DENV Detect™ IgG ELISA

For In Vitro Diagnostic Use
Not for Sale or Distribution in the United States of America

INTENDED USE

The DENV *Detect*™ IgG ELISA test for exposure to Dengue virus (DENV) is an ELISA assay system for the detection of IgG antibodies in human serum to Dengue-derived recombinant antigen (DENRA) (1-4). This test is to aid in the diagnosis of human exposure to the Dengue virus. It is not intended to screen blood or blood components, and is for *in vitro* diagnostic use only.

SUMMARY AND EXPLANATION OF THE TEST

Dengue is an acute viral disease of man, which is transmitted by *Aedes aegypti* mosquitoes. Dengue is characterized clinically by biphasic fever, rash and hematopoietic depression, and by constitutional symptoms such as malaise, arthralgia, myalgia and headache (1). Infrequently, more severe disease is seen, manifested by hemorrhagic fever which may progress to lethal shock (2, 3). It is endemic in the tropics and subtropics, worldwide, where an estimated 100,000,000 cases occur annually (4). It has been estimated that about 50 to 100 million cases of Dengue Fever (DF) occur every year with about 250,000 to 500,000 cases of Dengue Hemorrhagic Fever (DHF). During 2002, more than 30 Latin American countries reported over 10,000,000 DF cases, with large number of DHF cases. This has been followed by extensive epidemics of DHF in several parts of India during 2003 through 2005. In the Americas, the reported incidence has more than tripled from 1996 to 2002. The incidence of Dengue outbreak has been reported in Hawaii (5), and in Laredo, Texas. The potential for the virus to cause a severe disease has also resulted in the inclusion of this pathogen on the CDC “category A” list for potential biological warfare and bioterrorism agents.

PRINCIPLE OF THE TEST

The DENV *Detect*™ IgG ELISA consists of one enzymatically amplified "two-step" sandwich-type immunoassay.

In this assay, Dengue IgG Negative Control (represents non-reactive serum), Dengue IgG Positive Control and unknown serum samples are incubated in microtitration wells which have been coated with monoclonal antibody bound to recombinant Dengue antigen. The serum samples are diluted with Sample Dilution Buffer for Dengue IgG. After incubation and washing, the wells are treated with an antibody specific for human IgG labeled with the enzyme horseradish peroxidase (HRP). After a second incubation and washing step, the wells are incubated with the tetramethylbenzidine (TMB) substrate.

An acidic stopping solution is then added and the degree of enzymatic turnover of the substrate is determined by absorbance measurement at 450 nanometers. Above a certain threshold, the ratio of the absorbencies of the DENRA and the control wells accurately determines whether antibodies to Dengue are present.

Note: *A set of negative and positive controls are provided as internal controls in order to monitor the integrity of the kit components.*

MATERIALS SUPPLIED

Warning: Do not use any reagents where damage to the packaging has occurred.

The DENV *Detect*™ IgG ELISA Kit contains sufficient reagents for one plate of 96 wells (12 x 8 strips) each. The kit contains the following reagents:

1. **COATED MICROTITER STRIPS FOR DENGUE IGG:** Strip holder in ziplock foil, containing 96 polystyrene microtiter wells coated with monoclonal antibody bound to recombinant Dengue antigen in each well. Store at 2-8°C until expiry.
   Note: The DENRA and Normal Cell Antigen (NCA) are already bound to plates**.**

2. **SAMPLE DILUTION BUFFER FOR DENGUE IGG:** One bottle, 25 mL (contains 0.05% Proclin as preservative). Store at 2-8°C until expiry.
   Note: *If any precipitate is seen, vortex the bottle very well to obtain a homogeneous solution and then use.*

3. **DENGUE IGG NEGATIVE CONTROL:** One vial, 50 µL. The Negative Control will aid in monitoring the integrity of the kit. Store at 2-8°C until expiry. Quick spin the vial briefly before use to collect contents at the bottom.

