. The test is intended to aid in the diagnosis of Influenza B (Flu B) viral infections. Negative tests should be confirmed by cell culture.

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

The following commercial rapid test kit has been used in house as predicate methods:
Remel X/pect Flu A & B

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

A biotin-labeled anti-Influenza B antibody coated on an avidin surface serves as the capture surface for the sandwich ELISA. The nasal swab is subjected to an extraction process. This extracted material is then mixed with alkaline phosphatase-labeled anti-influenza B antibody. This mixture is then incubated with capture surface for 5 minutes. After the incubation, the surface is washed and the alkaline phosphatase substrate is incubated on the surface for 5 minutes, and then the resulting chemiluminescence is read in Relative Light Units (RLU).

Table [SEQ Table * ARABIC]: Materials

Name	**Supplier**	**Catalog #**
Carbonate-bicarbonate buffer	Sigma	C3041
Alkaline Phosphatase Labeling Kit (SH)	Dojindo	LK13
Biotin Labeling Kit (SH)	Dojindo	LK10
Capture Antibody	US Biological	I7650-01L
Detection Antibody	US Biological	I7650-16D
Phospho Glo Substrate	In House	n/a

Table [SEQ Table * ARABIC]: Antigen List

ID#	Vendor	Catalog
1	Southern Biotech	10885-01
2	US biological	I7651-01W
3	US biological	I7651-53C
4	US biological	I7651-01X
5	US biological	I7651-01L
6	US biological	I7651-01S
7	US biological	I7651-01K
8	US biological	I7651-16D
9	US biological	I7651-01Q
10	US biological	I7651-16C
11	US biological	I7651-01V
12	US biological	I7651-01R

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

2.1 Capture Surface: Antigen Screen on MTP

To determine the optimal pair for the Flu B antibody ELISA, all combinations of 12 Flu A antibodies were tested on a microtitre plate. The screening was performed using 5 ug/mL of CAb and 100 ng/mL of detection antibody in blocking buffer and a post sample wash. Flu B nucleoprotein calibrators and Flu A nucleoprotein calibrators were used to test for both modulation and specificity. No further sample dilution was performed.

Table [SEQ Table * ARABIC]: Summary of Antibody Screening Results

	DAb #											
Cab #	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
1												
2												
3												
4												
5												
6												
7												
8												
9												
10												
11												
12												

	Bad Response	Less than 200 fold and/or Cross Reactivity issues
	Fair Response	>200 with Low Cross Reactivity
	Good Response	>500 with Low Cross Reactivity

2.2 Extraction Buffer

To determine the optimal extraction buffer, different formulations were tested and compared to the Remel Xpect kit extraction buffer. The extraction buffer used for the Flu A assay is the same buffer that will be used for the Flu B assay. Antibody #3 was used as the capture antibody while antibody #8 was used as the detection antibody for this test. The conditions included 5 ug/mL of CAb and 100 ng/mL(final) of detection antibody in blocking buffer. Positive and Negative controls were obtained from either Microbix or the Virusys kit controls. Our extraction buffer consisted of 0.5% Tween 20, 0.1% sodium azide in 20mM phosphate buffer (pH 7.6). A volume of 50ul of sample was spiked in 400ul of extraction buffer for this experiment.

Table [SEQ Table * ARABIC]: Extraction Buffer evaluation

Extraction Process	Vendor	Sample	Type	Mean RLU	CV%	Modulation
Our Extraction buffer	Virusys	Pos	Flu B	89338	5.6	58
	Microbix	Pos	Flu B	87536	28.6	56
	Virusys	Neg	Flu B	1551	24.0	
Remel FDA Kit	Virusys	Pos	Flu B	5066	15.7	8.5
	Microbix	Pos	Flu B	15293	17.8	25.7
	Virusys	Neg	Flu B	594	24.3	

2.3 Antibody Screen on the Theranos system

The best pairs from the MTP Screen were evaluated on the Theranos system. The extraction buffer consisted of 0.5% Tween 20, 0.1% sodium azide in 20mM phosphate buffer (pH 7.6). A volume of 50ul of sample was spiked in 400ul of extraction buffer for this experiment. The conditions included 5 ug/mL of CAb and 100 ng/mL(final) of detection antibody in blocking buffer. Positive and Negative controls were obtained from either Microbix or the Virusys kit controls. The best pair was 5B-8B which has the best modulation and least cross reactivity.

