ELISA PRODUCT INFORMATION & MANUAL

Avian Influenza A H7N9 Hemagglutinin ELISA Kit (Colorimetric)

NBP2-80362

Sample Insert for Reference Only

Enzyme-linked Immunosorbent Assay for quantitative detection. For research use only.

Not for diagnostic or therapeutic procedures.

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BACKGROUND

On April 1, 2013, the World Health Organization (WHO) first reported 3 human infections with a new influenza A (H7N9) virus in China. Since then, additional cases have been reported. Most reported cases have severe respiratory illness and, in some cases, have died. At this time, no cases of H7N9 outside of China have been reported. The new H7N9 virus has not been detected in people or birds in the United States.

This new H7N9 virus is an avian (bird) influenza (flu) virus. Human infections with avian influenza (AI, or "bird flu") are rare but have occurred in the past, most commonly after exposure to infected poultry. However, this is the first time that this bird flu subtype (H7N9) has been found in people. This virus is very different from other H7N9 viruses previously found in birds.

H7N9 viruses have been detected in poultry in the same area where human infections have occurred. Many of the human cases of H7N9 are reported to have had contact with poultry. However some cases reportedly have not had such contact. Close contacts of confirmed H7N9 patients are being followed to see if any human-to-human spread of H7N9 might have occurred. Based on previous experience with other avian influenza viruses – most notably H5N1 – some limited human-to-human spread of this H7N9 virus would not be surprising. Most importantly, however, no sustained person-to-person spread of the H7N9 virus has been found at this time. Ongoing (sustained) person-to-person spread is necessary for a pandemic to occur.

The influenza viral Hemagglutinin (HA) protein is a homo trimer with a receptor binding pocket on the globular head of each monomer, and the influenza viral neuraminidase (NA) protein is a tetramer with an enzyme active site on the head of each monomer. Subtypes are further divided into strains; each genetically distinct virus isolate is usually considered to be a separate strain.

The influenza virus Hemagglutinin (HA) protein is translated in cells as a single protein, HA0, or hemagglutinin precursor protein. For viral activation, hemagglutinin precursor protein (HA0) must be cleaved by a trypsin-like serine endoprotease at a specific site, normally coded for by a single basic amino acid (usually arginine) between the HA1 and HA2 domains of the protein. After

cleavage, the two disulfide-bonded protein domains produce the mature form of the protein subunits as a prerequisite for the conformational change necessary for fusion and hence viral infectivity.