4. **DENGUE IGG POSITIVE CONTROL:** One vial, 50 µL. The Positive Control will aid in monitoring the integrity of the kit. Store at 2-8°C until expiry. Quick spin the vial briefly before use to collect contents at the bottom.

5. **READY TO USE ENZYME CONJUGATE-HRP FOR DENGUE IGG:**
   One bottle, 6 mL, ready to use, contains a pre-diluted goat anti-human IgG conjugate. Store at 2-8°C until expiry.

6. **10X WASH BUFFER:** One bottle, 120 mL, to be diluted before use. Store at 2-8°C until expiry.

7. **ENWASH:** One bottle, 20 mL, ready to use. Store at 2-8°C until expiry.

8. **LIQUID TMB SUBSTRATE:** One bottle, 9 mL, ready to use. Store at 2-8°C until expiry.
   Note: The substrate is to be kept in a light-protected bottle at all times as provided.

9. **STOP SOLUTION:** One bottle, 6 mL, ready to use. Store at 2-8°C until expiry.
   Caution: strong acid, wear protective gloves, mask and safety glasses. Dispose of all materials according to safety rules and regulations.

MATERIALS REQUIRED BUT NOT SUPPLIED

- ELISA spectrophotometer capable of absorbance measurement at 450 nm
- Biological or high-grade water
- Automatic plate washer
- 37°C incubator without CO_2 supply or humidification
- 1-10 µL single-channel pipetters, 50-200 µL single- and multi-channel pipetters
- Polypropylene tubes
- Parafilm
- Timer
- Vortex

PRECAUTIONS

- For *in vitro diagnostic use only. Not for sale or distribution in the United States of America.*
- All human source materials used in the preparation of controls have been heat-inactivated. However, all human controls and antigen should still be handled as potentially infectious material. The Centers for Disease Control and Prevention and the National Institutes of Health recommend that potentially infectious agents be handled at the Biosafety Level 2.
- A thorough understanding of this package insert is necessary for successful use of the product. Reliable results will only be obtained by using precise laboratory techniques and accurately following the package insert.
- Do not mix various lots of any kit component within an individual assay.
- Do not use any component beyond the expiration date shown on its label.
- Avoid exposure of the reagents to excessive heat or direct sunlight during storage and incubation.
- Some reagents may form a slight precipitate, mix gently before use.
- Incomplete washing will adversely affect the outcome and assay precision.
- To minimize potential assay drift due to variation in the substrate incubation time, care should be taken to add the stop solution into the wells in the same order and speed used to add the TMB solution.
- Avoid microbial contamination of reagents, especially of the Ready to Use Enzyme Conjugate-HRP for Dengue IgG. Avoid contamination of the TMB Substrate Solution with the Enzyme Conjugate-HRP.
- Wear protective clothing, eye protection and disposable gloves while performing the assay. Wash hands thoroughly afterwards.
- Use a clean disposable pipette tip for each reagent, Standard, Control or specimen.
- Cover working area with disposable absorbent paper.

WARNING:
POTENTIALLY BIOHAZARDOUS MATERIAL

This kit may contain reagents made with human serum or plasma. The serum or plasma used has been heat inactivated unless otherwise stated. Handle all sera and kits used as if they contain infectious agents. Observe established precautions against microbiological hazards while performing all procedures and follow the standard procedures for proper disposal of specimens.

CHEMICAL HAZARD

Safety Data Sheets (SDS) are available for all components of this kit. Review all appropriate SDS before performing this assay. Avoid all contact between hands and eyes or mucous membranes during testing. If contact does occur, consult the applicable SDS for appropriate treatment.

