Sample Manual

The following sample ELISA manual is based upon the Sandwich ELISA assay principle.

LSBio strives to develop all Custom ELISAbased upon the Sandwich assay principle; however, in the event that this is no possible, Competitive EIA assay principles may be used.

Specifications such as Range, Sensitivity, and Precision are defined upon delivery of the final product.

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 Manual

LSBio

LifeSpan BioSciences, Inc.

Chicken Glycated Albumin ELISAKit (Sandwich ELISA)

User Manual

Catalog No. LS-F44466

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

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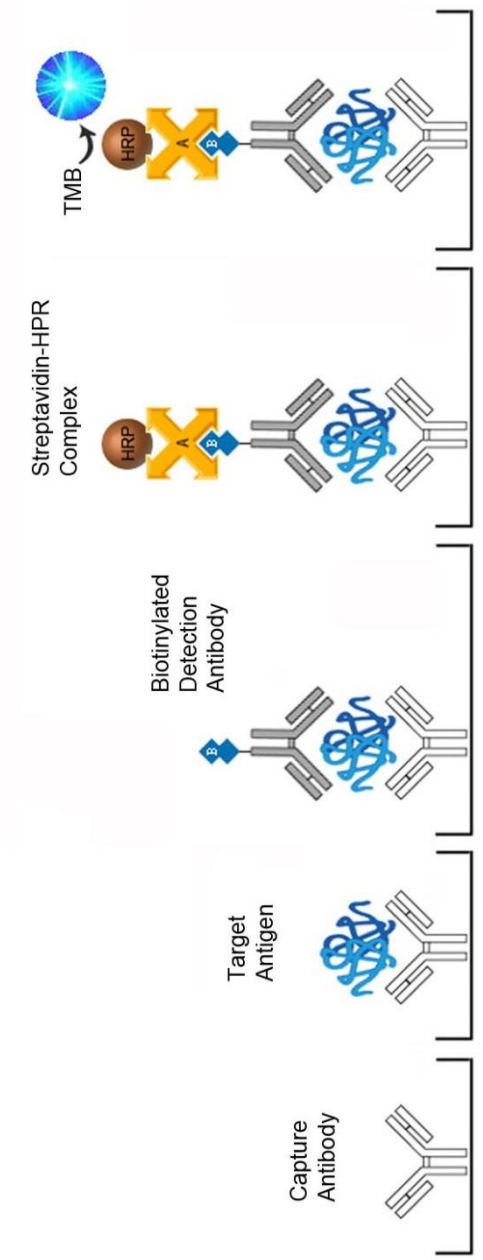
ASSAYSPECIFICATIONS

Target:	[Target Common Name]
Synonyms:	[Synonyms]
Specificity:	This kit is for the detection of [Target Species] [Target Common Name]. [Add cross-reactivity data]
Sample Types:	This kit is intended for use with samples such as [Target Species] [add samples types]. It has been empirically tested using the standard supplied with the kit (typically recombinant protein) and [add specific testing].
Detection:	Colorimetric - 450nm
Detection Range:	[add detection range]
Sensitivity:	[add sensitivity]
Performance:	IntraAssayCV; InterAssayCV[add values]
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.

ASSAYPRINCIPLE

This assay is based on the sandwich ELISA principle. Each well of the supplied microtiter plate has been pre-coated with atarget 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 TMB substrate is then added which reacts with the HRP enzymeresulting 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 measuredat a wavelength of 450 nm ± 2 nm. An OD standard curve is generated using known antigen concentrations; the OD of an unknown sample can then be compared to the standard curve in order to determine its antigen concentration.

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
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 TMB 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

ASSAYPLANNING

Before using this kit, researchers should consider the following:

1. Read this manual in its entirety in order to minimize the chance of error.
2. Confirm that you have the appropriate non-supplied equipment available.
3. Confirm that the species, target antigen, and sensitivity of this kit are appropriate for your intended application.
4. Confirm that your samples have been prepared appropriately based upon recommendations (see Sample Preparation) and that you have sufficient sample volume for use in the assay.
5. When first using a kit, appropriate validation steps should be taken before using valuable samples. Confirm that the kit adequately detects the target antigen in your intended sample type(s) by running control samples.
6. If the concentration of target antigen within your samples is unknown, a preliminary experiment should be run using a control sample to determine the optimal sample dilution (see Experimental Layout and Sample Preparation).
7. Ensure that the kit is properly stored and do not use it beyond its expiration date.
8. When using multiple lots of the same kit do not substitute reagents from one kit to another. Review each manual carefully as changes can occur between lots. To control for inter-assay variability include a carry-over control sample

EXPERIMENTALLAYOUT

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	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	…

SAMPLECOLLECTION

This assay is intended for use with samples such as [TargetSpecies] [Sample Types]. 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.

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.

