LSBio
an Absolute Biotech Company

Rat Orexin A ELISA Kit (Competitive EIA)

User Manual

Catalog No. LS-F4307

It is important that you read this entire manual carefully before starting your experiment.

SAMPLE ONLY

This kit is for **Research Use Only**. **Not for Diagnostic Use.**
This kit is not approved for use in humans or for clinical diagnosis.

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ASSAY SPECIFICATIONS

Target:	Orexin A
Synonyms:	Orexin A
Specificity:	This kit is for the detection of Rat Orexin A. No significant cross-reactivity or interference between Orexin A and analogs was observed. This claim is limited by existing techniques therefore cross-reactivity may exist with untested analogs.
Sample Types:	This kit is recommended for use with Rat Cell Culture Supernatants, Cell Lysates, Plasma, Serum, and Tissue Homogenates. Use with other sample types is not supported.
Detection:	Colorimetric - 450nm (TMB)
Detection Range:	12.35–1000 pg/ml
Sensitivity:	Typically less than 4.74 pg/ml
Performance:	Intra-Assay CV (<10%); Inter-Assay CV (<12%)
Limitations:	This kit is for **Research Use Only** and is not intended for diagnostic use. This kit is not approved for use in humans or for clinical diagnosis.

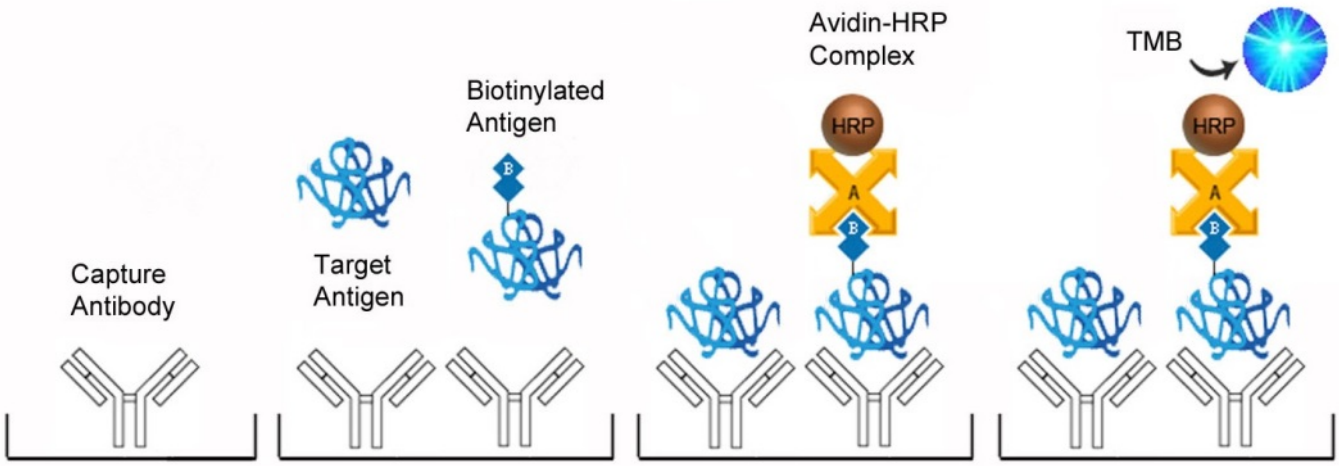
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ASSAY PRINCIPLE

This assay is based on the competition ELISA principle. Each well of the supplied microtiter plate has been pre-coated with a target specific capture antibody. Standards or samples are added to the wells as well as a fixed quantity of biotin-conjugated target antigen. The antigens in the standards or samples compete with the biotin-conjugated antigen to bind to the capture antibody. Unbound antigen is washed away. An Avidin-Horseradish Peroxidase (HRP) conjugate is then added which binds to the biotin. Unbound HRP-conjugate is washed away. A TMB substrate is then added which reacts with the HRP enzyme resulting in color development. A sulfuric acid stop solution is added to terminate color development reaction and then the optical density (OD) of the well is measured at a wavelength of 450 nm ± 2 nm. The OD of an unknown sample can then be compared to an OD standard curve generated using known antigen concentrations in order to determine its antigen concentration. In contrast to typical Sandwich ELISA assays, in competition assay the greater the amount of antigen in the sample, the lower the color development and optical density reading.