SPECIMEN COLLECTION AND PREPARATION

- Human serum must be used with this assay. Reagents have not been optimized or tested with whole blood or plasma so they cannot be tested directly.
- Remove serum from the clot of red cells as soon as possible to avoid hemolysis.
- Testing should be performed as soon as possible after collection. Do not leave sera at room temperature for prolonged periods.
- Serum should be used and the usual precautions for venipuncture should be observed. The samples may be stored at 2-8°C for up to 7 days, or frozen at -20°C or lower for up to 30 days. To maintain long-term longevity of the serum, store at -70°C. Avoid repeated freezing and thawing of samples.
- Frozen samples should be thawed to room temperature and mixed thoroughly by gentle swirling or inversion prior to use. Always quick spin before use.
- If sera are to be shipped, they should be packed in compliance with Federal Regulations covering transportation of infectious agents.
- Do not use sera if any indication of growth is observed.

TEST PROCEDURE

Caution: This kit has not been optimized by InBios for use with any particular automated ELISA processing system. Use with an automated ELISA processing system will require proper validation to ensure results are equivalent to the expectations described in this package insert. Modifications to the protocol of these systems and/or different volumes of reagents may be required.

Bring all kit reagents and specimens **to room temperature (~25°C) before use**. Thoroughly mix the reagents and samples before use by gentle inversion.

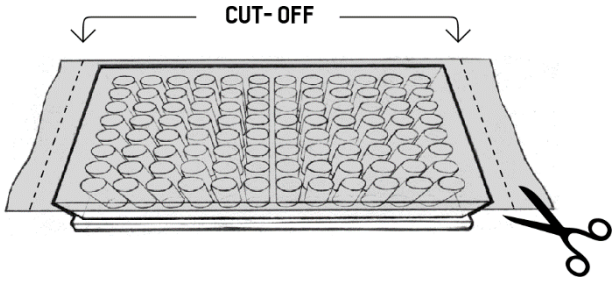
PREPARATION OF REAGENTS

- Preparation of 1X Wash Buffer
  Dilute the 10X Wash Buffer to 1X using Biological or High-Grade Water. To prepare a 1X wash buffer solution, mix 120 mL 10X wash buffer with 1080 mL distilled (or deionized) water and rinse out any crystals. Swirl until well mixed and all crystals are dissolved. After diluting to 1X, store at room temperature for up to 6 months. Check for contamination prior to use. Discard if contamination is suspected.
- Microtitration Wells
  Select the number of coated wells required for the assay. The remaining unused wells should be quickly placed back into the pouch and sealed, then stored at 2-8°C until ready to use or expiration.