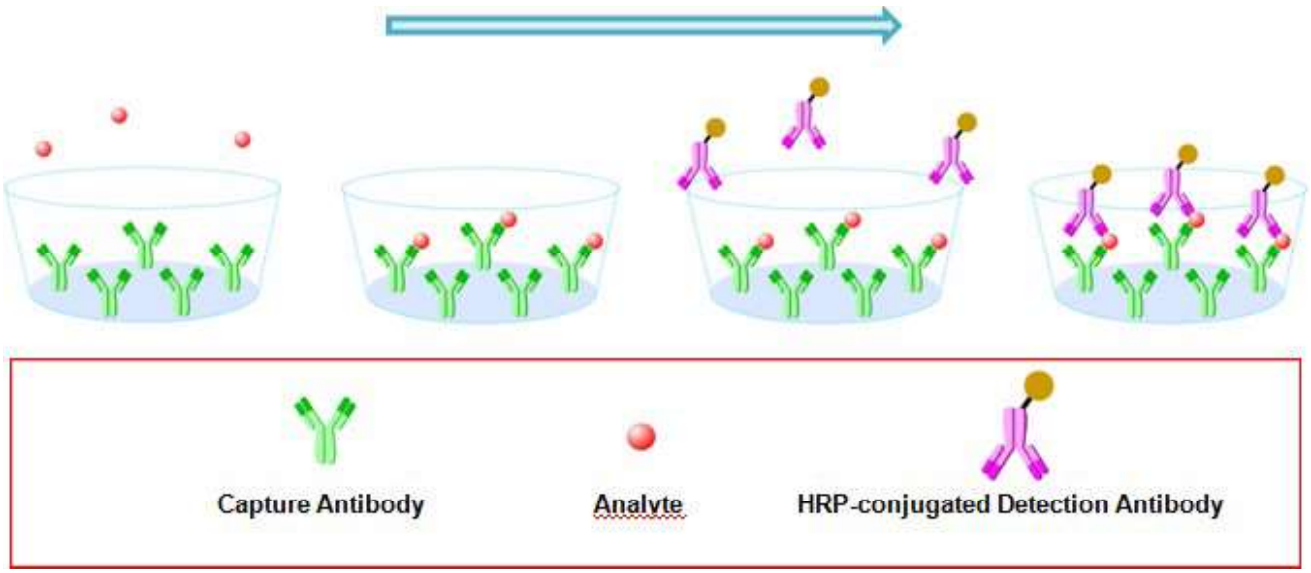
INTENDED USE

The kit has been verified by high purity Avian Influenza A H7N9 Hemagglutinin 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 Avian Influenza A H7N9 Hemagglutinin has been pre-coated onto well plate strips. Standards and samples are added to the wells and Avian Influenza A H7N9 Hemagglutinin present in the sample is bound by the immobilized antibody. After incubation the wells are washed and a horseradish peroxidase conjugated anti-Avian Influenza A H7N9 Hemagglutinin 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 Avian Influenza A H7N9 Hemagglutinin 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

Avian Influenza A H7N9 Hemagglutinin Microplate - 96 well polystyrene microplate (12 strips of 8 wells) coated with rabbit mAb antibody against Avian Influenza A H7N9 Hemagglutinin.

Avian Influenza A H7N9 Hemagglutinin Detection Antibody - 0.2 mg/mL of rabbit pAb antibody against Avian Influenza A H7N9 Hemagglutinin conjugated to horseradish peroxidase (HRP) with preservatives.

Avian Influenza A H7N9 Hemagglutinin Standard - Recombinant Influenza H7N9 (A/Shanghai/1/2013) Hemagglutinin 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

<table>
<tr><td>Unopened Kit</td><td colspan="2">Store at 2 - 8℃ and the kit is stable for 6 months upon receipt.</td></tr>
<tr><td rowspan="7">Opened/ Reconstituted Reagents</td><td>Diluted Wash Buffer</td><td rowspan="2">Stored for up to 1 week at 2 - 8℃</td></tr>
<tr><td>Diluted Dilution Buffer</td></tr>
<tr><td>Conjugate</td><td rowspan="3">Stored for up to 1 month at 2 - 8℃</td></tr>
<tr><td>Stop Solution</td></tr>
<tr><td>Unmixed Color Reagent A</td></tr>
<tr><td>Unmixed Color Reagent B</td><td rowspan="2">After reconstitution, store for up to 1 month at -80℃.
The reconstituted standards should be aliquoted and
avoid repeated freeze-thaw cycles.</td></tr>
<tr><td>Standard</td></tr>
<tr><td>Microplate Wells</td><td>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℃</td></tr>
</table>

OTHER SUPPLIES REQUIRED

·Microplate reader capable of measuring absorbance at 450 nm

·Pipettes and pipette tips

·Deionized or distilled water

·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.

TECHINICAL 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× 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 0.5 μg/mL in Dilution Buffer before use.

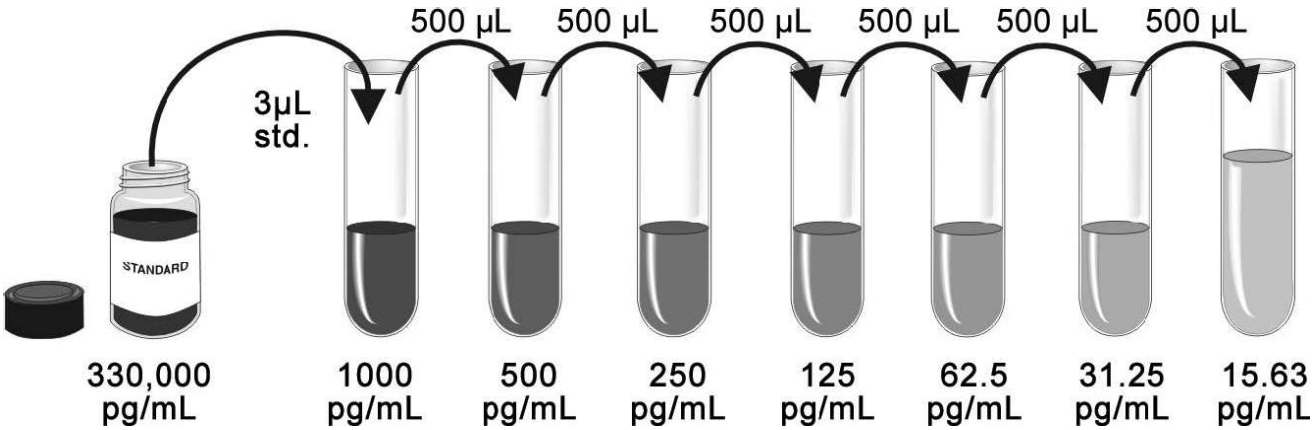
Substrate Solution **-** Color Reagents A and B should be mixed together in equal volumes within 15 minutes of use. Protect from light. 200 μL of the resultant mixture is required per well. **Take care not to contaminate the Color Reagent. If the mixed color reagent is blue. DO NOT USE.**