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 SampleDiluent before running.

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.

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.

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 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.

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.

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.

Urine - Aseptically collect the first urine of the day (mid-stream), voided directly into a sterile container. Centrifugeto remove particulate matter and collect the supernatant for assaying.

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.

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

SAMPLE COLLECTION NOTES

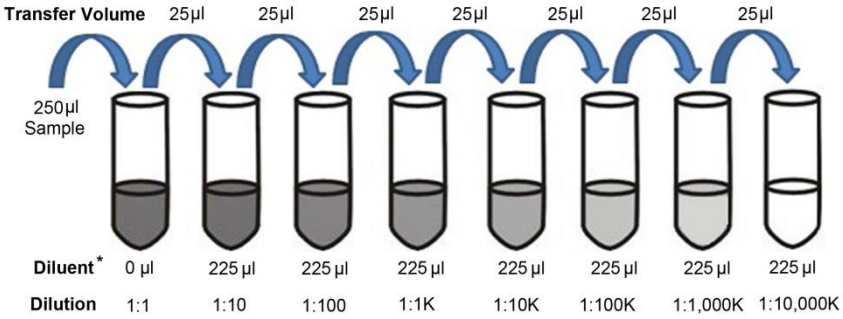
1. LifeSpan recommends that samples are used immediately upon preparation. Alternatively, samples stored at 2-8°C should be used within 5 days. For long-term storage sample aliquots should be prepared and stored at -20°C if used within 1 month, or -80°C if used within 6 months. Long term storage can result in protein degradation and denaturation, which may result in inaccurate results.
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. LifeSpan 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.

SAMPLEPREPARATION

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 triplicate. 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.



[List of any specific sample preparation details.]

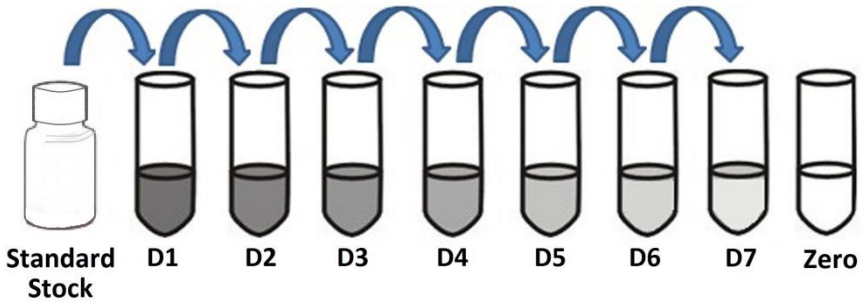
STANDARDPREPARATION

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. Reconstituted Standard and prepared standard dilutions should be used immediately and not stored for future use.

Standard Stock Solution (x Units): Reconstitute 1 tube of lyophilized Standard with x ml of Sample Diluent. Incubate at room temperature for 10 minutes with gentle agitation (avoid foaming).

D1 (x Units):	Pipette a µl of Stock Standard into x µl of Sample Diluent
D2 (x Units):	Pipette 250µl of D1 into 250µl of Sample Diluent
D3 (x Units):	Pipette 250µl of D2 into 250µl of Sample Diluent
D4 (x Units):	Pipette 250µl of D3 into 250µl of Sample Diluent
D5 (x Units):	Pipette 250µl of D4 into 250µl of Sample Diluent
D6 (x Units):	Pipette 250µl of D5 into 250µl of Sample Diluent
D7 (x Units):	Pipette 250µl of D6 into 250µl of Sample Diluent

Zero Standard (0 Units): Use Sample Diluent alone.



REAGENTPREPARATION

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.

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 by diluting them 1:100 with 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.

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 reconstituted standards is NOT recommended.
3. In order to avoid repeat freeze thaws, prepare one-time use aliquots of standard for long term storage at -20/-80°C.
4. All solutions prepared from concentrates are intended for one-time use. Do not reuse solutions.
5. Do not prepare Standard dilutions directly in wells.
6. Prepared Reagents may adhere to the tube wall or cap during transport; centrifuge tubes briefly before opening.
7. All solutions should be gently mixed prior to use.
8. Reconstitute stock reagents in strict accordance with the instructions provided.
9. To minimize imprecision caused by pipetting, ensure that pipettes are calibrated. Pipetting volumes of less than 10µL is not recommended.
10. TMB Substrate solution is easily contaminated; sterility precautions should be taken. TMB Substrate solution should also be protected from light.
11. Do not substitute reagents from one kit lot to another. Use only those reagents supplied within this kit.
12. 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.

ASSAYPROCEDURE

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 2 hours 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.

   Add 100µl of **Detection Reagent B** working solution to each well, cover with a new plate sealer, and incubate for 30-60 minutes at 37°C.

   Aspirate the liquid from each well and wash 5 times as outlined in step 4.
5. Add 90 µl of **TMB Substrate** solution to each well, cover with a new plate sealer, and incubate for 15-30 minutes at 37°C. Protect from light and monitor periodically until optimal color development has been achieved.
6. Add 50 µl of **Stop Solution** to each well. The blue color will change to yellow immediately. If color change does not appear uniform, gently tap the plate to ensure thorough mixing. The Stop Solution should be added to wells in the same order and timing as the TMB substrate solution.
7. Determine the optical density (OD value) of each well immediately using a microplate reader set to 450 nm.
8.

ASSAYPROCEDURENOTES

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**: 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 readings.

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. 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.

ASSAY PROCEDURE SUMMARY

Prepare all reagents, samples and standards.

Add 100 µl of **Sample**, **Standard**, or **Blank** to each well and incubate for 2 hours at 37°C.

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

Aspirate and wash 3 times.

Add 100 µl of **Detection Reagent B** and incubate for 30-60 minutes at 37°C.

Aspirate and wash 5 times.

Add 90 µl of **TMB Substrate** solution and incubate for 15-30 minutes at 37°C.

Add 50 µl of **Stop Solution**.

Read immediately at 450nm.

CALCULATIONOFRESULTS

Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density. Create a standard curve by reducing the data using computer software capable of generating a four parameter logistic (4-PL) curve-fit. As an alternative, construct a standard curve by plotting the mean absorbance for each standard on the x-axis against the 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 concentrations versus the log of the O.D. and the best fit line can be determined by regression analysis. Use of a commercial software program such as CurveExpert is recommended for performing this calculation. This procedure will produce an adequate but less precise fit of the data. 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.

Concentration (ng/ml)

[Standard Curve Image]

Optical Density

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.
		Turn on and warm-up the plate reader prior to use.

Troubleshooting Guide (continued)

Problem	Possible Cause	Solution
Large CV	Inaccurate pipetting	Check pipettes
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.
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.

ASSAY USAGE AND SUPPORT

This kit is for **Research Use Only** andis notintended for diagnostic use. This kit is not approved for use in humans or for clinical diagnosis. This kit should not be used beyond the expiration date printed on the lot specific kit label.

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.

The LifeSpan Guarantee: LifeSpan guarantees the integrity of all components contained with an immunoassay kit, and that the standards provided will produce a standard curve sufficient for the quantification of target antigen concentrations that fall within the specified range of the kit. Due to the variable nature of sample types and preparations, LifeSpan cannot guarantee that the target antigen will be detectable in customer-supplied samples. For this reason, LifeSpan strongly recommends that customers conduct validation experiments, using positive control samples generated in a similar manner to the experimental samples, before using valuable research specimens. Due to the perishable nature of ELISA kits, orders of greater than 5 units of a single catalog number cannot be returned upon shipment, and are not eligible for refund.

Technical Support: LifeSpan's knowledgeable staff scientists are available to answer any questions about this kit. Email your detailed questions to Technical.Support@LSBio.com.

RETURNS,REFUNDS,CANCELLATIONS

Any problems with LifeSpan products must be reported to LifeSpan within 10 days of product receipt. The customer must obtain written authorization from LifeSpan before returning items. To request that goods be returned, please contact LifeSpan Technical Support. If an error by LifeSpan Biosciences results in shipment of an incorrect order, LifeSpan will, at its option, either ship a replacement order at no charge, or credit the customer's account for the original product shipped in error. Returns and cancellations may be subject to a 30% restocking fee. Conditions & Warranty: All LifeSpan products are intended for Research Use Only and are not for use in human therapeutic or diagnostic applications. The information supplied with each product is believed to be accurate, but no warranty or guarantee is offered for the products, because the ultimate conditions of use are beyond LifeSpan's control. The information supplied with each product is not to be construed as a recommendation to use this product in violation of any patent, and LifeSpan will not be held responsible for any infringement or other violation that may occur with the use of its products. Under no event will LifeSpan be responsible for any loss of profit or indirect consequential damage, including, but not limited to, personal injuries resulting from use of these products. LifeSpan's liability to any user of Products for damages that do not result from any fault of the user, will be limited to replacement of the Product(s) only, and in no event shall LifeSpan's liability exceed the actual price received by LifeSpan for the Product(s) at issue. LifeSpan shall not be liable for any indirect, special, incidental or consequential damages. LIFESPAN FURTHER DISCLAIMS ANY AND ALL EXPRESS AND IMPLIED OR STATUTORY WARRANTIES WITH RESPECT TO THE PRODUCTS, INCLUDING BUT NOT LIMITED TO ANY IMPLIED WARRANTIES OF MERCHANTABILITY OR FITNESS FOR A PARTICULAR PURPOSE. LifeSpan disclaims any and all responsibility for any injury or damage which may be caused by the fault of the user.

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