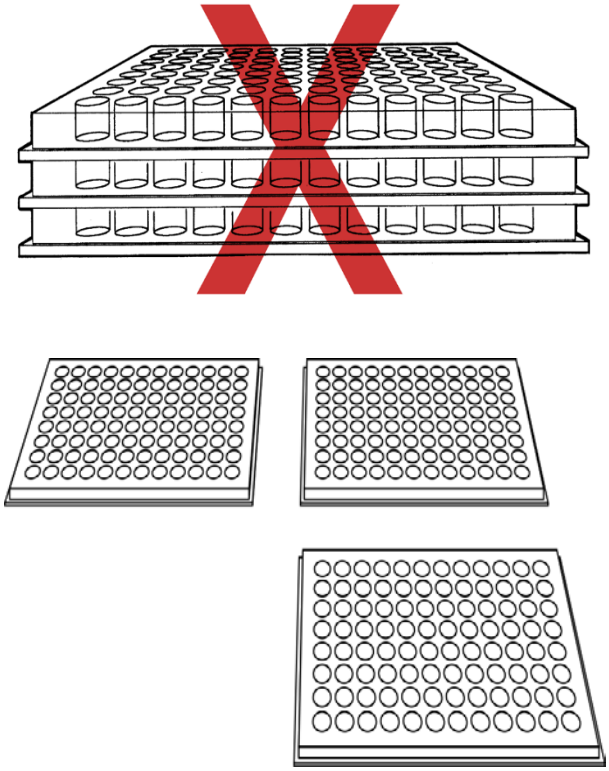
ASSAY PROCEDURE

1. Positive and negative controls should be assayed in duplicate for both DENRA and NCA portions of assay. Unknown serum samples to be tested can be assayed singly, but must be assayed for both DENRA and NCA portions of assay. Refer to flow chart at the end of this section for illustration of this procedure. Up to forty-four test specimens can be tested on one 96 well plate.
2. Mark the microtitration strips to be used.
   Note that the Dengue Antigens (DENRA) and control antigens (NCA) are already bound to the plate in this arrangement:
   DENRA: rows A-D
   NCA: rows E-H
3. Dilute test sera and controls to 1/100 using the provided Sample Dilution Buffer. Use small polypropylene tubes for these dilutions and use at least 4 µL of sera from positive and negative controls, and unknown samples. For example: mix 4 µL serum plus 396 µL of Sample Dilution Buffer for Dengue IgG to make 1/100 dilution.
4. Apply 50 µL per well of 1/100 diluted test sera, Dengue IgG Negative Control, and Dengue IgG Positive Control to the plate by single or multi-channel pipetter as appropriate. An exemplary arrangement for forty-four test serum samples is shown in "Example for Serum Sample Application" chart at the end of insert. Note: All serum samples are to be tested with DENRA and NCA.
5. Cover the plate with parafilm just on the well opening surface, so the bottom of the plate is not covered.

   ***Note**: This is to ensure the temperature distribution is evenly spread out in all wells from bottom and sides; any extra parafilm can be cut off once the top is sealed to block evaporation.*



6. Incubate the plate at 37°C for 1 hour in an incubator.
   ***Note**: Do not stack plates on top of each other. They should be spread out as a single layer. This is very important for even temperature distribution. Do not use CO_2 or other gases. Do not place plates in contact with any wet substances such as wet paper towels, etc.*



