LSBio
an Absolute Biotech Company

Human LCN2 / Lipocalin 2 / NGAL CLIA Kit (Sandwich CLIA)

User Manual

Catalog No. LS-F26058

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

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

SAMPLE ONLY

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ASSAY SPECIFICATIONS

Target:	LCN2 / Lipocalin 2 / NGAL
Synonyms:	LCN2 / Lipocalin 2 / NGAL, LCN2, lipocalin 2, 24p3, HNL, Lipocalin 2, Lipocalin-2, MSFI, Siderocalin LCN2, NGAL, p25, Lipocalin 2 (oncogene 24p3), Oncogene 24p3
Specificity:	This kit is for the detection of Human LCN2 / Lipocalin 2 / NGAL. No significant cross-reactivity or interference between LCN2 / Lipocalin 2 / NGAL 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 Human Cell Culture Supernatants, Cell Lysates, Plasma, Serum, Tissue Homogenates, and Urine. Use with other sample types is not supported.
Detection:	Chemiluminescent
Measurement:	Quantitative
Detection Range:	27.43–20000 pg/ml
Sensitivity:	Typically less than 27.43 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 sandwich CLIA 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 and the target antigen binds to the capture antibody. Unbound standard or sample is washed away. A biotin-conjugated detection antibody is then added which binds to the captured antigen. Unbound detection antibody is washed away. An Avidin-Horseradish Peroxidase (HRP) conjugate is then added which binds to the biotin. Unbound Avidin-HRP conjugate is washed away. A chemiluminescent substrate is then added which reacts with the HRP enzyme resulting in light development. The relative light units (RLU) of each well are measured using a luminometer. The RLU of an unknown sample can then be compared to an RLU standard curve generated using known antigen concentrations in order to determine its antigen concentration.

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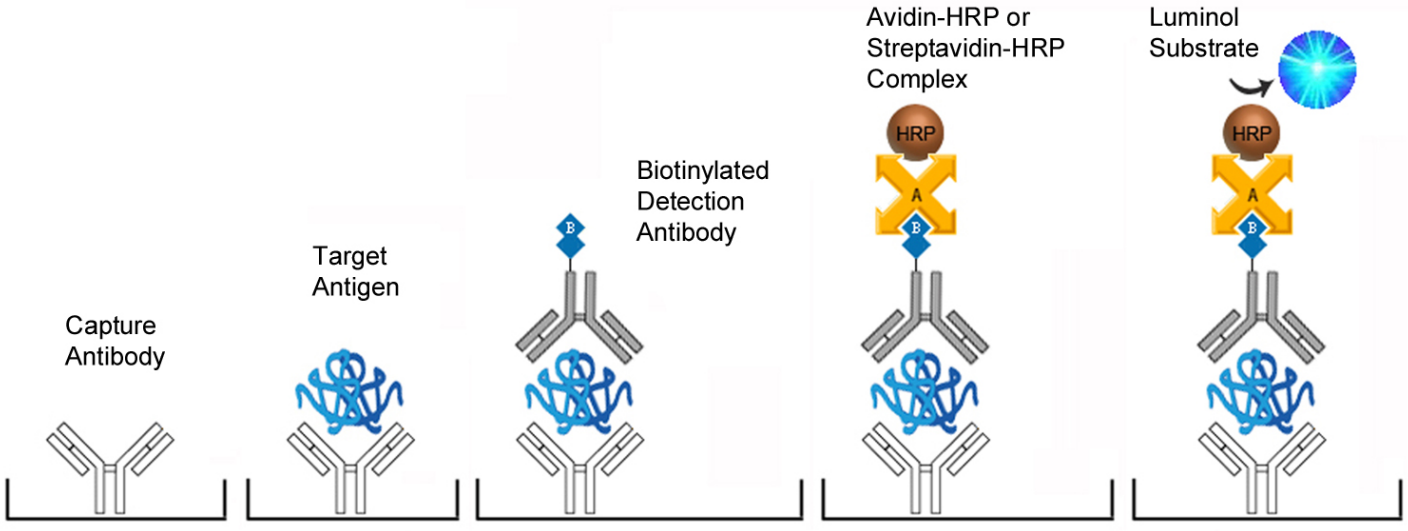
ASSAY PRINCIPLE IMAGE



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	1 vial x 120 µl
Detection Reagent B	1 vial x 120 µl
Wash Buffer (30x)	1 vial x 20 ml
Substrate A	1 vial x 10 ml
Substrate B	1 vial x 2 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 Substrates 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