Avian Influenza A H7N9 Hemagglutinin Standard - Reconstitute the Influenza H7N9 (A/Shanghai/1/2013) Hemagglutinin 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 μL of Dilution Buffer into the 1000 pg/mL tube. Pipette 500 μL 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 1000 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.


500 µL
500 µL
500 µL
500 µL
500 µL
500 µL
3µL
std.
STANDARD
330,000
pg/mL
1000
pg/mL
500
pg/mL
250
pg/mL
125
pg/mL
62.5
pg/mL
31.25
pg/mL
15.63
pg/mL

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 µL/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 µL 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 µL 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 µL of Substrate Solution to each well. Incubate for 20 minutes at room temperature**. Protect from light.**

9. Add 50 µL 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.

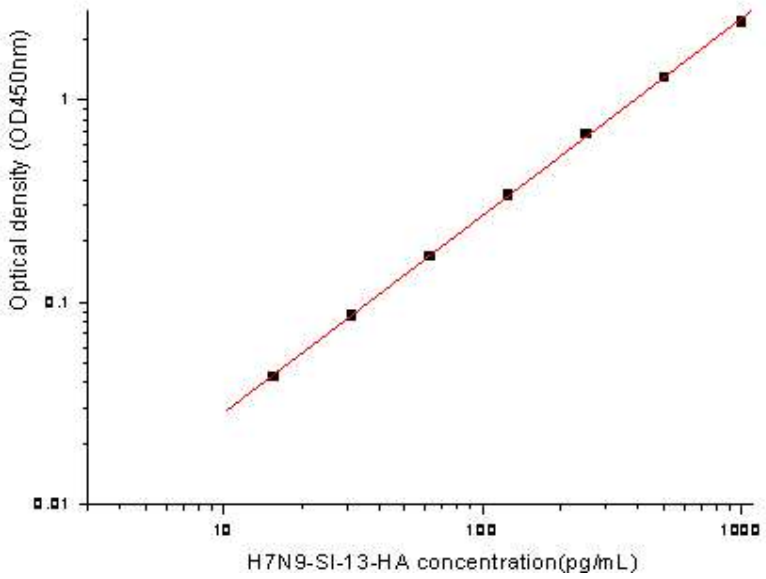
Calculate the mean absorbance for each standard, control and sample and subtract average zero standard optical density (O.D.) .

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
15.62	0.043
31.25	0.086
62.5	0.170
125	0.339
250	0.680
500	1.295
1000	2.415



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		
Sample	1	2	3	1	2	3
N	20	20	20	5	5	5
Mean(ng/mL)	0.224	0.462	0.87	0.220	0.458	0.867
SD	0.005	0.008	0.021	0.005	0.012	0.016
CV(%)	2.2%	1.6%	2.4%	2.1%	2.7%	1.8%

RECOVERY

The recovery of Avian Influenza A H7N9 Hemagglutinin spiked to different levels throughout the range of the assay in related matrices was evaluated.

Sample	Average % Recovery	Range
cell culture supernates(n=3)	99	97-100%

LINEARITY

		cell culture supernates
1:2	recovery of detected	112%
1:4	recovery of detected	110%
1:8	recovery of detected	103%
1:16	recovery of detected	91%

SENSITIVITY

The minimum detectable dose (MDD) of Avian Influenza A H7N9 Hemagglutinin is typically less than 3 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 H7N9 (A/Shanghai/1/2013) Hemagglutinin.