Table [SEQ Table * ARABIC]: Antibody Screen on the Theranos system

CAb-DAb	Vendor	Sample Type	Sample	Mean RLU	CV%	Modulation
3B-8B	Virusys	Pos	Flu B	62644	14.3	51.5
	Microbix	Pos	Flu B	67612	16.4	55.6
	Virusys	Neg	Flu B	1215	9.5	
	Microbix	Pos	Flu A	5538	27.0	4.6
2B-5B	Virusys	Pos	Flu B	1507	9.9	2.0
	Microbix	Pos	Flu B	6852	9.1	9.3
	Virusys	Neg	Flu B	736	25.3	
	Microbix	Pos	Flu A	2176	32.2	3.0
4B-5B	Virusys	Pos	Flu B	3481	18.4	2.8
	Microbix	Pos	Flu B	7069	18.5	5.6
	Virusys	Neg	Flu B	1255	32.4	
	Microbix	Pos	Flu A	3047	30.5	2.4
8B-13B	Virusys	Pos	Flu B	1433	28.6	1.6
	Microbix	Pos	Flu B	5303	2.2	5.7
	Virusys	Neg	Flu B	924	23.3	
	Microbix	Pos	Flu A	3484	10.4	3.8
5B-8B	Virusys	Pos	Flu B	86214	26.4	63.8
	Microbix	Pos	Flu B	81412	2.8	60.2
	Virusys	Neg	Flu B	1352	10.2	
	Microbix	Pos	Flu A	2707	21.6	2.0

2.4 Capture Surface Titration on the Theranos Syste

The capture surface was titrated at the following concentrations: 10ug/ml, 5ug/ml, and 1ug/ml. Controls from the Virusys kit and Microbix were used for this screening. The blank refers to the background control when no sample (only blocking buffer) is added. The detection antibody is maintained at 100ng/ml final in regular 3% BSA blocking buffer. The optimal capture antibody concentration which was determined to be 5ug/ml.

Table [SEQ Table * ARABIC]: Capture Surface Titration-Theranos system

[Cab] ug/ml	Sample	Type	Mean RLU	CV%	Modulation
10ug/ml	Positive- Flu A	Microbix	103328	9.1	66.4
	Negative	Virusys-Flu B	1556	12.7	
	Negative	Blank	3725	11.8	
5ug/ml	Positive- Flu A	Microbix	90390	10.0	60.1
	Negative	Virusys-Flu B	1504	23.0	
	Negative	Blank	3347	16.0	
2.5ug/ml	Positive- Flu A	Microbix	62865	18.3	35.8
	Negative	Virusys-Flu B	1754	23.2	
	Negative	Blank	3135	19.9	

2.5 Alkaline Phosphatase Stabilizer

Two alkaline phosphatase stabilizers were tested as detection antibody (DAb) diluents. The In House AP stabilizer is prepared by adding 0.1mM zinc chloride and 5mM magnesium chloride to the 3% BSA blocking buffer. Both In House AP Stabilizer and Stabilzyme AP worked well. Here, 50ul of sample is added to 500ul of our extraction buffer. The capture antibody was at 5ug/ml while detection antibody was maintained at 100ng/ml final after protocol run.

Table [SEQ Table * ARABIC]: Alkaline Phosphatase Stabilizer

Stabilizer	Sample Type	Sample	Mean RLU	CV%	Modulation
In House AP Stabilizer	Microbix	Flu B Pos	90390	10.0	60.1
	Virusys	Flu B Neg	1504	23.0	
	Blank	Blocking Buffer	3347	16.0	
Biostab	Blank	Flu B Pos	81223	17.1	44.5
	Positive	Flu B Neg	1827	26.5	
	Neg	Blocking Buffer	2083	8.0	
Stabilzyme AP	Blank	Flu B Pos	115032	3.2	59.3
	Positive	Flu B Neg	1940	21.1	
	Neg	Blocking Buffer	2396	7.5	

2.6 Detection Antibody Titration

The AP conjugated detection antibodies were titrated in In House AP Stabilizer. The best modulation between the positive and negative controls was observed at 50ng/ml final. The positive controls were from Microbix and Virusys.

Table [SEQ Table * ARABIC]: Detection Antibody Titration

Final [DAb], ng/ml	Sample Type	Type	Mean RLU	CV%	Mod
150	Microbix	Flu B Pos	81726	5.3	36.0
	Virusys	Flu B Neg	2272	23.6	
	Blank	Blocking Buffer	4470	14.5	
100	Microbix	Flu B Pos	90390	10.0	60.1
	Virusys	Flu B Neg	1504	23.0	
	Blank	Blocking Buffer	3347	16.0	
50	Microbix	Flu B Pos	44003	8.9	40.0
	Virusys	Flu B Neg	1101	7.8	
	Blank	Blocking Buffer	1351	8.8	

2.7 Specificity tests on the Theranos System

Specificity and cross reactivity studies were performed on the Theranos system. All these cross reactants listed in the table did not show any cross reactivity with the Flu B assay. Briefly, capture was at 5ug/ml while detection was maintained at 100 ng/ml final after protocol run.

