Human Serum Albumin (HSA)
ELISA Quantitation Kit

Manual

Catalog number : NB-06-0036

For the quantitative determination of human serum albumin levels in plasma or other biological samples.

This kit is for research use only, and is not for use in diagnostic procedures.

Kit Contents: (enough for ten 96-well plates)

Coating Antibody
Affinity purified Chicken IgY against Human Serum Albumin

Concentration:	2.7 mg/mL
Volume:	75 µL
Working concentration:	1.8 µg/mL
Lot:	130520A-10067F

Protein Calibrator
Purified Human HSA Antigen

Concentration:	30 µg/mL
Working concentration:	3,000-4 ηg/mL
Volume:	1 mL
Lot:	130520C-10067F

Detection Antibody
Affinity purified Chicken IgY against Human HSA – Horseradish Peroxidase (HRP) Conjugate

Concentration:	0.44 mg/ml
Working concentration:	200 ηg/mL
Volume:	50 µL
Lot:	130520D-10067F

Notes:

1. Range of Detection: 3,000 – 4 ηg/ml.
2. Shelf life: One year from date of receipt.
3. Storage: -20°C.
4. Assay Condition:
   The kit performance has been optimized for the stated protocol using the materials listed and standard dilutions from 3,000-4 ηg/ml of HSA. For alternative assay conditions, the operator must determine appropriate dilutions of reagents. ELISA assay reactivity is sensitive to any variation in operator, pipetting and washing techniques, incubation time and temperature, composition of reagents, and kit age. Adjustments may be required to position the standard curve and/or samples in the desired detection range.
4. Country of Origin: United States of America.
5. Assay Use: For in vitro laboratory use only. Not for any clinical, therapeutic, or diagnostic use or consumption in human or animals.

HSA Quantitative ELISA Protocol

Materials

1. TMB Peroxidase Substrate System. KPL, Cat. # 50-76-00.
2. Costar 96 well EIA/RIA high binding plate (Corning, Inc. Cat #. 3590)
3. Sodium Bicarbonate. MW 84.01, Sigma-Aldrich, Cat. # S6014-5kg.
4. Tris Hydroxvmethy Aminomethano Hydrochloride. MW 157.6, Fisher. Cat. # BP153-1.
5. Tris Crystallized free base. MW 121.14, Fisher. Cat. # BP 152-5.
6. NaCl, MW 58.44, Fisher. Cat. # BP 358-10.
7. Tween 20, Sigma. Cat. # P-1379.
8. BSA, Sigma. Cat. # A3059-100G.
9. Sulfuric Acid, 4.0N. Labchem Inc., Cat. # LC 25830-2.

Buffer Preparation

1. Coating Buffer: 0.05 M Carbonate-Bicarbonate, pH 9.6
2. Wash Solution: 0.05% Tween 20 in PBS, pH 7.4
3. Blocking Solution: 50 mM Tris, 0.14 M NaCl, 1% BSA, pH 8.0
4. Sample/Conjugate Diluents: 50 mM Tris, 0.14 M NaCl, 1% BSA, 0.05% Tween 20, pH 8.0

Step-by-Step Method (Perform all steps at room temperature)

1. Coat with Capture Antibody

    1) Determine the number of single wells needed. Standards, samples, blanks and/or controls should be analyzed in triplicates.
    2) Dilute capture antibody with Coating Buffer to 1.8 ug/ml. Transfer 100 µl to each well.
    3) Incubate coated plate for 60 minutes at room temperature/37 °C or overnight at 4 °C.
    4) After incubation, aspirate the Capture Antibody solution from each well.
    5) Wash each well with Wash Solution as follows:

        a. Fill each well with Wash Solution
        b. Remove Wash Solution by aspiration
        c. Repeat for a total of 3 washes.

2. Blocking

    1) Add 200 µl of Blocking Solution to each well.
    2) Incubate 60 minutes at room temperature.
    3) After incubation, remove the Blocking Solution and wash each well three times as in Step 1.5.

3. Standards and Samples

    1) Dilute the standards (calibrator) in Sample Diluent to a concentration of 3000 ηg/ml, then do a 1/3 serial dilutions down to 4.11 ηg/ml
    2) Dilute the samples, based on the expected concentration of the analyte, to fall within the concentration range of the standards.
    3) Transfer 100 µl of standard or sample to assigned wells.
    4) Incubate plate 60 minutes at room temperature.
    5) After incubation, remove samples and standards and wash each well 5 times as in Step 1.5.

4. HRP Detection Antibody
    1) Dilute the HRP conjugate in dilution buffer to a concentration of 200 ηg/ml. (Note: Adjustments in dilution may be needed depending on substrate used, incubation time, and age of kit).
    2) Transfer 100 µl to each well.
    3) Incubate 60 minutes at room temperature.
    4) After incubation, remove HRP Conjugate and wash each well 5 times as in Step 1.5.

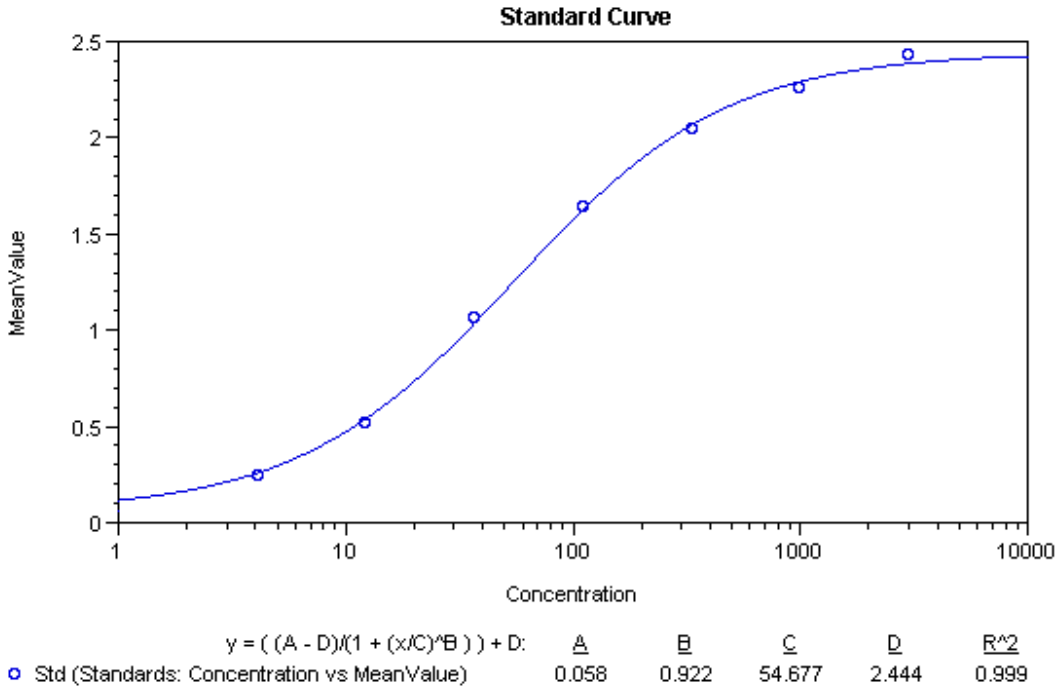
5. Enzyme Substrate Reaction

    1) Prepare the substrate solution according to the manufacturer's recommendation.
    2) Transfer 100 µl of substrate solution to each well.
    3) Incubate plate for 5-15 minutes.
    4) To stop the TMB reaction, apply 100 µl of 2.0N Sulfuric Acid to each well. If using another substrate, use the stop solution recommended by manufacturer.

6. Plate Reading
   Using a microtiter plate reader, read the plate at the wavelength that is appropriate for the substrate used (450 nm for TMB).

Calculation of Results

1. Average the readings from each standard, control, and sample.
2. Subtract the zero reading from each averaged value above.
3. Create a standard curve by reducing the data using computer software capable of generating a four parameter logistic (4-PL) curve-fit. Other curve fits may also be used.
4. A standard curve should be generated for each set of samples. See example below:



Standard	Concentration (ηg/mL)	Calculated Concentration	Abs (450 nm)	Average Abs	Standard Deviation	% CV
St01	3,000	14,448.078	2.430	2.426	0.018	0.7
		Range?	2.447			
		4,535.930	2.404			
		9,749.622	2.424			
St02	1,000	776.940	2.254	2.258	0.012	0.5
		786.689	2.256			
		740.004	2.246			
		885.265	2.274			
St03	333.333	320.025	2.053	2.040	0.013	0.6
		312.730	2.046			
		291.466	2.024			
		302.766	2.036			
St04	111.111	112.268	1.633	1.640	0.017	1
		111.589	1.630			
		111.815	1.631			
		119.864	1.665			
St05	37.037	39.673	1.076	1.068	0.015	1.4
		38.151	1.055			
		38.151	1.055			
		40.342	1.085			
St06	12.346	11.632	0.520	0.516	0.007	1.3
		11.363	0.512			
		11.262	0.509			
		11.734	0.523			
St07	4.115	4.105	0.259	0.245	0.011	4.3
		3.533	0.235			
		3.627	0.239			
		3.769	0.245			
St08	0	0.404	0.084	0.076	0.006	7.3
		0.234	0.074			
		0.185	0.071			
		0.268	0.076			

Technical Hints

1. The Capture antibody should be diluted with coating buffer immediately prior to its addition to the wells. Coated plates are stable overnight at 4ºC when covered.
2. Change pipette tips between each addition of standard, sample and reagents to avoid cross-contamination.
3. Standards and samples should be pipetted to the bottom of the wells and all other reagents should be added to the side of the wells to avoid contamination.
4. Ensure that all buffers are not contaminated or expired. When troubleshooting ELISA results, it is recommended to prepare all new buffers in new vessels.
5. Do not add Sodium Azide to any of the buffers.
6. Sample and Conjugate dilutions should be made shortly before use.
7. Wash buffer should be aspirated from wells, as pouring wash buffer from wells may cause cross-contamination.
8. When preparing dilutions, wipe excess antibody/analyte from pipette tips to ensure accurate dilutions.
9. Incubation time of the Enzyme Substrate will depend on the substrate used and the intensity of the color change. The high standard should have an O.D. reading of between 1 and 3. The low standard should have an O.D. reading above background.
10. The Stopping solution should be added to the wells in the same order as the Enzyme Substrate.

Troubleshooting

The following are some common problems encountered with the use of ELISA kits, and some of the causes of these problems.

1. Problem: Low absorbance
   - ☐ Incorrect dilutions or pipetting errors.
   - ☐ Improper incubation times
   - ☐ Improper mixing of the TMB substrate. Each component is mixed in equal parts.
   - ☐ Wrong filter on microtiter reader. Wavelength should be 450 nm for TMB, 490 nm for OPD, or 405 nm for ABTS.
   - ☐ Kit materials or reagents are contaminated or expired.
   - ☐ Incorrect reagents used.

2. Problem: High Absorbance
   - ☐ Cross contamination from other samples or positive control.
   - ☐ Incorrect dilutions or pipetting errors.
   - ☐ Improper washing.
   - ☐ Wrong filter on microtiter reader.
   - ☐ Contaminated buffers or enzyme substrate.
   - ☐ Improper incubation times.
   - ☐ Kit materials or reagents are contaminated or expired.

3. Problem: Poor Duplicates
   - ☐ Poor mixing of specimens.
   - ☐ Incorrect dilutions or pipetting errors.
   - ☐ Technical error.
   - ☐ Inconsistency in following ELISA protocol.
   - ☐ Inefficient washing.

4. Problem: All wells are positive
   - ☐ Contaminated buffers or enzyme substrate.
   - ☐ Incorrect dilutions or pipetting errors.
   - ☐ Kit materials or reagents are contaminated or expired.
   - ☐ Inefficient washing.

5. Problem: All wells are negative
   - ☐ Procedure not followed correctly.
   - ☐ Contaminated buffers or enzyme substrate.
   - ☐ Contaminated conjugate.
   - ☐ Kit materials or reagents are contaminated or expired.