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ELISA PRODUCT INFORMATION & MANUAL

Influenza A H1N1 Hemagglutinin ELISA Kit (Colorimetric) NBP2-80333 Sample Insert for Reference Only

Enzyme-linked Immunosorbent Assay for quantitative detection. For research use only. Not for diagnostic or therapeutic procedures.

www.novusbio.com - P: 303.730.1950 - P: 888.506.6887 - F: 303.730.1966 - technical@novusbio.com

Novus kits are guaranteed for 6 months from date of receipt

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BACKGROUND

Hemagglutinin (HA) is a single-pass type I integral membrane glycoprotein from the influenza virus, and comprises over 80% of the envelope proteins present in the virus particle. In natural infection, inactive HA is matured into HA1 and HA2 outside the cell by one or more trypsin-like, arginine-specific endoprotease secreted by the bronchial epithelial cells. Binding of HA to sialic acid-containing receptors on the surface of its target cell brings about the attachment of the virus particle to the cell and forms a endosome. Low pH in endosomes induce an irreversible conformational change in HA2, releasing the hydrophobic portion "fusion peptide". After which, virus penetrates the cell and pours its contents including the RNA genome into the cytoplasm mediated by fusion of the endocytosed virus particle's own membrane and the endosomal membrane. Hemagglutinin plays a major role in the determination of host range restriction and virulence. H1N1 is a subtype of influenza virus A and the most common cause of influenza in humans.

INTENDED USE

The kit has been verified by high purity Influenza H1N1 Hemagglutinin / HA recombinant protein.

The use of this kit for natural samples need be validated by the end user due to the complexity of natural targets and unpredictable interference.

PRINCIPLE OF THE ASSAY

The principle of this ELISA kit is based on the solid phase sandwich enzyme immunoassay technique. A monoclonal antibody specific for Influenza H1N1 Hemagglutinin / HA has been pre-coated onto well plate strips. Standards and samples are added to the wells and Influenza H1N1 Hemagglutinin / HA present in the sample is bound by the immobilized antibody. After incubation the wells are washed and a horseradish peroxidase conjugated anti-Influenza H1N1 Hemagglutinin / HA antibody is added, producing an antibody-antigen-antibody "sandwich complex". Following a wash to remove any unbound antibody a TMB substrate solution is loaded and color develops in proportion to the amount of Influenza H1N1 Hemagglutinin / HA bound. The reaction is stopped by the addition of a stop solution and the intensity of the color can be measured at 450 nm (See schematics below).



MATERIALS PROVIDED

Influenza H1N1 Hemagglutinin / HA Microplate - 96 well polystyrene microplate (12 strips of 8 wells) coated with mouse mAb antibody against Influenza H1N1 Hemagglutinin / HA.

Influenza H1N1 Hemagglutinin / HA Detection Antibody - 0.2 mg/mL of rabbit pAb antibody against Influenza H1N1 Hemagglutinin / HA conjugated to horseradish peroxidase (HRP) with preservatives.

Influenza H1N1 Hemagglutinin / HA Standard - Recombinant Influenza H1N1 (A/California/04/2009) Hemagglutinin / HA in a buffer with preservatives, lyophilized. The amount of standard is lot specific and indicated on the label of standard vial.

Wash Buffer Concentrate - 25 mL of a 20-fold concentrated solution of buffered surfactant with preservatives.

Dilution Buffer Concentrate - 8 mL of a 20-fold concentrated dilution buffer with preservatives.

Color Reagent A - 13 mL of stabilized hydrogen peroxide.

Color Reagent B - 13 mL of stabilized chromogen (tetramethylbenzidine).

Stop Solution - 8 mL of 2 N sulfuric acid.

