MPS-005
ALBUMIN ELISA

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University of Pittsburgh Drug Discovery Institute

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Revision history			
Version	***By***	***Date***	***Revisions***
EPA 1.3	---	7-Dec-17	
1.0	Rich DeBiasio	24-Sept-20	Reformat for MPS
1.1	Rich DeBiasio	12-Oct-20	Incorporate G. LaRocca comments
1.2	Rich DeBiasio	2-Feb-21	New standards incorporated Updated volumes
1.2	Rich DeBiasio	5-Jan-22	New conjugate dilution scheme
1.3	Richard DeBiasio	4-Sept-24	TRACE, minor edits to QC, qualification etc., remove ref to HMM

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A. PURPOSE AND APPLICABILITY

Hepatocytes are the major source of serum albumin *in vivo*. The albumin produced and secreted by human hepatocytes into culture medium was shown to be a reliable measure of the functional status of the hepatocytes [1, 3]. Viable, functional hepatocytes are expected to produce albumin throughout the period of their culture. ELISA is a sensitive and specific method for measuring protein levels in cell culture media. The ELISA kit described in this protocol uses anti-human antibody to bind albumin to the plate, then detect the bound albumin using HRP conjugated to a second antibody to measure human albumin levels. The assay incubations, wash steps, and readouts can be completed in 3.5 hours. Albumin concentration in the samples is calculated by regression analysis from a standard curve.

B. SUMMARY OF METHOD

Efflux media is collected from Liver MPS on collection days as defined in experimental protocols. This SOP details the analysis of efflux samples to determine albumin secretion levels as determined by ELISA.

A standard curve is generated using human albumin standard and run in parallel with efflux samples. The ELISA method uses HRP and TMB to detect bound albumin from standard or samples. The ELISA assay is generally run as per manufacturer recommendations.

C. DEFINITIONS

ABS	Absorbance
ELISA	Enzyme-Linked Immunosorbent Assay
HRP	Horseradish Peroxidase
TMB	3, 3', 5, 5'-Tetramethylbenzidine

D. QUALIFICATIONS

- Laboratory personnel will be trained on this SOP and essential instrumentation by qualified experienced personnel. Successful completion of training will be indicated by initialing and dating of this SOP by the Trainer and Trainee.
- Successful completion of training is indicated by initialing and dating of the training record by the trainer and the trainee.

E. HEALTH AND SAFETY WARNINGS

- Chemicals, media, reagents, and laboratory materials are handled according to the manufacturer's instructions and/or information stated in the Safety Data Sheets (SDS).
- All chemicals must be handled in compliance with the University of Pittsburgh's Chemical Hygiene Plan Safety Manual.
- All biological material must be handled in compliance with the University of Pittsburgh's Biosafety Guidelines.

F. CAUTIONS

- Store all reagents at 2-8°C. Do not freeze reagents.
- All reagents must be at room temperature (20-25°C) before use.
- Vigorous plate washing is essential (a multichannel aspirator and manual multichannel pipettor are used).
- Do not allow wells to dry between wash steps.

- Working dilutions should be prepared and used immediately.
- Tap plate gently after reagent additions to ensure well coverage.
- Use new disposable pipette tips for each sample transfer to avoid cross-contamination.

G. INTERFERENCES

- Avoid microbial contamination of reagents and equipment. Take care not to contaminate the TMB Solution.
- Do not expose TMB Substrate solution to glass, foil, or metal. If the solution is blue before use, do not use it.

H. EQUIPMENT AND SUPPLIES

ELISA reagents and starter kit are manufactured by Bethyl Labs and purchased through Fisher Scientific.

- Experimental sample medium
  - Defined in experimental protocol
  - Serves as a sample blank
- ELISA Starter Kit
  - Fisher, cat no. E101
  - Store 2-8°C for up to one year
- Sulfuric Acid (18 M)
  - Aldrich, cat no. 25810-5
  - Used for stop solution
- Goat anti-Human Albumin
  - Fisher, cat no. A80-129A
- Human Albumin Standard
  - Sigma, cat no. 126658
- Prepared at 10 mg/mL
- HRP Conjugated Goat anti-Human Albumin Detection Antibody
  - Fisher, cat no. A80-129P
- 96-well Polypropylene V-Bottom Plate
  - Greiner, cat no. 652161
  - For dilution of standards and samples
- Multichannel Pipettor – 8 Channel
  - Various manufacturers
  - 50-300µL
- Aspiration Manifold – 8 Channel
  - Drummond, cat no. 3-000-093
- M5 Spectrophotometer
  - Molecular Devices

I. PROCEDURAL STEPS

I1. REAGENT PREPARATION

Coating Buffer	Dissolve contents of starter kit capsule in 100mL MilliQ H_2O Do not add capsule itself
Wash Solution	Dissolve one packet (T9039) in 1L MilliQ H_2O
Blocking Buffer	Dissolve one packet (T6789) in 1L MilliQ H_2O
Sample/Conjugate Diluent	Add 0.5mL of 10% Tween 20 to 100mL blocking buffer
Enzyme Substrate	Ready to use as supplied
Stop Solution	Dilute 18M sulfuric acid 1:100 in demineralized water to make 0.18M solution Store 2-8°C
Goat anti-Human Albumin	Dilute 1:100 in coating buffer
Human Albumin Standard	Prepare at 10mg/mL in water Store 20µL aliquots at -80°C Dilute to 400ng/mL in sample/conjugate diluent (5µL into 5mL and then → 100µL per 2.4mL) In-depth guide below
HRP Conjugated Goat anti-Human Albumin Detection Antibody	Dilute stock 1:75,000 in sample/conjugate diluent 10uL into 0.99mL and then → 1:750 dilution into appropriate volume as needed for the number of wells being used.