- Luminometer capable of reading 96-well microplates with the following parameters: lag time 30.0secs; read time 1.0 sec/well.
- 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 RLU reading within the RLU 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	Standard Dilution 6	Standard Dilution 6	Sample (1:100k)	Sample (1:100k)	...
G	Standard Dilution 7	Standard Dilution 7	Sample (1:1,000k)	Sample (1:1,000k)	...
H	Negative Control	Negative Control	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	Standard Dilution 6	Standard Dilution 6	Sample C	Sample G	...
G	Standard Dilution 7	Standard Dilution 7	Sample D	Sample H	...
H	Negative Control	Negative Control	Sample D	Sample H	...

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SAMPLE COLLECTION

This assay is recommended for use with Human Cell Culture Supernatants, Cell Lysates, Plasma, Serum, Tissue Homogenates, and Urine. 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 Sample Diluent before running. Store un-diluted samples at -20°C or below. Avoid repeated freeze-thaw cycles.

Plasma - Collect plasma using EDTA or heparin 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 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.

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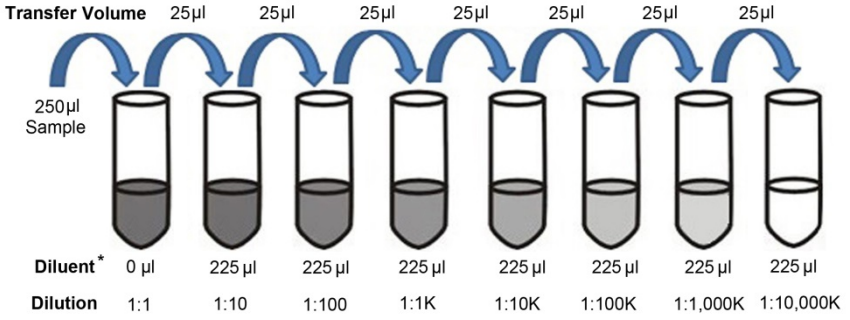
SAMPLE PREPARATION

The resulting RLU values of your sample must fall within the RLU 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, a preliminary step dilution, such as 1:5 or 1:10, can be made using PBS (0.02mol/L pH7.0-7.2) as the diluent.

* **The final dilution should always be made using the same buffer that is used to dilute the Standards, and/or generate the Standard Curve.** Running duplicate or triplicate wells for each sample is recommended.



Special Cases

Serum and plasma typically require a 100-fold dilution in order for their OD readings to fall within the Standard Curve of this assay. However, due to sample variation, it is recommended that researchers determine the optimal dilution for their samples as described above.

STUDY ONLY

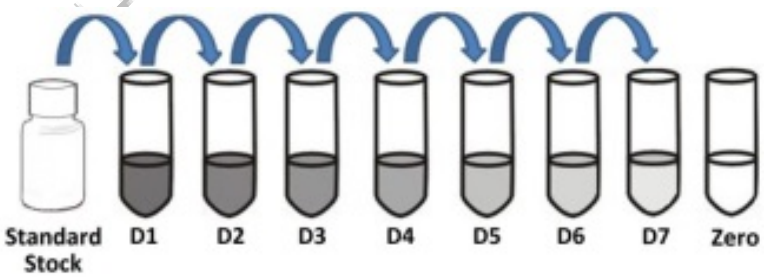
STANDARD PREPARATION

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). Prepare standards 15 minutes before running the assay. The following will prepare sufficient volumes to run the Standard dilution series in duplicate. Resuspended standard and prepared standard dilutions should be used immediately and not stored for future use.

Standard Stock Solution (20,000 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 (20000 pg/ml): Pipette 900µl ofStandard Stock Solution into 0µl of Sample Diluent
D2 (6667 pg/ml): Pipette 300µl of D1 into 600µl of Sample Diluent
D3 (2222 pg/ml): Pipette 300µl of D2 into 600µl of Sample Diluent
D4 (740.7 pg/ml): Pipette 300µl of D3 into 600µl of Sample Diluent
D5 (246.9 pg/ml): Pipette 300µl of D4 into 600µl of Sample Diluent
D6 (82.3 pg/ml): Pipette 300µl of D5 into 600µl of Sample Diluent
D7 (27.43 pg/ml): Pipette 300µl of D6 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: 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.