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ASSAY PRINCIPLE IMAGE



KIT COMPONENTS

Component	Quantity
Coated 96-well Strip Plate	1
Standard (Lyophilized)	2 vials
Sample Diluent	1 vial x 20 ml
Assay Diluent A	1 vial x 12 ml
Assay Diluent B	1 vial x 12 ml
Detection Reagent A (Lyophilized)	1 vial
Detection Reagent B	1 vial x 120 µl
Reagent Diluent	1 vial x 300 µl
Wash Buffer (30x)	1 vial x 20 ml
TMB Substrate	1 vial x 9 ml
Stop Solution	1 vial x 6 ml
Adhesive Plate Sealers	4
Instruction Manual	1

KIT STORAGE

Upon receipt the kit should be stored at 4°C if intended for use within 24 hours. Otherwise the Assay Plate, Standard, Detection Reagent A, and Detection Reagent B should be stored at -20°C. Avoid repeated freeze-thaw cycles. Store all other kit components at 4°C. The Substrate should never be frozen. Once individual reagents are opened it is recommended that the kit be used within 1 month. Unused Strip Plate wells should be stored at -20°C in a sealed bag containing desiccant in order to minimize exposure to moisture. Do not use the kit beyond its expiration date.

OTHER REQUIRED SUPPLIES

- Microplate reader with 450nm wavelength filter
- High-precision pipette and sterile pipette tips
- Eppendorf tubes
- 37°C incubator
- Deionized or distilled water
- Absorbent paper

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EXPERIMENTAL LAYOUT

The following is an example of how to layout a study. A dilution series of the positive control Standard should be run in duplicate or triplicate, with the last well in each series being the negative control blank. Samples should also be run in duplicate or triplicate. Unknown samples should be run as a dilution series in order to identify the optimal dilution that produces an OD reading within the OD range of the positive control Standard dilution series.

Example 1: Standard Curve and dilution series of an unknown sample.

	1	2	3	4	...
A	Standard Dilution 1	Standard Dilution 1	Sample (1:1)	Sample (1:1)	...
B	Standard Dilution 2	Standard Dilution 2	Sample (1:10)	Sample (1:10)	...
C	Standard Dilution 3	Standard Dilution 3	Sample (1:100)	Sample (1:100)	...
D	Standard Dilution 4	Standard Dilution 4	Sample (1:1k)	Sample (1:1k)	...
E	Standard Dilution 5	Standard Dilution 5	Sample (1:10k)	Sample (1:10k)	...
F	Negative Control	Negative Control	Sample (1:100k)	Sample (1:100k)	...
G			Sample (1:1,000k)	Sample (1:1,000k)	...
H			Sample (1:10,000k)	Sample (1:10,000k)	...

Example 2: Standard Curve and samples run in duplicate.

	1	2	3	4	...
A	Standard Dilution 1	Standard Dilution 1	Sample A	Sample E	...
B	Standard Dilution 2	Standard Dilution 2	Sample A	Sample E	...
C	Standard Dilution 3	Standard Dilution 3	Sample B	Sample F	...
D	Standard Dilution 4	Standard Dilution 4	Sample B	Sample F	...
E	Standard Dilution 5	Standard Dilution 5	Sample C	Sample G	...
F	Negative Control	Negative Control	Sample C	Sample G	...
G			Sample D	Sample H	...
H			Sample D	Sample H	...