1. Albumin Standard Preparation

1. Dilute the human albumin standard by adding 5 µL of albumin standard to 5 mL of sample diluent (1:1,000) then dilute again 1:25 into sample diluent (100 µL into 2.4 ml).
2. Using the 1:25 dilution (400 ng/ml) prepared above prepare a 1:2 dilution series from 200 through 3.125 ng/ml in sample diluent.
3. In a 96 well PP v bottom plate add 125 µL of sample diluent to column 1 and 2 from wells A-H.
4. Add 125 µL of the 400 ng/mL human reference standard dilution from steps 1 and 2, starting at well A1 and A2 and ending at G1 and G2, serially transfer 125 µL to make a 1:2 dilution (100 µL minimum needed per dilution).
5. The eighth well (H1) contains sample diluent only.

I2. ASSAY PROCEDURE

1. Warm buffers to room temperature before beginning.
2. Determine the volume of the efflux samples tested as this value will be used along with the dilution factor, if any in the final calculation.
3. Determine the number of days the efflux samples were collected as this value will be used

in the final calculation.

4. Assemble strips of wells into plate using two strips for standard and one well for each sample and three wells to serve as a sample blank.
5. Coat wells with diluted anti-human antibody (1:100 in coating buffer) allowing 1 well for each sample and 16 wells for duplicate standards plus blank.
    a. Add 100 µL of diluted goat anti human albumin antibody to each well. Tap the plate gently to disperse the antibody solution.
6. Incubate at room temperature for 60 min.
7. Prepare albumin standard dilutions (see guide in *Albumin Standard Preparation*) while plate with coating buffer is incubating.
8. Using the aspirating manifold, aspirate antibody from all wells and add 200 µL of wash buffer, then repeat by aspirating and adding buffer for a total of 5 washes.
9. Aspirate final wash, then add 200 µl of blocking solution to each well. Tap the plate gently to disperse the blocking solution.
10. Incubate for 30 min at room temperature.
11. Dilute samples in sample diluent to the required level based upon prior testing. *Mix thoroughly by pipetting up and down 5 times.*
12. Dilute sample blanks (media used in experiment with no exposure to cells) in sample diluent to the same level as samples are diluted to serve as a blank for the samples.

    Note: samples may need to be diluted in order to obtain absorbance within the linear range of the standard. Typical samples are diluted 1:10 to 1:200 into sample diluent but this will vary depending on hepatocyte experimental conditions.
13. Aspirate the blocking solution.
14. Add 100 µl of diluted samples into one well and diluted standards into duplicate wells. Tap the plate gently to disperse the samples and standards.
    a. Standards are put into col 1 and 2, samples begin in col 3.
15. Incubate for 60 min at room temperature.
16. Wash all wells as in step 5.
17. Aspirate the final wash then add 100 µl of diluted HRP detection antibody (step H.4.c). Tap the well gently to disperse the HRP solution.
18. Incubate for 60 min at room temperature.
19. Wash all wells as in step 5.
20. Aspirate the final wash then add 100 µl of TMB solution. Tap the well gently to disperse the TMB solution.

21. Incubate at room temperature in the dark for 15 minutes.
22. Stop the reaction by adding 50 µl of Stop solution. Mix gently by tapping the plate.
23. Dry the bottom of the plate thoroughly using a Kimwipe in the event that it had become wet during wash steps. Ensure that the bottom of the well plate is clean to ensure that they are optically clear.
24. Ensure that there are no bubbles in the wells. bubbles can be removed by piercing with a needle.
25. Read the absorbance of each well at 450 nm using the M5 spectrophotometer.
    a. The plate must be read within 30 minutes of stop solution addition.
26. Dispose of the plate into the biological waste after reading.

J. DATA AND RECORDS MANAGEMENTALL DATA MUST BE ENTERED INTO THE EVE ANALYTICS DATABASE

- Records related to the performance of the assay such as date performed, lot numbers o fkit/reagents, assay plate layouts, etc. Are recorded in laboratory notebooks assigned to laboratory personnel.
- Calculate results using the MPS efflux Excel spreadsheet to determine the ng/day/1M hepatocytes for each sample based on comparison of ABS to standard curve, sample volume, sample dilution and number of days of collection.

K. QUALITY ASSURANCE AND QUALITY CONTROL

- Standard curve values should be within 20% of historical values with acceptable R^2 values. Should these conditions not be met the assay should be repeated. If consistent out of control results are seen the kit should not be used and the manufacturer notified.
- R^2 values of the standard curve should be in the range of 0.985 to 1.0.
- Sample values should be within the acceptable range of standard concentrations to be considered valid and if not should be repeated with dilution if necessary.
- The assay should be repeated if the criteria above are not met.
- Pipets used in performing the assay are calibrated yearly.
- The kit is stored as per manufacturer's recommendations and used within the expiration date.

L. REFERENCES

1. Vernetti L*, Senutovitch N*, Boltz R, DeBiasio R, Gough A, Shun TY, Taylor DL (*co-first authors). A Human Liver Microphysiology Platform for Investigating Physiology, Drug Safety and Disease Models. Exp Biol Med, 2016 Jan; 241.1: 101-114.
2. Kostadinova, R. et al. Liver Co culture. Tox App Pharm. 268:1-16. 2013