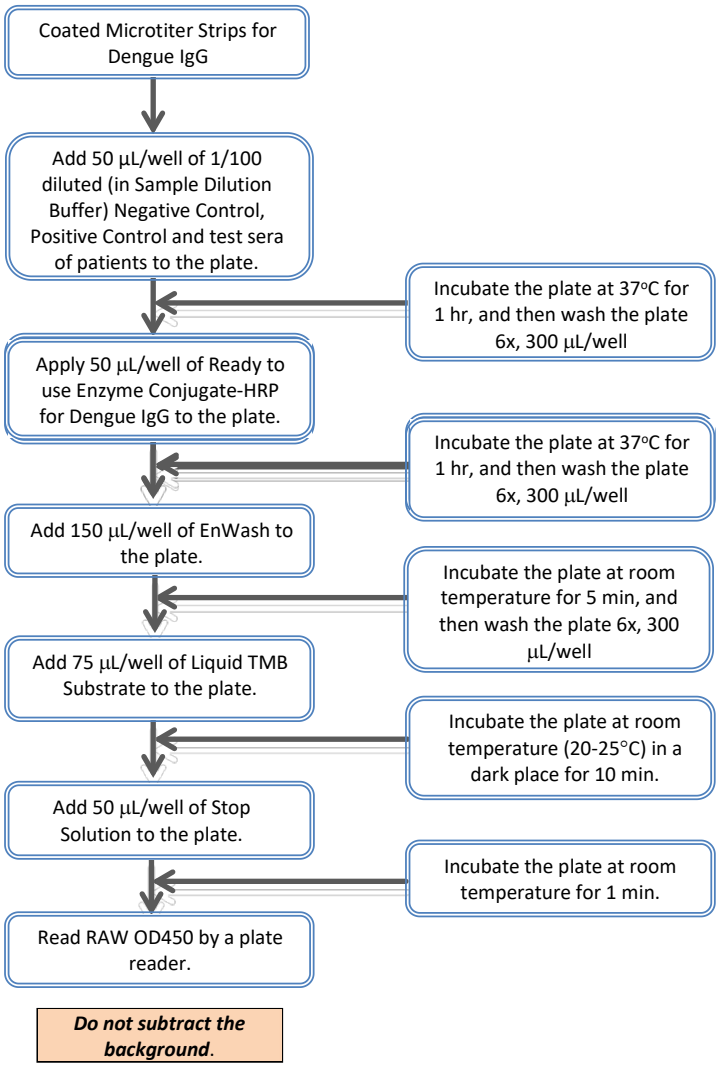
CORRECT METHOD

7. After the incubation, wash the plate 6 times with an automatic plate washer using 1X wash buffer. Use 300 µL per well in each wash cycle.
8. Add 50 µL per well of ready to use Enzyme-HRP conjugate into all wells by multi-channel pipetter.
9. Cover the plate with parafilm just on the well opening surface. The bottom of the plate should not be covered (see step 5).
10. Incubate the plate at 37°C for 1 hour in an incubator (see step 6).
11. After the incubation, wash the plate 6 times with an automatic plate washer using 1X wash buffer.
12. Add 150 µL per well of EnWash into all wells by multi-channel pipetter.
13. Incubate the plate at room temperature for 5 minutes without any cover on the plate.
14. After the incubation, wash the plate 6 times with an automatic plate washer using 1X wash buffer.

15. Add 75 µL per well of Liquid TMB substrate into all wells by multi-channel pipetter.
16. Incubate the plate at room temperature in a dark place (or container) for 10 minutes without any cover on the plate.
17. After the incubation, add 50 µL per well of Stop solution into all wells by multi-channel pipetter and incubate at room temperature for 1 minute without any cover on the plate.
18. After the incubation, read the **RAW** OD 450nm values with a microplate reader.

Ensure that the microplate reader does NOT subtract or normalize for any blank values or wells.

DENV Detect™ IgG ELISA Procedure Flowchart:



Do not subtract the background.

QUALITY CONTROL

Each kit contains positive and negative control sera. Acceptable Immune Status Ratio (ISR) values for these controls are found on specification table below. The negative and positive controls are intended to monitor for substantial reagent failure. The positive control will not ensure precision at the assay cut-off. The test is invalid and must be repeated if the ISR value of either of the controls does not meet the specifications below. If the test is invalid, patient results cannot be reported. Quality control requirements must be performed in conformance with local, state, and/or federal regulations or accreditation requirements and your laboratory's standard Quality Control procedures. It is recommended that the user refer to CLSI C24 and 42 CFR 493.1256 for guidance on appropriate QC practices. The results below are given strictly for guidance purposes only. Applicable for spectrophotometric readings only.

Calculation of the Negative Control: Calculate the mean Dengue IgG Negative Control values with DENRA and with NCA:

Example 1: Dengue IgG Negative Control

	OD450	
	DENRA	***NCA***
Replicate 1	0.098	0.101
Replicate 2	0.096	0.103
Sum	0.194	0.204

Average DENRA = 0.194 ÷ 2 = 0.097
Average NCA = 0.204 ÷ 2 = 0.102

Calculate the DENRA/NCA ratio (ISR): 0.097 ÷ 0.102 = 0.95

Any Dengue IgG Negative Control ISR greater than 2.0 indicates that the test procedure must be repeated.

Calculation of the Positive Control: Calculate the mean Dengue IgG Positive Control values with DENRA and with NCA:

Example 2: Dengue IgG Positive Control

	OD450	
	DENRA	***NCA***
Replicate 1	1.374	0.034
Replicate 2	1.380	0.036
Sum	2.754	0.070

Average DENRA = 2.754 ÷ 2 = 1.377
Average NCA = 0.070 ÷ 2 = 0.035

Calculate the DENRA/NCA ratio (ISR): 1.377 ÷ 0.035 = 39.3

Any Dengue IgG Positive Control ISR less than 5.0 indicates that the test procedure must be repeated.

The values in the table below must be obtained in order that the results of the assay may be reported. Non-fulfillment of these criteria is an indication of deterioration of reagents or an error in the test procedure and the assay must be repeated.