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SAMPLE COLLECTION

This assay is recommended for use with Rat Cell Culture Supernatants, Cell Lysates, Plasma, Serum, and Tissue Homogenates. Use with other sample types is not supported. The sample collection protocols below have been provided for your reference.

Breast Milk - Centrifuge samples for 20 minutes at 1000×g to remove particulates. Collect the supernatant for assaying. Store un-diluted samples at -20°C or below. Avoid repeated freeze-thaw cycles.

Cell Lysates - Collect and pellet the cells by centrifugation and remove the supernatant. Wash the cells 3 times with PBS* then resuspend in PBS*. Lyse the cells by ultrasonication 4 times. Alternatively freeze the cells to -20°C and thaw to room temperature 3 times. Centrifuge at 1500×g for 10 minutes at 2 - 8°C to remove cellular debris. Collect the supernatant for assaying. Store un-diluted samples at -20°C or below. Avoid repeated freeze-thaw cycles.

Erythrocyte Lysates - Centrifuge whole blood for 20 minutes at 1000×g to pellet the cells and remove the supernatant. Wash the cells 3 times with PBS* then resuspend in PBS*. Freeze (-20°C)/thaw (room temperature) the cells 3 times. Centrifuge at 5,000×g for 10 minutes at 2-8°C to remove cellular debris. Collect the supernatant for assaying. Erythrocyte lysates must be diluted with Standard Diluent before running. Store un-diluted samples at -20°C or below. Avoid repeated freeze-thaw cycles.

Plasma - Collect plasma using heparin or EDTA as an anticoagulant. Centrifuge samples for 15 minutes at 1000×g at 2–8°C within 30 minutes of collection. Collect the supernatant for assaying. Store un-diluted samples at -20°C or below. Avoid repeated freeze-thaw cycles.

Platelet-Poor Plasma - Collect plasma using heparin or EDTA as an anticoagulant. Centrifuge samples for 15 minutes at 1000×g at 2–8°C within 30 minutes of collection. It is recommended that samples should be centrifuged for 10 minutes at 10,000×g for complete platelet removal. Collect the supernatant for assaying. Store un-diluted samples at -20°C or below. Avoid repeated freeze-thaw cycles.

Sperm and Seminal Plasma - Allow semen to liquefy at room temperature or 37°C. After liquefaction, centrifuge at 2,000×g for 10-15 minutes. Collect seminal plasma supernatant for assaying. Wash the

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precipitated protein 3 times with PBS* then resuspend in PBS*. Lyse the cells by ultrasonication then centrifuge at 2,000×g for 10-15 minutes. Collect the supernatant for assaying. Store un-diluted samples at -20°C or below. Avoid repeated freeze-thaw cycles.

Serum - Use a serum separator tube and allow samples to clot for 2 hours at room temperature or overnight at 4°C before centrifugation for 20 minutes at approximately 1000×g. Collect the supernatant for assaying. Store un-diluted samples at -20°C or below. Avoid repeated freeze-thaw cycles.

Tissue Homogenates - Because preparation methods for tissue homogenates vary depending upon tissue type, users should research tissue specific conditions independently. The following is one example only. Rinse tissues in PBS* to remove excess blood and weigh before homogenization. Finely mince tissues and homogenize them in 5-10mL of PBS*with a glass homogenizer on ice. Lyse the cells by ultrasonication or freeze (-20°C)/thaw (room temperature) 3 times. Centrifuge homogenate at 5000×g for 5 minutes. Collect the supernatant for assaying. Store un-diluted samples at -20°C or below. Avoid repeated freeze-thaw cycles.

Urine - Aseptically collect the first urine of the day (mid-stream), voided directly into a sterile container. Centrifuge to remove particulate matter and collect the supernatant for assaying. Store un-diluted samples at -20°C or below. Avoid repeated freeze-thaw cycles.