STORAGE

Unopened Kit	Store at 2 - 8°C and the kit is stable for 6 months upon receipt.		
	Diluted Wash Buffer Diluted Dilution Buffer	Stored for up to 1 week at 2 - 8°C	
Opened/ Reconstituted Reagents	ConjugateConjugateStop SolutionUnmixed ColorReagent AReagentsUnmixed ColorReagent B	Stored for up to 1 month at 2 - 8 $^{\circ}$ C	
	Standard	After reconstitution, store for up to 1 month at -80°C. The reconstituted standards should be aliquoted and avoid repeated freeze-thaw cycles.	
	Microplate Wells	Return unused strips to the foil pouch containing the desiccant pack and reseal along entire edge of zip-seal. Stored for up to 1 month at 2 - 8°C	

OTHER SUPPLIES REQUIRED

·Microplate reader capable of measuring absorbance at 450 nm

•Pipettes and pipette tips

·Deionized or distilled water

 \cdot Multi -channel pipette, squirt bottle, manifold dispenser, or automated microplate washer

·500 mL graduated cylinder

•Tubes for standard dilution

·Well plate cover or seals

PRECAUTIONS

- 1. This kit is **for research use only** and is not for use in diagnostic or therapeutic procedures.
- 2. The kit should not be used beyond the expiration date.
- 3. Do not mix reagents from different lots.
- 4. The kit is designed and tested to detect the specific targets and samples shown in the manual. The use of this kit for other purpose should be verified carefully by the end user.

SAFETY INSTRUCTIONS

- 5. The Stop Solution provided with this kit is an acid solution. Take care when using the reagent to avoid the risk.
- 6. All biological materials should be handled and discarded as potentially hazardous following local laws and regulations.
- 7. Personal protective equipments such as lab coats, gloves, surgical masks and goggles are necessary in experiments for safety reasons.

TECHNICAL TIPS

- 8. Bring all reagents and samples to room temperature before use.
- 9. Samples should be thawed completely and mixed well prior to analysis. Avoid repeated freeze-thaw cycles of frozen samples.
- 10.A standard curve should be generated for each set of sample assayed. DO NOT USE the standard curves from other plates or other days.
- 11.Use a new disposable reagent reservoir and new disposable pipette tips for each transfer to avoid cross-contamination.
- 12.Read the absorbance of each well within 20 minutes after adding the stop solution.

REAGENT PREPARATION

Bring all reagents to room temperature before use. If crystals have formed in buffer solution, warm to room temperature and mix gently until the crystals have completely dissolved.

Wash Buffer - Prepare 1× wash buffer by adding 20 mL of Wash Buffer Concentrate to deionized or distilled water to prepare 400 mL of Wash Buffer.

Dilution Buffer - Prepare $1 \times$ dilution buffer by adding 5 mL of Dilution Buffer Concentrate to deionized or distilled water to prepare 100 mL of Dilution Buffer.

Detection Antibody - Centrifuge at 10,000 x g for 20 seconds. Dilute to **work** concentration of 1 μ g/mL in Dilution Buffer before use.

Substrate Solution - Color Reagents A and B should be mixed together in equal volumes within 15 minutes of use. Protect from light. 200 μ L of the resultant mixture is required per well. Take care not to contaminate the Color Reagent. If the mixed color reagent is blue. DO NOT USE.

Influenza H1N1 Hemagglutinin / HA Standard - Reconstitute the Influenza H1N1 (A/California/04/2009) Hemagglutinin / HA Standard with 1 mL of Dilution Buffer to make stock solution. Shake the vial gently until the lyophilized powder totally dissolved (**Do not turn the vial upside down**). Mix the standard to ensure complete reconstitution prior to making dilutions.

Prepare serially diluted standards as described in the following step:

Pipette 1000 μ L of Dilution Buffer into the 1200 pg/mL tube. Pipette 500 μ L of Dilution Buffer into the remaining tubes. Use the stock solution to produce a dilution series as the following figure. Mix each tube thoroughly before the next transfer. The 1200 pg/mL standard serves as the high standard. The Dilution Buffer serves as the zero standard (0 pg/mL). Ensures each assay has a standard curve. DO NOT USE the standard curve on other plates or other days.

The following graph is only for demonstration purposes. The concentration of stock solution is lot specific and need be calculated with the actual amount of standard labeled on the standard vial.



ASSAY PROCEDURE

Bring all reagents and samples to room temperature before use. It is recommended that all samples and standards be assayed in duplicate.