SPECIFICITY

This assay recognizes most of recombinant H7N9 proteins.Influenza A H7N9 (A/Shanghai/2/2013) Hemagglutinin is used as the standard. Crossreaction with other H7N9 strains are as follows:

Expressed Host:	Strain	Concentration (pg/mL)	Observed Value (pg/mL)	Cross reaction rates
Human Cells	H7N9 (A/Anhui/1/2013) HA Protein	500	611	122%
Human Cells	H7N9 (A/Anhui/1/2013) HA Protein (HA1 Subunit)	500	232	46%
Insect Cells	H7N9 (A/Anhui/1/2013) HA Protein	500	621	124%
Human Cells	H7N9 (A/Anhui/1/2013) HA Protein (HA1+HA2, cleavage)	500	124	25%
Human Cells	H7N9 (A/Shanghai/1/2013) HA Protein	500	720	144%
Human Cells	H7N9 (A/Shanghai/1/2013) HA Protein (HA1 Subunit)	500	250	50%
Insect Cells	H7N9 (A/Shanghai/1/2013) HA Protein	500	722	144%
Insect Cells	H7N9 (A/Shanghai/1/2013) HA Protein (HA1 Subunit)	500	352	70%
Human Cells	H7N9 (A/Shanghai/1/2013) HA Protein (HA1+HA2, cleavage)	500	282	56%
Human Cells	H7N9 (A/Hangzhou/1/2013) HA Protein	500	10	2%
Human Cells	H7N9 (A/Hangzhou/1/2013) HA Protein (HA1 Subunit)	500	299	60%
Insect Cells	H7N9 (A/Hangzhou/1/2013) HA Protein	500	611	122%
Human Cells	H7N9 (A/Pigeon/Shanghai/S1069/2013) HA Protein	500	660	132%
Human Cells	H7N9 (A/Pigeon/Shanghai/S1069/2013) HA Protein (HA1 Subunit)	500	228	46%
Insect Cells	H7N9 (A/Pigeon/Shanghai/S1069/2013) HA Protein	500	883	177%
Insect Cells	H7N9 (A/Pigeon/Shanghai/S1069/2013) HA Protein (HA1 Subunit)	500	134	27%
Insect Cells	H7N9 (A/Hangzhou/3/2013) HA Protein	500	404	81%
Insect Cells	H7N9 (A/Zhejiang/1/2013) HA Protein	500	564	113%
Insect Cells	H7N9 (A/Shanghai/4664T/2013) HA Protein	500	401	80%
Human Cells	H7N9 (A/Shanghai/2/2013) HA Protein	500	445	89%
Insect Cells	H7N9 (A/Shanghai/2/2013) HA Protein	500	375	75%
Human Cells	H7N9 (A/Zhejiang/DTID-ZJU10/2013) HA Protein	500	192	38%
Insect Cells	H7N9 (A/Zhejiang/DTID-ZJU10/2013) HA Protein	500	632	126%
Human Cells	H7N7 (A/Netherlands/219/03) HA Protein	1000	140	14%

The representative strains that belonged to other subtype 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	H1N1 (A/Brisbane/59/2007) HA Protein
Human Cells	H1N1 (A/Puerto Rico/8/34) HA Protein
Human Cells	H1N1 (A/California/04/2009) HA Protein
Human Cells	H2N2 (A/Canada/720/2005) 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	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
No signal	Incorrect or no Detection Antibody was added	Add appropriate Detection Antibody and continue
	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
Poor Standard Curve	Standard was incompletely reconstituted or was inappropriately stored	Aliquot reconstituted standard and store at -80 ℃. The reconstituted standards should be aliquoted and avoid repeated freeze-thaw cycles.
	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
Poor detection value	The concentration of antigen in samples was too low	Enriching samples to increase the concentration of antigen
	Samples were ineffective	Check if the samples are stored at cold environment. Detect samples in timely manner
High Background	Insufficient washes	Use multichannel pipettes without touching the reagents on the plate
		Increase cycles of washes and soaking time between washes
	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
Non-specificity	Samples were contaminated	Avoid cross contamination of samples
	The concentration of samples was too high	Try higher dilution rate of samples

ASSAY SUMMARY