Cell culture supernatants, cerebrospinal, follicular, and lung lavage fluids, saliva, sweat, tears, and other biological fluids - Centrifuge samples for 20 minutes at 1000×g to remove particulates. Collect the supernatant for assaying. Store un-diluted samples at -20°C or below. Avoid repeated freeze-thaw cycles.

* 1xPBS (0.02mol/L pH7.0-7.2)

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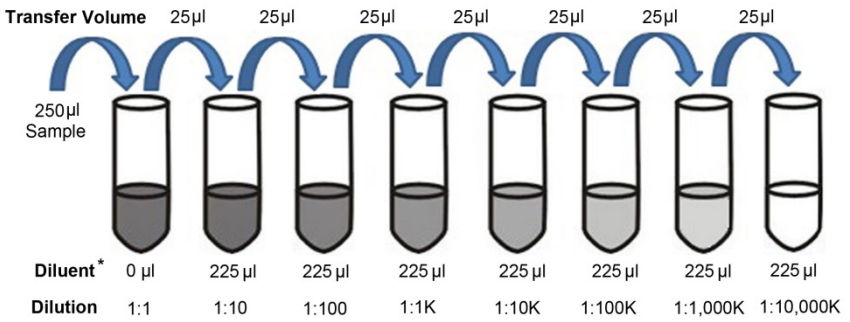
SAMPLE COLLECTION NOTES

1. LSBio recommends that samples are used immediately upon preparation.
2. Avoid repeated freeze/thaw cycles for all samples.
3. In the event that a sample type not listed above is intended to be used with the kit, it is recommended that the customer conduct validation experiments in order to be confident in the results.
4. Due to chemical interference, the use of tissue or cell extraction samples prepared by chemical lysis buffers may result in inaccurate results.
5. Due to factors including cell viability, cell number or sampling time, samples from cell culture supernatant may not be detected by the kit.
6. Samples should be brought to room temperature (18-25°C) before performing the assay without the use of extra heating.
7. Sample concentrations should be predicted before being used in the assay. If the sample concentration is not within the range of the standard curve, users must determine the optimal sample dilutions for their particular experiments.
8. LSBio is responsible for the quality and performance of the kit components but is NOT responsible for the performance of customer supplied samples used with the kit.

SAMPLE ONLY

SAMPLE PREPARATION

The resulting Optical Density (OD) values of your sample must fall within the OD values of the standard curve in order for the calculated antigen concentration to be accurate. In many cases samples will need to be diluted in order to lower the antigen concentration to sufficient levels. Information about antigen concentrations within various sample types may be available from the published literature; however, it is often necessary to run a dilution series of each sample type. The following will prepare sufficient volumes to run the Sample dilution series in duplicate. In the case of small volume samples the first step in the series can be a dilution, like 1:5 or 1:10, rather than undiluted sample. Running duplicate or triplicate wells for each sample is recommended. * **Always dilute samples in the same buffer as the Standard used to generate the Standard Curve**.



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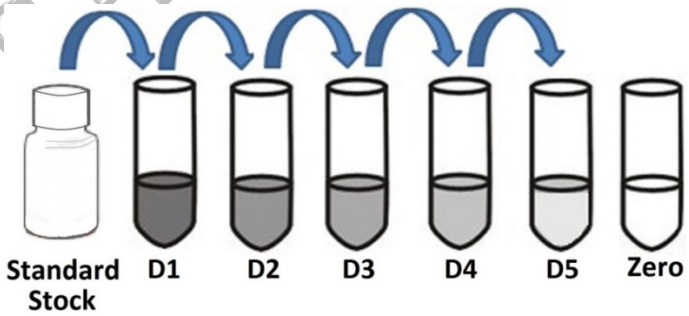
STANDARD PREPARATION

The following are instructions for the preparation of a Standard dilution series which will be used to generate the standard curve. The standard curve is then used to determine the concentration of target antigen in unknown samples (see the **Calculation of Results** section). The following will prepare sufficient volumes to run the Standard dilution series in duplicate. ResuspendedStandard and prepared standard dilutions should be used immediately and not stored for future use.