1. Prepare all reagents, working standards, and samples as directed in the previous

sections.

2. Remove unused microplate strips from the plate frame, return them to the foil pouchcontaining the desiccant pack, and reseal.

3. Wash each well three times with Wash Buffer (300 μ L/well) using a squirt bottle, multi-channel pipette, manifold dispenser or autowasher. Complete removal of liquid at each step is essential to good performance. Remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.

4. Add 100 μ L of each serially diluted protein standard or test sample per well including a zero standard. **Ensure reagent addition is uninterrupted and completed within 15 minutes.** Cover/seal the plate and incubate for 2 hours at room temperature.

5. Repeat the aspiration/wash as in Step 3.

6. Add 100 μ L of Detection Antibody in working concentration to each well. Cover/seal the plate and incubate for 1 hour at room temperature.

7. Repeat the aspiration/wash as in Step 3.

8. Add 200 μ L of Substrate Solution to each well. Incubate for 20 minutes at room temperature. **Protect from light.**

9. Add 50 μ L of Stop Solution to each well. If color change does not appear uniform, gently tap the plate to ensure thorough mixing.

10. **Determine the optical density of each well within 20 minutes**, using a microplate reader set to 450 nm.

CALCULATION OF RESULTS

If samples generate values higher than the highest standard, dilute the samples and repeat the assay.

Calculate the mean absorbance for each standard, control and sample and subtract average zero standard optical density (O.D.) .

Construct a standard curve by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis and draw a best fit curve through the points on the graph. Most graphing software can help make the curve and a four parameter logistic (4-PL) usually provide the best fit, though other equations (e.g. linear, log/log) can also be tried to see which provides the most accurate. Extrapolate the target protein concentrations for unknown samples from the standard curve plotted.

TYPICAL DATA

This standard curve is only for demonstration purposes. A standard curve should be generated for each assay.

Concentration (pg/mL)	Zero standard subtracted OD
0	0
18.75	0.033
37.5	0.077
75	0.142
150	0. 288
300	0.559
600	1.126
1200	2,149



PRECISION

Intra-assay Precision (Precision within an assay)

Three samples of known concentration were tested twenty times on one plate to assess intra-assay precision.

Inter-assay Precision (Precision between assays)

Three samples of known concentration were tested in five separate assays to assess inter-assay precision.

	Intra -assay Precision		Inter -assay Precision		ion	
Sample	1	2	3	1	2	3
N	20	20	20	5	5	5
Mean (pg/mL)	209	386	722	198	372	702
SD	6.21	7.50	16.09	18.55	33.80	56.90
CV (%)	3.0%	1.9%	2.2%	9.4%	9.1%	8.1%

RECOVERY

The recovery of Influenza H1N1 Hemagglutinin / HA spiked to different levels throughout the range of the assay in related matrices was evaluated.

Sample	Average % Recovery	Range
Cell culture supernates (n=3)	103	92 -111%

LINEARITY

14		Cell culture supernates
1:2	recovery of detected	94%
1:4	recovery of detected	96%
1:8	recovery of detected	92%
1:16	recovery of detected	118%

SENSITIVITY

The minimum detectable dose (MDD) of Influenza H1N1 Hemagglutinin / HA is typically less than 7.91 pg/mL. The MDD was determined by adding three standard deviations to the mean optical density value of twenty zero standard replicates and calculating the corresponding concentration.

CALIBRATION

This immunoassay is calibrated against a highly purified recombinant Influenza H1N1 (A/California/04/2009) Hemagglutinin / HA.

SPECIFICITY

This assay recognizes most of recombinant H1N1 HA proteins. Influenza A H1N1 (A/California/04/2009) hemagglutinin / HA was served as the reference standard, and reaction of other H1N1 strains were as follows.

