MPS-008
COLLAGEN IA1 ELISA

September 10, 2024
University of Pittsburgh Drug Discovery Institute

AUTHOR:

Richard DeBiasio — Date: 10/17/24

APPROVED:

Greg LaRocca — Date: 10/19/24

Lawrence Vernetti — Date: 10-18-24

Revision history			
Version	***By***	***Date***	***Revisions***
1.0	Rich DeBiasio	01-Oct-21	
1.1	Richard DeBiasio	10-Sept-24	Trace edits Reagent prep table added Standard preparation edits Text edits to QC and data records Added Purpose, Summary, and Definition sections

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A. PURPOSE AND APPLICABILITY

Collagen Ia1 is produced by liver stellate ells in response to stress in the liver which can lead to MAFLD. This protein is responsible for the fibrotic state of liver tissue which is a hallmark of MAFLD. Col Ia1 is assayed ion efflux samples from liver MPS in vitro models as a marker of a fibrotic state developing in the model.

B. SUMMARY OF METHOD

Efflux samples from MPS models are assayed for Collagen Ia1 using an ELISA method in 96 well format. Samples are typically diluted before being tested and results are reported in ng/day.

A standard curve is generated using a Collagen Ia1 standard run in parallel with efflux samples. The ELISA method uses HRP and TMB to detect bound Collagen Ia1 from standard or samples. The ELISA assay is generally run as per manufacturer recommendations.

C. DEFINITIONS

MAFLD	Metabolic associated fatty liver disease
MPS	Microphysiology systems
CoA	Certificate of analysis
ABS	Absorbance
ELISA	Enzyme linked immunofluorescent assay
PBS	Phosphate buffered saline

D. QUALIFICATIONS

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

E. HEALTH AND SAFETY WARNINGS

- 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

- Check the certificate of analysis for each kit to double check reagent dilutions. Dilutions indicated are determined by previous CoA and experience.
- Do not allow wells to dry between wash steps.
- Vigorous plate washing is essential (a multichannel aspirator and manual multichannel pipettor are used).
- Bring all reagents to room temperature before use.
- Working dilutions should be prepared and used immediately.
- Tap plate gently after reagent additions to ensure well coverage.

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

- Human pro-collagen Ia1 ELISA Kit
  - R&D Systems, cat no. DY6220-05
- Duoset Ancillary Reagent Kit 2
  - R&D Systems, cat no. DY008B
- 96-well Polypropylene V-Bottom Plate
  - Greiner, cat no. 652161
- Multichannel Pipettor – 8 Channel
  - Various manufacturers
  - 50-300µL
  - For dilution of standards and samples
- Aspiration Manifold – 8 Channel
  - Drummond, cat no. 3-000-093
- M5 Spectrophotometer
  - Molecular Devices

I. PROCEDURAL STEPS

I1. REAGENT PREPARATION

Wash Buffer	Dilute concentrate 1:25 in MilliQ water
Reagent Diluent 2	Dilute concentrate 1:10 in MilliQ water
Coating Buffer	Ready to use as supplied
Capture Antibody	Reconstitute stock with 0.5mL of coating buffer Dilute stock 1:60 in coating buffer Store stock at 4°C for up to 8 weeks after reconstitution
Detection Antibody	Reconstitute stock with 1.0mL of reagent diluent Dilute stock 1:60 in reagent diluent Store stock at 4°C for up to 8 weeks after reconstitution
Streptavidin-HRP	Dilute as indicated on the vial (typically 1:40 in reagent diluent)
Substrate Solution	TMB
Stop Solution	Ready to use
Col Ia1 Standard	Follow guide below

1. Col Ia1 Standard Preparation

Refer to the CoA for each new kit lot as the amount per vial of the standard varies. Usually, 0.5 ml of reagent diluent will yield 200 ng/mL, but this volume will need to be adjusted if the amount per vial is not 100 ng.

For example, if 90 ng is provided the volume used would be 0.45 ml to yield 200 ng/ml. Store 50 µl aliquots of the 200 ng.ml solution at –80°C for up to 8 weeks.

1. Place 250µL of 2ng/mL standard in well A1.
2. Place 125µL of reagent diluent in wells B1 through H1.
3. Serially dilute by transferring 125µL from A1 to B1 to C1 … to G1.
4. Well H1 should contain only reagent diluent as standard blank.

I2. Assay Procedure

1. 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.
2. Determine the number of days the efflux samples were collected as this value will be used in the final calculation.
3. Assemble strips of wells into the plate using two strips for standard and one well for each sample and three wells for sample blank.
4. Dilute capture antibody 1:60 in plate coating buffer (PBS without protein)
5. Add 50 µl of diluted capture antibody to each well. Tap the plate gently to ensure that the bottom of the well is covered with diluted antibody.
6. Incubate overnight at room temp.
7. Wash wells with wash buffer 5x using 100 µl buffer and aspirating between washes.
8. Remove last wash add 100 µl of reagent diluent (blocking) and incubate at room temp for 1 hour.
9. Dilute standard (see above) and samples as needed in reagent buffer. Mix thoroughly by pipetting up and down 5 times.
10. Aspirate blocking buffer and add 50 µl of standard or samples.
11. Incubate for 2 hours at room temperature.
12. Wash wells with wash buffer 5x using 100 µl buffer and aspirating between washes.
13. Aspirate last wash and add 50 µl of diluted detection antibody.
14. Incubate for 2 hours at room temperature.
15. Wash wells with wash buffer 5x using 100 µl buffer and aspirating between washes.
16. Aspirate last wash and add 50 µl of diluted streptavidin-HRP
17. Incubate for 20 minutes at room temperature
18. Wash wells with wash buffer 5x using 100 µl buffer and aspirating between washes.
19. Aspirate last wash and add 50 µl of TMB.
20. Incubate for 20 minutes at room temp in the dark.

21. Add 25 µl of stop solution and gently tap to ensure thorough mixing.
22. 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 the wells are optically clear.
23. Ensure that there are no bubbles in the wells. bubbles can be removed by piercing with a needle.
24. Read absorbance of each well at 450 nm using the M5 spectrophotometer.
25. The plate must be read within 30 minutes of stop solution addition.

J. DATA AND RECORDS MANAGEMENT

- All data must be entered into the Eve Analytics database
- Records related to the performance of the assay such as date performed, lot numbers of kit/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