MPS-007
LACTATE DEHYDROGENASE CYTOTOXICITY ASSAY

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Revision history			
Version	***By***	***Date***	***Revisions***
		4-Aug-16	Original EPA Document
1.0	Rich DeBiasio	6-Oct-20	Reformat for MPS
1.1	Rich DeBiasio	6-April-21	Change media type (eliminate HMM)
1.2	Richard DeBiasio	11-Sept-24	TRACE, edit records and QC section, minor text edits.

TABLE OF CONTENTS

A. PURPOSE AND APPLICABILITY

Lactate dehydrogenase (LDH) is an indicator of toxicity in cell cultures as it is released when the cell membrane is compromised. As this enzyme is relatively stable, it can be measured in cell culture fluids as a reliable indicator of its release. In the assay reaction NADH is produced from lactate and NAD+ by LDH which is then used by the enzyme diaphorase to convert it into formazan which is red and can be detected spectrophotometrically by absorbance at 490 nm.

LDH measurements are used in MPS to measure the viability of the cells comprising the in vitro model.

B. SUMMARY OF METHOD

Efflux media is collected from Liver MPS on collection days as defined in experimental protocol. This SOP details the analysis of those samples to determine toxicity as indicated by LDH release. A standard curve is generated, and sample values are compared to this curve to determine concentrations of LDH.

C. DEFINITIONS

ABS	Absorbance
LDH	Lactate Dehydrogenase
NADH	Nicotinamide Adenine Dinucleotide, Reduced
NAD+	Nicotinamide Adenine Dinucleotide
MPS	Microphysiology systems

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 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 or information stated in the Safety Data Sheets (SDS).
- All biological materials are handled in compliance with the University of Pittsburgh's Biosafety Guidelines.

F. CAUTIONS

- All reagents must be stored according to manufacturer's instructions.
- All steps must be performed according to assay protocol.
- Bubble formation in wells is a common occurrence and they need to be removed before reading ABS. Bubbles can be removed by streaming CO2 gas over the surface of the wells.

G. INTERFERENCES

- Serum may contribute to background noise in the assay.

H. EQUIPMENT AND SUPPLIES

- ProMega 96 Non-Radioactive Cytotoxicity Assay
  - Promega, cat no. G1782
- Experimental sample medium Medium
  - Defined in experimental protocol
  - Serves as a sample blank
- Williams' E medium without glucose
  - Gibco (special order), cat no. ME18082L1
- 96-well Polypropylene V-Bottom Plate
  - Greiner, cat no 651261
- 384-well Polystyrene Clear Bottom Plate
  - Greiner, cat no. 781091
- SpectraMax M5 or M5e Spectrophotometer
  - Molecular Devices

I. PROCEDURAL STEPS

1. Prepare substrate by adding 12 ml of Assay Buffer to a vial of Substrate Mix.
   a. Store unused reagent at -20°C in using amber vials. The frozen solution is stable for 8 weeks. *Aliquots may be defrosted one time.*
2. Prepare LDH standard dilutions:
   a. Dilute LDH positive control 1:1000 (1 µl/1 ml in a 1.5 ml vial) in Williams' E medium then perform serial 2X dilutions in a 96 well pp plate for a total of 11 dilutions.
   b. Place 100 µl of Williams' E medium into wells A2-A12. No diluent in well A1 which will receive 200 µl of diluted positive control.
   c. Williams' E medium placed into the 12th well is used as a standard blank.
   d. Serially dilute the standard by transferring 100 µl from well to well (A1-A11).
3. Transfer 10 µl of each standard dilution and blank into each of two wells of a 384 well clear bottom plate.
4. Transfer 10 µl of each sample to be tested into a well of the 384 well plate.
5. Transfer 10 µl of experimental medium into three wells of a 384 well plate to serve as sample blanks.
6. Add 10 µl of prepared substrate into each well of standard and samples using a multichannel pipettor.
7. Spin the plate at 50g for 1 minute to mix reagents and reduce bubbles.
8. Incubate the plate in the dark for 30 minutes.
9. Add 10 µl of undiluted stop solution to each well and gently tap the plate to mix.
10. Read absorbance at 490 nm within 60 minutes after removing any bubbles in the wells.

Bubbles can be removed by streaming CO2 gas over the surface of the wells and/or piercing with a needle.

11. If the absorbance value of the sample is greater than the standard ABS value for the highest standard concentration the LDH determination should be repeated after diluting the sample. A 1:10 dilution will usually give an acceptable absorbance.
12. 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.

J. DATA AND RECORDS MANAGEMENT

- All data must be entered into the Eve Analytics database software.
- 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 kit should not be used and the manufacturer notified.
- R^2 values of the standard curve should be in the range of 0.98 to 1.0. Some values may need to be excluded to achieve these R^2 values especially at the upper end of the curve. 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 1 and 2 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. CytoTox 96 Non-Radioactive Cytotoxicity Assay, Technical Bulletin, TB163, revised 12/12, ProMega Corporation.
2. Decker, T and Lohmann-Matthes, ML. A quick and simple method for quantitation of lactate dehydrogenase release in measurement of cellular cytotoxicity and tumor necrosis factor activity. J Immunol. Meth. 115, 61-9.