Expressed Host	Strain	Concentration (pg/mL)	Observed Value (pg/mL)	Crossreaction rates
Human Cells	H1N1 (A/Brevig Mission/1/1918) HA Protein	800	152	19%
Human Cells	H1N1 (A/California/04/2009) HA Protein	800	480	60%
Human Cells	H1N1 (A/California/04/2009) Protein (HA1 Subunit)	800	569	71%
Insect Cells	H1N1 (A/California/04/2009) HA Protein	800	1196	149%
Human Cells	H1N1 (A/California/07/2009) HA Protein	800	1508	188%
Human Cells	H1N1 (A/Brevig Mission/1/1918) Protein (HA1 Subunit)	800	26	3%
Human Cells	H1N1 (A/Ohio/07/2009) HA Protein	800	1430	179%
Human Cells	H1N1 (A/England/195/2009) HA Protein	400	707	177%
Human Cells	H1N1 (A/England/195/2009) Protein (HA1 Subunit)	800	1049	68%
Human Cells	H1N1 (A/Texas/05/2009) HA Protein	800	2694	176%
Human Cells	H1N1 (A/Texas/05/2009)Protein (HA1 Subunit)	800	1050	68%
Human Cells	H1N1 (A/New York/18/2009) HA Protein	800	1801	117%
Human Cells	H1N1 (A/New York/18/2009) Protein (HA1 Subunit)	800	1204	78%
Human Cells	H1N1 (A/Beijing/22808/2009) Protein (HA1 Subunit)	800	956	62%
Human Cells	H1N1 (A/Beijing/22808/2009)HA Protein	800	1651	108%
Human Cells	H1N1 (A/Ohio/07/2009) Protein (HA1 Subunit)	800	967	63%
Insect Cells	H1N1 (A/New York/1/1918) HA Protein	800	444	29%
Human Cells	H1N1 (A/New York/1/1918) Protein (HA1 Subunit)	800	47	3%
Insect Cells	H1N1 (A/California/07/2009) HA Protein	800	2476	162%
Insect Cells	H1N1 (A/New York/18/2009)HA Protein	800	2471	179%
Human Cells	H1N1 (A/California/06/2009) HA1 Protein	800	1006	69%
Insect Cells	H1N1 (A/California/04/2009)HA Protein	800	414	28%
Human Cells	H1N1 (A/NewJersey/8/1976) HA1 Protein	800	69	4%
Insect Cells	H1N1 (A/California/06/2009) HA Protein	800	2304	160%
Insect Cells	H1N1 (A/England/195/2009) HA Protein	800	2143	148%
Human Cells	H1N1 (A/Brisbane/59/2007) HA Protein	1200	<7.91	<0.1%
Human Cells	H1N1 (A/Brisbane/59/2007) Protein (HA1 Subunit)	1200	<7.91	<0.1%
Human Cells	H1N1 (A/New Caledonia/20/99) Protein (HA1 Subunit)	1200	<7.91	<0.1%

Human Cells	H1N1 (A/Puerto Rico/8/34) Protein (HA1 Subunit)	1200	<7.91	<0.1%
Human Cells	H1N1 (A/Ohio/UR06-0091/2007) Protein (HA1 Subunit)	1200	<7.91	<0.1%
Human Cells	H1N1 (A/WSN/33) Protein (HA1 Subunit)	1200	<7.91	<0.1%
Human Cells	H1N1 (A/WSN/33) HA Protein	1200	<7.91	<0.1%
Human Cells	H1N1 (A/Puerto Rico/8/34) HA Protein	1200	<7.91	<0.1%
Human Cells	H1N1 (A/New Caledonia/20/99)HA Protein	1200	<7.91	<0.1%
Human Cells	H1N1 (A/Ohio/UR06-0091/2007) HA Protein	1200	<7.91	<0.1%
Human Cells	H1N1 (A/Solomon Islands/3/2006) HA Protein	1200	<7.91	<0.1%
Human Cells	H1N1 (A/Solomon Islands/3/2006) Protein (HA1 Subunit)	1200	<7.91	<0.1%
Human Cells	H1N1 (A/California/04/2009) Protein (HA2 Subunit)	1200	<7.91	<0.1%
Insect Cells	H1N1 (A/USSR/90/77) HA Protein	1200	<7.91	<0.1%
Insect Cells	H1N1 (A/Beijing/262/1995) HA Protein	1200	<7.91	<0.1%
Insect Cells	H1N1 (A/Taiwan/01/1986) HA Protein	1200	<7.91	<0.1%
Human Cells	H1N1 (A/Texas/36/1991) Protein (HA1 Subunit)	1200	<7.91	<0.1%
Human Cells	H1N1 (A/USSR/90/197) Protein (HA1 Subunit)	1200	<7.91	<0.1%
Insect Cells	H1N1 (A/WSN/1933) HA Protein	1200	<7.91	<0.1%
Insect Cells	H1N1 (A/Puerto Rico/8/1934) HA Protein	1200	<7.91	<0.1%
Insect Cells	H1N1 (A/Texas/36/1991) HA Protein	1200	<7.91	<0.1%
Human Cells	H1N1 (A/Beijing/262/1995) HA Protein	1200	<7.91	<0.1%