Factor (For Assay Verification)	Tolerance
Mean Dengue IgG Negative Control OD in DENRA	< 0.300
Mean Dengue IgG Positive Control OD in DENRA	> 0.350
Dengue IgG Positive Control Immune Status Ratio (ISR)	> 5.000
Dengue IgG Negative Control Immune Status Ratio (ISR)	< 2.000

CALCULATIONS FOR UNKNOWN SAMPLE ANALYSIS

Calculation of the Immune Status Ratio (ISR): If unknown samples were assayed in duplicate, then compute the average of the two replicates with the DENRA, and the two replicates with the NCA, then calculate the DENRA/NCA ratio (ISR) by dividing the average DENRA OD value by the average NCA OD value. If unknown samples were assayed singly, divide the DENRA OD value by the NCA OD value.

Selection of the Cut-off: The cut-off was selected using values from a small set of field data and is an estimate only.

Interpretation of Results: The table below shows how the results should be interpreted.

ISR	Results	Interpretation
≤1.65	Negative	No detectable IgG antibody
1.65-2.84	Equivocal	Need confirmatory test
≥2.84	Positive	Presence of detectable IgG antibody. Supplemental confirmatory testing is recommended

PERFORMANCE CHARACTERISTICS

In-House Sensitivity Study:
In order to evaluate sensitivity of the DENV *Detect*™ IgG ELISA kit, one hundred and thirty two dengue suspected sera (Brazil- various regions), kit controls and a panel of positive and negative samples were tested in duplicate. Sera were collected from all subjects at two different times (initial visit and repeat visit). ELISA tests are performed as described in the product insert. The results of this study are summarized in the table below.

Table: Brazil Dengue suspected disease panel

Dengue suspected serum origin	# of samples	DENV *Detect*™ IgG ELISA	
		Negative	Positive
Brazil	132	20	112
Total	132	20/132	112/132

Serological Sensitivity= 112/132= 84.84%

55 patient samples were evaluated by Plaque Reduction Neutralization Test (PRNT) and 39/55 subjects were considered PRNT positive. All 39 PRNT positive samples were identified with a positive ISR value with the DENV *Detect*™ IgG ELISA kit.

In-House Specificity Study:
A panel of fifty-four normal human sera (NHS) was used to evaluate specificity of the DENV *Detect*™ IgG ELISA kit. ELISA tests were performed as described in the product insert and included kit controls and a panel of positive and negative samples. All samples were tested in duplicate at each time point. The results of this study are shown in the table below.

Category	DENV *Detect*™ IgG ELISA kit		
	Negative	Equivocal	Positive
NHS	52	1	1
Total	52/54	1/54	1/54

Serological Specificity= 96.3%

In-House Cross-Reactivity Study:
Two panels of dengue-negative sera were used to evaluate cross-reactivity of the DENV *Detect*™ IgG ELISA kit. The flavivirus (closely-related species) panel consisted of thirty West Nile (WN) Virus IgG positive sera, three Saint Louis encephalitis (SLE) and one Japanese encephalitis (JE) positive sera. The unrelated diseases panel consisted of sera from patients infected with Epstein Barr Virus (EBV), Rheumatoid factor (RF), anti-nuclear antibodies (ANA), Varicella zoster virus (VZV) and Cytomegalovirus (CMV). ELISA tests were performed as described in the product insert. Kit controls and all samples were tested in duplicate. The tables below summarize the results of this study. Significant cross-reactivity was observed with West Nile virus.