Working Substrate Solution: Substrate should be prepared no more than 15 minutes before running the assay. Estimate the volume of Working Substrate Solution for the number of wells you are planning to run. Prepare this volume by combining Substrate A and Substrate B by a ratio of 99:1 and mix thoroughly. For example, to prepare 1,000 µL of Working Substrate Solution, combine 990 µL of Substrate A with 10 µL of Substrate B. Dispose of unused Working Substrate Solution rather than returning it to the stock container.

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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. Substrate solutions are easily contaminated; sterility precautions should be taken. Substrate solutions 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 100µl of **Standard, Blank, or Sample** per well, cover with a plate sealer, and incubate for 1 hour at 37°C.
2. Aspirate the liquid of each well, don't wash.
3. Add 100µl of **Detection Reagent A** working solution to each well, cover with a plate sealer, and gently agitate to ensure thorough mixing. Incubate for 1 hour at 37°C.
4. 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.
5. Add 100µl of **Detection Reagent B** working solution to each well, cover with a new plate sealer, and incubate for 30 minutes at 37°C.
6. Aspirate the liquid from each well and wash 5 times as outlined in step 4.
7. Add 100µl of **Working Substrate Solution** to each well, cover with a new plate sealer, and incubate for 10 minutes at 37°C. Do not exceed 10 minutes and protect from light.
8. Determine the Relative Light Units (RLU) of each well immediately using a microplate luminometer.

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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 RLU 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. **Reading**: The microplate reader should be preheated and programmed prior to use. Prior to taking RLU readings, remove any residual liquid or fingerprints from the underside of the plate and confirm that there are no bubbles in the wells.
8. **Reaction Time Control**: Control reaction time should be strictly followed as outlined.

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9. **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.
10. 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.
11. Due to inter- and intra-assay variability, it is recommended that appropriate carry-over controls be included between assays.
12. Prior to running valuable samples, LSBio recommends that the user run a preliminary experiment using the supplied controls in order to validate the assay.
13. To minimize external influence on the assay performance, operational procedures and lab conditions (such as room temperature, humidity, 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.
14. The kit should not be used beyond the expiration date on the kit label.

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ASSAY PROCEDURE SUMMARY

Prepare all reagents, samples and standards.

Add 100 µl **Standard, Sample, or Blank** to each well and incubate for 1 hour at 37°C.

Aspirate and add 100 µl prepared **Detection Reagent A** and incubate of 1 hour at 37°C.

Aspirate and wash 3 times.

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

Aspirate and wash 5 times.

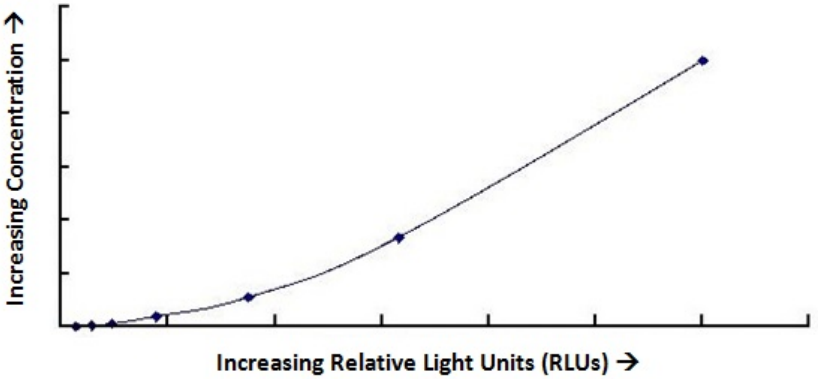
Add 100 µl **Substrate Solution** and incubate for 10 minutes at 37°C.

Read luminescence immediately.

CALCULATION OF RESULTS

Average the duplicate RLU readings for each standard, control, and sample and subtract the average blank standard RLU value. Create a standard curve by plotting the mean RLU 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 RLU 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 RLU 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.
Large CV	Inaccurate pipetting	Check pipettes.
Low sensitivity	Improper storage of the ELISA kit	All the reagents should be stored according to the instructions.
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	

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Troubleshooting Guide (continued)

Problem	Possible Cause	Solution
High background	Concentration of detector too high	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.

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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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CAUTION RESEARCH ONLY

LS Bio
an Absolute Biotech Company

2 Shaker Rd Suite B101 Shirley, MA 01464
Tel: 206.374.1102
Fax: 206.577.4565
Technical.Support@LSBio.com