Table [SEQ Table * ARABIC]: Specificity Tests on the Theranos System

Type	Sample	Mean RLU	CV%
Microbix CTL	Flu B Pos	120127	11.7
Virusys CTL	Flu B Pos	95127	12.1
	Mean Positive	107627	
Negative CTL	Negative Flu B Virusys CTL	1965	18.3
Cross Reactant	Parainfluenza 1	1257	19.3
Cross Reactant	Parainfluenza 2	1509	19.3
Cross Reactant	Parainfluenza 3	1496	5.4
Cross Reactant	Adenovirus	1169	23.8
Cross Reactant	M. Pneumoniae	1979	6.5
Cross Reactant	Respiratory Syncytial Virus	1313	25.1
Cross Reactant	Corynebacterium diptheriae	3081	22.0
Cross Reactant	Streptococcus pyrogenes	4388	24.8
Cross Reactant	Streptococcus pneumoniae	6902	25.5
Cross Reactant	CMV	534	11.5
Cross Reactant	N.meningitis	3455	14.8
Cross Reactant	Epstein Barr Virus	1938	8.2
Cross Reactant	Measles	1710	23.6
Cross Reactant	Mumps	2423	10.0
Cross Reactant	E.coli	2291	9.2
	Mean RLU of cross reactants	2363	
	Modulation	45.5	

2.8 Clinical evaluation of the Flu B assay on the Theranos system

An evaluation of the Flu B assay's performance on the Theranos system compared to the Remel FDA kit was performed. The assay works excellent. Briefly, capture was at 5ug/ml while detection was maintained at 50 ng/ml final after protocol run. For the NIBSC strains, 50ul is added to the swab and treated like a sample swab. For the Zeptometrix panel controls (prediluted samples), 200ul of sample is mixed with 200ul of extraction buffer. For any sample swabs, swab is subjected to 500ul of extraction buffer and incubated for 3-5 minutes. This extracted sample is then used on the Theranos system. Swabs and samples were processed on the Remel kit as

directed on the FDA kit. The Cutoff was determined to be Means of Normals + 4 * Stdev(Normals).

Table [SEQ Table * ARABIC]: Clinical Evaluation on the Theranos System

Type	ID#	Theranos System	Remel FDA
Normal Clinicals	1	0.02	
	6	0.03	
	7	0.02	
	8	0.02	
	10	0.04	
	11	0.02	
	12	0.03	
	13	0.01	
	15	0.02	
	16	0.06	
	17	0.02	
	18	0.03	
	2	0.11	
	3	0.03	
	4	0.04	
	9	0.05	
	14	0.36	
	19	0.79	
REMEL	FDA Pos B Swab	10.78	
Zeptometric QC panel			
Flu A	Flu A POS	0.02	
Flu A	Brisbane/10/07	0.03	
Flu A	Solomon Islands/03/2006	0.01	
Flu A	New Caledonia/20/99	0.02	
Flu A	Brisbane/59/07	0.03	
NIBSC STANDARDS			
	Panama 45/90	14.38	
	Influenza Antigen B-Johannesburg	2.58	
	Influenza Antigen B-Guangdong	21.28	
	Influenza Antigen B/Yamanashi/166/98	6.05	
	Influenza Antigen B/Malaysia/2506/2004	7.53	
	Influenza Antigen B/Harbin/7/94	18.21	
	B /Florida 4/2006	19.27	
	Influenza Antigen A/California /7/2009-H1N1	0.36	
	Influenza Antigen A/HongKong/1073/99 (H9N2)	0.50	
	Influenza Antigen A/Cambodia/RO405050/2007 (H5N1)	0.61	
	Influenza Antigen A/mallard/England/727/2006 (H2N3)	0.50	
	Influenza Antigen A/New York/107/2003 (H7N2) (NIBRG-109)	0.39	
	Influenza Antigen A/New York/55/2004 (H3N2) (NYMC X-157)	0.12	
Zeptometrix Panel			
Flu B	Lee/40	11.31	
Flu B	Florida/02/2006	2.57	
Flu B	Brisbane/33/2008	12.36	
Flu B	Panama/45/90	5.93	
Flu B	Panama/45/90	4.64	

2.9 Stability Studies

Stability monitoring is ongoing for the the assay reagents stored at 4°C and protected from light. Different detection antibody stabilizers will be evaluated throughout this study. These include detection antibody in either Stabilzyme AP or the Theranos In-house AP stabilizer.