Standard Stock Solution (1000 pg/ml): Briefly centrifuge the vial to ensure that all lyophilisate is collected at the bottom of the vial. Resuspend 1 tube of Standard with 1.0 ml of Sample Diluent. Incubate at room temperature for 10 minutes with gentle agitation (avoid foaming).

D1 (1000 pg/ml): Pipette 400µl of Stock Standard into 0µl of Sample Diluent
D2 (333.3 pg/ml): Pipette 300µl of D1 into 600µl of Sample Diluent
D3 (111.1 pg/ml): Pipette 300µl of D2 into 600µl of Sample Diluent
D4 (37.03 pg/ml): Pipette 300µl of D3 into 600µl of Sample Diluent
D5 (12.34 pg/ml): Pipette 300µl of D4 into 600µl of Sample Diluent

Zero Standard (0 pg/ml): Use Sample Diluent alone



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REAGENT PREPARATION

Bring all reagents to room temperature (18-25°C) before use.

Detection Reagent A and B: Reconstitute Detection Reagent A in 150 µl of Reagent Diluent at room temperature for 10 minutes with gentle agitation. Use the Detection Reagent A and B stocks to prepare sufficient volumes of Detection Reagent A and B Working Solution for the number of wells you are planning to run. Dilute Detection Reagent A and B to a ratio of 1:100 using Assay Diluent A and B respectively.

Wash Buffer: If crystals have formed in the concentrate, warm to room temperature and mix it gently until crystals have completely dissolved. Prepare 600 ml of Working Wash Buffer by diluting the supplied 20 ml of 30x Wash Buffer Concentrate with 580 ml of deionized or distilled water. Wash Buffer can be stored at 4°C once prepared.

TMB Substrate Solution: Using sterile techniques remove the needed volume of TMB Substrate Solution for the number of wells you are planning to run. Dispose of unused TMB Substrate Solution rather than returning it to the stock container.

SAMPLE ONLY

REAGENT PREPARATION NOTES

1. It is highly recommended that standard curves and samples are run in duplicate within each experiment.
2. Once resuspended, standards should be used immediately, and used only once. Long-term storage of resuspended standards is NOT recommended.
3. All solutions prepared from concentrates are intended for one-time use. Do not reuse solutions.
4. Do not prepare Standard dilutions directly in wells.
5. Prepared Reagents may adhere to the tube wall or cap during transport; centrifuge tubes briefly before opening.
6. All solutions should be gently mixed prior to use.
7. Reconstitute stock reagents in strict accordance with the instructions provided.
8. To minimize imprecision caused by pipetting, ensure that pipettes are calibrated. Pipetting volumes of less than 10µL is not recommended.
9. TMB Substrate solution is easily contaminated; sterility precautions should be taken. TMB Substrate solution should also be protected from light.
10. Do not substitute reagents from one kit lot to another. Use only those reagents supplied within this kit.
11. Due to the antigen specificity of the antibodies used in this assay, native or recombinant proteins from other manufacturers may not be detected by this kit.

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ASSAY PROCEDURE

Bring all reagents and samples to room temperature without additional heating and mix thoroughly by gently swirling before pipetting (avoid foaming). Prepare all reagents, working standards, and samples as directed in the previous sections.