The representative strains of other influenza subtypes listed below were prepared at 50 ng/mL in dilution buffer and assayed for cross-reactivity. No cross-reactivity was observed.

Expressed Host	Strain		
Human Cells	Influenza B virus (B/Florida/4/2006) HA Protein		
Human Cells	Influenza B (B/Brisbane/60/2008) HA Protein		
Human Cells	H3N2 (A/Brisbane/10/2007) HA Protein		
Human Cells	H3N2 (A/Aichi/2/1968) HA Protein		
Human Cells	H4N6 (A/Swine/Ontario/01911-1/99) HA Protein		
Insect Cells	H5N1 (A/Anhui/1/2005) HA Protein		
Insect Cells	H5N1 (A/chicken/VietNam/NCVD-016/2008) HA Protein		
Human Cells	H6N1 (A/northern shoveler/California/HKWF115/2007) HA Protein		
Human Cells	H7N7 (A/Netherlands/219/03) HA Protein		
Human Cells	H8N4 (A/pintail duck/Alberta/114/1979) HA Protein		
Human Cells	H9N2 (A/Hong Kong/1073/99)HA Protein		
Insect Cells	H10N8 (A/Jiangxi-Donghu/346/2013) HA Protein		
Human Cells	H11N2 (A/duck/Yangzhou/906/2002)HA Protein		
Human Cells	H12N5 (A/green-winged teal/ALB/199/1991) HA Protein		
Human Cells	H13N8 (A/black-headed gull/Netherlands/1/00) HA Protein		
Human Cells	H15N8 (A/duck/AUS/341/1983) HA Protein		
Human Cells	H16N3 (A/black-headed gull/Sweden/5/99) HA Protein		
Insect Cells	H18N11 (A/flat-faced bat/Peru/033/2010) HA Protein		

TROUBLE SHOOTING

Problems	Possible Sources	Solutions	
	Incorrect or no Detection Antibody was added	Add appropriate Detection Antibody and continue	
No signal	Substrate solution was not added	Add substrate solution and continue	
	Incorrect storage condition	Check if the kit is stored at recommended condition and used before expiration date	
	Standard was incompletely reconstituted or was inappropriately stored	Aliquot reconstituted standard and store at -80 °C. The reconstituted standards should be aliquoted and avoid repeated freeze-thaw cycles.	
Poor Standard Curve	Imprecise / inaccurate pipetting	Check / calibrate pipettes	
	Incubations done at inappropriate temperature, timing or agitation	Follow the general ELISA protocol	
	Background wells were contaminated	Avoid cross contamination by using the sealer appropriately	
	The concentration of antigen in samples was too low	Enriching samples to increase the concentration of antigen	
value	Samples were ineffective	Check if the samples are stored at cold environment. Detect samples in timely manner	
		Use multichannel pipettes without touching the reagents on the plate	
	Insufficient wasnes	Increase cycles of washes and soaking time between washes	
High Background	Color Reagent should be clear and colorless prior to addition to wells	Color Reagent should be clear and colorless prior to addition to wells	
	Use clean tubes and pipettes tips	Use clean plates, tubes and pipettes tips	
	Samples were contaminated	Avoid cross contamination of samples	
non-specificity	The concentration of samples was too high	Try higher dilution rate of samples	

ASSAY SUMMARY

