Gro*Pep* Bioreagents Pty Ltd

Procedure for LR^3 IGF-I ELISA (MAN100/MAO100)

ASSAY SHOULD BE VALIDATED FOR SPECIFIC PURPOSE IN THE USER LABORATORY

1. Principle of the Assay

The assay employs a quantitative 'sandwich' enzyme linked immunoassay technique (ELISA) to measure LR^3IGF-I. A monoclonal antibody against LR^3IGF-I is coated into wells of an ELISA plate. Standards and samples are pipetted into the wells and LR^3IGF-I is bound by the immobilized antibody. A biotin-labelled monoclonal antibody against LR^3IGF-I is added to the wells to form a complex with the immobilized antibody and LR^3IGF-I. Avidin- labelled horseradish peroxidase is added to the wells followed by a chromogenic substrate for HRP. Colour develops in proportion to the amount of LR^3IGF-I bound to the immobilized monoclonal antibody. The colour development is stopped, and colour intensity is measured by absorbance on a microplate reader. A calibration curve is prepared, plotting the absorbance versus the concentration of LR^3IGF-I. The concentration of LR^3IGF-I in the unknown samples is then determined by comparing the absorbance of the samples to the calibration curve.

2. Gro*P*ep Bioreagents Products Required:

Store reagents refrigerated at 2 - 8°C

Reagent	Product Code	Quantity
Coating Antibody LR^3IGF-I monoclonal antibody	MAN100	1 x 100µg
Detection Antibody Biotin-labelled LR^3IGF-I monoclonal antibody	MAO100	1 x 100µg
LR^3IGF-I	AM001	1 x 1mg

3. Reagents and Equipment Required:

- **ELISA plates** (Nunc MaxiSorp™, 96 wells, Catalogue Code: 468667)
- **Streptavidin-HRP** (R&D Systems, Catalogue Code: DY998)
- **Tetramethylbenzidine Substrate Solution (TMB)** (SIGMA, Catalogue Code: T0440)
- **PBS-Tween (for blocking, all dilutions, reagents and washing steps)**
  Phosphate buffered saline containing 0.5 ml Tween 20 per litre PBS. Store at 4°C for a maximum of 14 days.
- **Stop Solution**
  2M H_2SO_4 - Sulphuric Acid (2 M)

- **Plate reader**
  Configured for ELISA plates.
- **Plate shaker**
  A shaking platform capable of holding ELISA plates and operating at approximately 500 oscillations per minute.
- **Plate washer**
  Manifold dispenser, multi-channel pipette, squirt bottle or automated ELISA plate washer.
- **Pipettes**
  Adjustable pipettes able to deliver 10 µl to 1 ml.
  Multichannel pipettes to deliver 50, 100 and 250 µl
- **Water**
  Milli-Q or equivalent standard water is required.
- **Laboratory items**
  Tubes, racks, reagent reservoirs and other general laboratory items are required for preparation steps in the assay.

4. Sample Collection and Storage

The reagents have been developed as a support product for companies using LR^3IGF-I in serum-free media for cell culture. It is likely to be used to measure LR^3IGF-I in media and during downstream processing of media following a production cycle and is not intended for other use. Normal precautions should be taken for sample collection and storage. Cell culture supernatants should be centrifuged and stored frozen at -20°C. Avoid freeze- thaw cycles. Samples at extremes of pH should be buffered to neutral pH before adding to the assay wells. