Title: HSA ELISA SOP

Approvals:
Preparer:_____Bob O'Brien_____ Date ____01Apr09_____
Reviewer:_____Kari Britt_____ Date ____01Apr09_____

1. **Purpose:**
   1.1. To detect Human Serum Albumin (HSA) via Enzyme Linked Immunosorbent Assay (ELISA) and quantify the concentration of HSA in each sample.
2. **Scope:**
   2.1. To detect and quantify the Human Serum Albumin concentration of a given sample using the Human Albumin ELISA Quantitation Set by Bethyl Laboratories, Inc.
3. **Responsibilities:**
   3.1. It is the responsibility of the course instructor/lab assistant to ensure that this SOP is performed as described and to update the procedure when necessary.
   3.2. It is the responsibility of the students/technicians to follow the SOP as described and to inform the instructor about any deviations or problems that may occur while performing the procedure.
4. **References:**
   4.1. Human Albumin ELISA Quantitation Set manual
   4.2. plate reader SOP
5. **Definitions:** N/A
6. **Precautions:**
   6.1. Albumin standards are of human origin and should be treated as Biosafety Level 2. Dispose of waste in biohazard containers.
   6.2. Do not expose TMB Substrate solution to glass, foil or metal. Do not use if solution is blue.
7. **Materials:**
   7.1. Human Albumin ELISA Quantitative set from Bethyl Laboratories (cat #: E80-129)
      7.1.1. ELISA Coating buffer (cat# E107)
      7.1.2. ELISA Wash solution (cat# E106)
      7.1.3. ELISA Blocking buffer (cat# 104)
      7.1.4. Sample/ Conjugate Diluent (ELISA Blocking Buffer + Tween 20)
      7.1.5. 10% Tween 20 (cat#E108)
      7.1.6. Enzyme substrate , TMB (cat# E102)
      7.1.7. 96 well plates
      7.1.8. Human Albumin Standards
   7.2. micropipettors (P-100 or P-200) and tips
   7.3. biopure water
   7.4. paper towels
   7.5. containers to prepare buffers
   7.6. containers to prepare reagents
   7.7. microfuge tubes
   7.8. micro titer plate reader operable at 450 nm
   7.9. 0.18M H_2SO_4 (sulfuric acid)

Title: HSA ELISA SOP

8. Process:

8.1. **Assay Preparation**

8.1.1. Equilibrate all reagents to room temperature before use.

8.2. **Reagent preparation:**

8.2.1. **Wash Solution Buffer**: Tris buffer saline with Tween 20 (cat # E106)

8.2.1.1. Dilute contents of Tris buffer saline with Tween 20 packet in 1L of ultra pure water in an appropriate vessel. Mix thoroughly until the contents go into solution.

8.2.1.2. Label the vessel as: Wash Solution, Tris buffer saline with Tween 20, Store: Room Temperature, Dispose: Drain, [Date], [Initials].

8.2.2. **Blocking Buffer:** Tris buffer saline with 1% BSA. (cat# E104)

8.2.2.1. Dilute contents of Tris buffer saline with 1% BSA packet in 1L of ultra pure water in an appropriate vessel. Mix thoroughly until the contents go into solution.

8.2.2.2. Label vessel as: Blocking Buffer, Tris buffered saline with 1% BSA, Store: 2-8°C, Dispose: Drain, [Date], [Initials].

8.2.3. **Sample/Conjugate Diluent**

8.2.3.1. Combine 500mL of the blocking buffer with 2.5mLof 10% Tween in an appropriate vessel.

8.2.3.2. Label vessel as: Sample/Conjugate Diluent, Tris buffered saline with 1% BSA and 10% Tween, Store: 2-8°C, Dispose: Drain, [Date], [Initials].

8.2.4. **Coating Solution**

8.2.4.1. Break apart the Coating Solution gel capsule and pour contents into 100mL of ultrapure water in an appropriate vessel.
Note: Do not place capsule into the water without breaking it apart. The gelatin from the capsule interferes with the binding of the coating antibody to the plate.

8.2.4.2. Label vessel as: Coating Solution for HSA ELISA, Store: 2-8°C, Dispose: Drain, [Date], [Initials].

8.2.5. **Diluted Coating Antibody Buffer**

8.2.5.1. Dilute 1µL of affinity purified antibody (A80-129A) to 100µL Coating solution buffer for each well to be coated. (Example: for 96 wells, dilute 96µL antibody to 9600µL (9.6mL) ultrapure water).
Note: It is better to mix excess solution than to not have enough. Therefore, for the above example it would be better to add 100µL antibody to 10mL ultrapure water.

8.2.5.2. Place solution in appropriate sized tube. Label tube as: Diluted Coating Antibody, Store: 2-8°C, Dispose: Biohazard waste container, [Date], [Initials].

8.2.6. **Standard Serial Dilutions**

Note: Refer to Figure 1: Standards Dilution Table, on the following page, at the end of this section.

8.2.6.1. Label nine (9) test tubes, one for initial dilution and one for each standard curve point:

Title: HSA ELISA SOP

1. HSA Standard Initial Dilution (15mL test tube)
2. HSA 400ng/mL (15 mL test tube)
3. HSA 200 ng/mL (2mL test tube)
4. HSA 100 ng/mL (2mL test tube)
5. HSA 50 ng/mL (2mL test tube)
6. HSA 25 ng/mL (2mL test tube)
7. HSA 12.5 ng/mL a (2mL test tube)
8. HSA 6.25 ng/mL a (2mL test tube)
9. HSA 0 ng/mL, Blank (2mL test tube)

8.2.6.2. **HSA Standard Initial Dilution:** Prepare initial dilution of the 10,000 ng/mL by diluting 5µL of Human Reference Serum (RS10-110-3) with 12.5 mL of Sample/Conjugate Diluent. Mix well by closing the tube securely and inverting several times.

8.2.6.3. Pipette 2.4mL of Sample/Conjugate Diluent into the 15mL test tube labeled 400ng/mL.

8.2.6.4. Pipette 500µL of Sample/Conjugate Diluent into all the other standard tubes: 200 ng/mL, 100 ng/mL, 50 ng/mL, 25 ng/mL, 12.5 ng/mL, 6.25 ng/mL and a 0 ng/mL.

8.2.6.5. Pipette 100µL from the HSA Standard Initial Dilution tube into the 400ng/mL tube.
Note: Wipe excess antibody/ analyte solution from pipette tips between tubes when making dilutions.

8.2.6.6. Pipette 500µL from the 400ng/mL tube into the 200ng/mL tube.

8.2.6.7. Pipette 500µL from the 200ng/mL tube into the 100ng/mL tube.

8.2.6.8. Pipette 500µL from the 100ng/mL tube into the 50ng/mL tube.

8.2.6.9. Pipette 500µL from the 50ng/mL tube into the 25ng/mL tube.

8.2.6.10. Pipette 500µL from the 25ng/mL tube into the 12.5ng/mL tube.

8.2.6.11. Pipette 500µL from the 12.5ng/mL tube into the 6.25ng/mL tube.

8.2.6.12. Do not pipette into the 0ng/mL tube. Use Sample/conjugate diluents only as the blank.

Standard (Std.)	ng/mL	RS10-110-3 (25 mg/mL Human Albumin)	Sample/Conjugate Diluent
Initial	10,000	5µL	12.5mL
1	400	100µL from initial	2.4mL
2	200	500µL from std. 1	500µL
3	100	500µL from std. 2	500µL
4	50	500µL from std. 3	500µL
5	25	500µL from std. 4	500µL
6	12.5	500µL from std. 5	500µL
7	6.25	500µL from std. 6	500µL
8	0	Blank	500µL

Figure 1: Standards Dilutions Table

Title: HSA ELISA SOP

8.2.7. **Dilute HRP Detection Antibody** (cat # A80-129P)

8.2.7.1. Place 12mL of ultrapure water in a 15mL test tube.

8.2.7.2. Dispense 1µL of HRP detection antibody into the 12mL of water.
Note: Be sure to dispense the antibody below the surface of the water. After dispensing pipette up and down several times to rinse the inside of the pipette tip.

8.2.7.3. Close the tube securely and invert several times to mix.

8.2.7.4. Label the tube as: HRP Detection Antibody Solution, 1:12,000, Store: 2 8°C, Dispose: Drain, [Date], [Initials].

8.2.8. **TMB**

Note: Do not use glass pipettes to measure TMB substrate reagents and do not use the TMB if it is blue at any point before adding to the ELISA plate.

8.2.8.1. Prepare the TMB substrate solution in a test tube by mixing equal volumes of the two individual substrate reagents based on the amount of wells used. (Example: For 96 wells and 100µL per well a minimum of 9.6mL will be needed. Therefore, make 10mL of TMB substrate by mixing 5mL of each reagent).

8.2.8.2. Label container containing the mixed TMB substrate as: TMB, Hydrogen Peroxide, Store: 2-8°C and protect from light, Dispose: Drain, [Date], [Initials].

8.2.8.3. Wrap test tube with aluminum foil to block light and refrigerate until needed.
Note: Aluminum foil should not be used to cover the ELISA plate during the reaction.

8.2.9. **Stop Solution**

8.2.9.1. Prepare a 20-100mL solution of 2M sulfuric acid (H_2SO_4) and label as: Stop Solution, 2M sulfuric acid (H_2SO_4), Store: acid cabinet, room temperature, Dispose: neutralize pH then drain, [Date], [Initials].

8.3. **Assay**

8.3.1. **Coating wells**

8.3.1.1. Equilibrate all reagents to room temperature before use.

8.3.1.2. Obtain an ELISA plate.

8.3.1.3. Dispense 100µL of diluted coating antibody to each well that will be used (Extra wells can be coated if excess coating antibody solution remains after the minimum number of wells has been coated.).

8.3.1.4. Incubate at room temperature (20-25°C) for at least 60 minutes**.**

8.3.1.5. Remove the coating antibody solution mixture by turning the plate upside down on a lab towel and tapping the liquid out on to the towel several times.

8.3.1.6. Wash plate per plate washing direction (step 8.3.2.).

8.3.2. **Plate Washing**

Title: HSA ELISA SOP

8.3.2.1. Rinse the wells five times with ELISA Wash solution as follows:
Fill each well with ELISA Wash solution (approximately 200µL).
Remove the ELISA Wash solution by aspirating with a pipette.

8.3.2.2. Repeat step 8.3.2.1 four additional times.

8.3.2.3. After the fifth wash, blot the plate dry by turning it upside down on a lab towel and tapping several times, to remove visible liquid.
Note: Visible liquid should be removed from the wells, but the operator should also minimize lag time between plate washing and the following assay step to ensure that the plate does not completely dry out during the assay.

8.3.3. **Blocking**

8.3.3.1. Dispense 200µL of blocking solution into each well.

8.3.3.2. Cover the ELISA plate with laboratory film such as Parafilm.

8.3.3.3. Incubate for 30 minutes at room temperature.

8.3.3.4. Wash plate five times per plate washing procedure (step 8.3.2).

8.3.4. **Adding samples and standards**

8.3.4.1. If not done already, centrifuge the samples to remove cells from the media and remove the supernatant to a new tube. Use the supernatant in the assay. This step is not necessary if the cells were removed while performing a previous SOP or if the sample has been eluted from a chromatography column.

8.3.4.2. Add100µL of standard or sample to appropriate wells.

8.3.4.3. Record positions of standards and samples.

8.3.4.4. Cover the ELISA plate with a new sheet of laboratory film.

8.3.4.5. Incubate for 60 minutes at room temperature.

8.3.4.6. Wash plate five times per plate washing procedure (step 8.3.2).

8.3.5. **Adding diluted HRP detection antibody**

8.3.5.1. Add 100µL of diluted HRP detection antibody to each well.

8.3.5.2. Cover the ELISA plate with a new sheet of laboratory film.

8.3.5.3. Incubate for 60 minutes at room temperature.

8.3.5.4. Wash plate five times per plate washing procedure (step 8.3.2).

8.3.6. **Adding TMB Enzyme**

8.3.6.1. Add 100µL of TMB substrate solution to each well. Take care not contaminate the TMB.
Note: Do not expose TMB or ELISA plate wells to glass, aluminum foil, or metal. Also, if the TMB substrate solution is blue before adding to the plate, DO NOT USE IT!

8.3.6.2. Cover the ELISA plate with a new sheet of laboratory film.

8.3.6.3. Develop in a dark room (or area not exposed to light) for approximately 15 minutes.

8.3.6.4. After 15 minutes stop reaction by adding 100µL of stop solution to each well. With the bottom of the plate laying flat on a hard surface, swirl the plate gently to mix solution.

Title: HSA ELISA SOP

Note: Wells with blue solution should turn from blue to yellow after adding the stop solution.

8.3.6.5. Wipe the underside of the plate with a lab tissue.

8.3.7. **Evaluating the ELISA Plate**

Note: The plate must be evaluated on the plate reader within 30 minutes of stopping the reaction.

8.3.7.1. Measure the absorbance of the wells at 450nm per plate reader SOP.

8.3.8. **Generate a Standard Curve and Calculate Results**

Note: For detailed directions on how to generate a standard curve using Microsoft's Excel 2007 see section 9 (attachments). Other appropriate software programs may be used to generate the standard curve.

8.3.8.1. Plot absorbance at 450nm against the standard concentrations.

8.3.8.2. Fit a trend line through the points.

8.3.8.3. Include the R-squared value and linear equation on the graph.

8.3.8.4. Use the equation to calculate the concentration of HSA in the samples.

Note: If sample absorbance values are higher than the range encompassed by the standard curve, they will need to be diluted and the ELISA assay repeated (A 1:10 dilution is recommended in this case.). After calculating the concentration using the equation, multiply by the dilution factor to get the actual concentration.

9. Attachments:

9.1. Figure 1: Standards dilution table located in section 8.2.6.

9.2. Directions for generating a standard curve using Microsoft's Excel 2007

9.2.1. Open a new spread sheet in Excel 2007.

9.2.2. Enter the concentration data (X axis) from top to bottom into a column starting with 0 and ending with the number value of the highest concentration.

9.2.3. Enter the corresponding absorbance value (Y axis) generated by the plate reader in the column directly to the right of the column used to enter the concentration data.

For example:

X-value	Y-value
ng/mL	Absorbance
0	0
6.25	0.092
12.25	0.179
25	0.320

Note: Enter as many standard concentration values as were used in the assay.

9.2.4. Highlight the cells containing number values only.

9.2.5. Click on the "Insert" tab.

9.2.6. Click on "Scatter" in the "Charts" section.

9.2.7. Choose the chart-type at the top of the left column called "Scatter with only Markers" when you mouse over the choice. The chart will appear.

Title: HSA ELISA SOP

9.2.8. Right click on one of the data points in the chart and choose "Add Trendline…". The "Trendline Options" dialog box will appear.
9.2.9. Select "Linear" and check off "Display Equation on chart" and "Display R-squared value on Chart" in the "Trendline Options" dialog box.
9.2.10. Click on "Close". The line, equation and R-squared value will appear in the chart.
9.2.11. To calculate the concentration of HSA in the sample, substitute the absorbance value for "y" in the equation and solve for "x".

10. History:

Name	Date	Amendment
Bob O'Brien	01Apr09	Initial release