Table: Flavivirus disease panel

Disease	Number of Samples	DENV *Detect*™ IgG ELISA		Total # of Positive or Equivocal
		Equivocal	Positive	
WN	30	6	11	17/30
SLE	3	0	0	0/3
JE	1	1	0	1/1
Total	34	7	11	18/34

Table: Non-flavivirus disease panel

Disease	Number of Samples	DENV *Detect*™ IgG ELISA		Total # of Positive or Equivocal
		Equivocal	Positive	
EBV	1	0	0	0/1
RF	1	0	0	0/1
ANA	1	0	0	0/1
VZV	1	0	0	0/1
CMV	1	0	0	0/1
Total	5	0	0	0/5

LIMITATIONS

- Since this is an indirect screening method, the presence of false positive and negative results must be considered.
- All reactive samples must be evaluated by a confirmatory test.
- The reagents supplied in this kit are optimized to measure DENRA-reactive antibody levels in serum.
- Serological cross-reactivity across the flavivirus group is common. Certain sera from patients infected with Japanese Encephalitis, West Nile, and/or Saint Louis viruses may give false positive results. Therefore any Dengue-positive sera must be confirmed with other tests.
- The assay performance characteristics have not been established for visual result determination.
- Results from immunosuppressed patients must be interpreted with caution.
- Assay results should be interpreted only in the context of other laboratory findings and the total clinical status of the patient.

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EC REP

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IVD

CE

Example for Serum Sample Application												
	1	2	3	4	5	6	7	8	9	10	11	12
A	Negative Control	Sample # 1	Sample # 5	Sample # 9	Sample # 13	Sample # 17	Sample # 21	Sample # 25	Sample # 29	Sample # 33	Sample # 37	Sample # 41
B	Negative Control	Sample # 2	Sample # 6	Sample # 10	Sample # 14	Sample # 18	Sample # 22	Sample # 26	Sample # 30	Sample # 34	Sample # 38	Sample # 42
C	Positive Control	Sample # 3	Sample # 7	Sample # 11	Sample # 15	Sample # 19	Sample # 23	Sample # 27	Sample # 31	Sample # 35	Sample # 39	Sample # 43
D	Positive Control	Sample # 4	Sample # 8	Sample # 12	Sample # 16	Sample # 20	Sample # 24	Sample # 28	Sample # 32	Sample # 36	Sample # 40	Sample # 44
E	Positive Control	Sample # 4	Sample # 8	Sample # 12	Sample # 16	Sample # 20	Sample # 24	Sample # 28	Sample #32	Sample # 36	Sample # 40	Sample # 44
F	Positive Control	Sample #3	Sample # 7	Sample # 11	Sample # 15	Sample # 19	Sample # 23	Sample # 27	Sample # 31	Sample # 35	Sample # 39	Sample # 43
G	Negative Control	Sample # 2	Sample # 6	Sample # 10	Sample # 14	Sample # 18	Sample # 22	Sample # 26	Sample # 30	Sample # 34	Sample # 38	Sample # 42
H	Negative Control	Sample # 1	Sample # 5	Sample # 9	Sample # 13	Sample # 17	Sample # 21	Sample # 25	Sample # 29	Sample # 33	Sample # 37	Sample # 41

Layout of DENRA and NCA Plate Coating												
	1	2	3	4	5	6	7	8	9	10	11	12
A	DENRA	DENRA	DENRA	DENRA	DENRA	DENRA	DENRA	DENRA	DENRA	DENRA	DENRA	DENRA
B	DENRA	DENRA	DENRA	DENRA	DENRA	DENRA	DENRA	DENRA	DENRA	DENRA	DENRA	DENRA
C	DENRA	DENRA	DENRA	DENRA	DENRA	DENRA	DENRA	DENRA	DENRA	DENRA	DENRA	DENRA
D	DENRA	DENRA	DENRA	DENRA	DENRA	DENRA	DENRA	DENRA	DENRA	DENRA	DENRA	DENRA
E	NCA	NCA	NCA	NCA	NCA	NCA	NCA	NCA	NCA	NCA	NCA	NCA
F	NCA	NCA	NCA	NCA	NCA	NCA	NCA	NCA	NCA	NCA	NCA	NCA
G	NCA	NCA	NCA	NCA	NCA	NCA	NCA	NCA	NCA	NCA	NCA	NCA
H	NCA	NCA	NCA	NCA	NCA	NCA	NCA	NCA	NCA	NCA	NCA	NCA