1. Add 50µl of **Standard, Blank, or Sample** per well.
2. Immediately add 50µl of **Detection Reagent A** working solution to each well, cover with a plate sealer, gently agitate to ensure thorough mixing, and incubate for 1 hour at 37°C.
3. Aspirate the liquid from each well and wash 3 times. Wash by adding approximately 350 µl of Wash Buffer using a squirt bottle, multi-channel pipette, manifold dispenser or automated washer. Allow each wash to sit for 1-2 minutes before completely aspirating. After the last wash, aspirate to remove any remaining Wash Buffer then invert the plate and tap against clean absorbent paper.
4. Add 100µl of **Detection Reagent B** working solution to each well, gently agitate to ensure thorough mixing, cover with a new plate sealer, and incubate for 30 minutes at 37°C.
5. Aspirate the liquid from each well and wash 5 times as outlined in step 3.
6. Add 90µl of **TMB Substrate solution** to each well, gently agitate to ensure thorough mixing, cover with a new plate sealer, and incubate for 10-20 minutes at 37°C. Protect from light and monitor periodically until optimal color development has been achieved.
7. Add 50µl of **Stop Solution** to each well in the same order and timing as the TMB Substrate solution and gently agitate to ensure thorough mixing. The blue color will change to yellow immediately.
8. Determine the optical density (OD value) of each well immediately using a microplate reader set to 450 nm.

SAMPLE ONLY

ASSAY PROCEDURE NOTES

1. **ELISA Plate**: Keep appropriate numbers of strips for 1 experiment and remove extra strips from microtiter plate. Removed strips should be placed in a sealed bag containing desiccant and stored at -20°C.
2. **Solutions**: In the event that Detection Reagent A working solution appears cloudy, warm to room temperature and mix gently until solution appears uniform. To avoid cross-contamination, change pipette tips between additions of each standard, between sample additions, and between reagent additions. Also, use separate reservoirs for each reagent.
3. **Applying Solutions**: All solutions should be added to the bottom of the ELISA plate well. Avoid touching the inside wall of the well. Avoid foaming when possible.
4. **Assay Timing**: The interval between adding sample to the first and last wells should be minimized. Delays will increase the incubation time differential between wells, which will significantly affect the experimental accuracy and repeatability. For each step in the procedure, total dispensing time for addition of reagents or samples should not exceed 10 minutes.
5. **Incubation**: To prevent evaporation and ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary. Do not allow wells to sit uncovered for extended periods of time between incubation steps. Do not let wells dry out at any time during the assay. Strictly observe the recommended incubation times and temperatures.
6. **Washing**: Proper washing procedure is critical. Insufficient washing will result in poor precision and falsely elevated absorbance readings. Residual liquid in the reaction wells should be patted dry against absorbent paper during the washing process. Do not put absorbent paper directly into the reaction wells.
7. **Controlling Substrate Reaction Time**: After the addition of the TMB Substrate, periodically monitor the color development. Stop color development before the color becomes too deep by adding Stop Solution. Excessively strong color will result in inaccurate absorbance reading.

SAMPLE ONLY

8. **Reading**: The microplate reader should be preheated and programmed prior to use. Prior to taking OD readings, remove any residual liquid or fingerprints from the underside of the plate and confirm that there are no bubbles in the wells.
9. **Reaction Time Control**: Control reaction time should be strictly followed as outlined.
10. **Stop Solution**: The Stop Solution contains an acid, therefore proper precautions should be taken during its use, such as protection of the eyes, hands, face, and clothing.
11. **Mixing**: During incubation times, the use of a micro-oscillator at low frequency is recommended. Sufficient and gentle mixing is particularly important in producing reliable results.
12. Kits from different batches may be a little different in detection range, sensitivity, and color developing time. Please perform the experiment exactly according to the supplied instructions.
13. Due to inter- and intra-assay variability, it is recommended that appropriate carry-over controls be included between assays.
14. Prior to running valuable samples, LSBio recommends that the user run a preliminary experiment using the supplied controls in order to validate the assay.
15. To minimize external influence on the assay performance, operational procedures and lab conditions (such as room temperature, humidity, and incubator temperature) should be strictly controlled. It is also strongly suggested that the whole assay is performed by the same operator from the beginning to the end.
16. The kit should not be used beyond the expiration date on the kit label.

SAMPLE ONLY

ASSAY PROCEDURE SUMMARY

Prepare all reagents, samples and standards.

Add 50µl of **Sample**, **Standard**, or **Blank** to each well.

Immediately add 50µl of **Detection Reagent A** and incubate for 1 hour at 37°C.

Aspirate and wash 3 times.

Add 100µl of **Detection Reagent B** and incubate for 30minutes at 37°C.

Aspirate and wash 5 times.

Add 90µl of **TMB Substrate** solution and incubate for 10-20 minutes at 37°C.

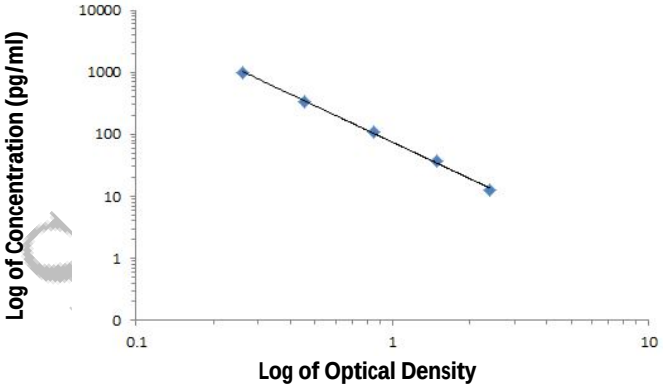
Add 50µl of **Stop Solution**.

Read immediately at 450nm.

CALCULATION OF RESULTS

Average the duplicate readings for each standard, control, and sample. Create a standard curve by plotting the mean absorbance for each standard on the x-axis against the target antigen concentration on the y-axis and draw a best fit curve through the points on the graph. The data may be linearized by plotting the log of the target antigen concentration on the Y axis versus the O.D. of the standards on the X axis and the best fit line can be determined by regression analysis. The linear equation (Y = mx + b) can be used to calculate the standard curve where y is the log of the concentration of the standard and x is the OD value of the standard. If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

Typical Data: The following standard curve is an example only and should not be used to calculate results for tested samples. A new standard curve must be generated for each set of samples tested.



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TROUBLESHOOTING GUIDE

Problem	Possible Cause	Solution
Poor standard curve	Inaccurate pipetting	Check pipettes.
	Improper standard dilution	Briefly spin the vial of standard and dissolve the powder thoroughly by a gentle mix.
	Wells not completely aspirated	Completely aspirate wells between steps.
Low signal	Too brief incubation times	Ensure sufficient incubation time.
	Incorrect assay temperature	Use recommended incubation temperature. Bring substrate to room temperature before use.
	Inadequate reagent volumes	Check pipettes and ensure correct preparation.
	Improper dilution	
Deep color but low value	Plate reader settings not optimal	Verify the wavelength and filter setting in the plate reader.
		Open the Plate Reader ahead to pre-heat.

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TROUBLESHOOTING GUIDE (continued)

Problem	Possible Cause	Solution
Large CV	Inaccurate pipetting	Check pipettes.
High background	Concentration of detector too hig	Use recommended dilution factor.
	Plate is insufficiently washed	Review the manual for proper washing instructions. If using a plate washer, check that all ports are unobstructed.
	Contaminated wash buffer	Make fresh wash buffer.
Low sensitivity	Improper storage of the ELISA kit	All the reagents should be stored according to the instructions.
	Stop solution not added	Stop solution should be added to each well before measurement.

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Important Note: During shipment, small volumes of product will occasionally become entrapped in the seal of the product vial. We recommend briefly centrifuging the vial to dislodge any liquid in the container's cap prior to opening.

Warning: This reagent may contain sodium azide and sulfuric acid. The chemical, physical, and toxicological properties of these materials have not been thoroughly investigated. Standard Laboratory Practices should be followed. Avoid skin and eye contact, inhalation, and ingestion. Sodium azide forms hydrazoic acid under acidic conditions and may react with lead or copper plumbing to form highly explosive metal azides. On disposal, flush with large volumes of water to prevent accumulation.

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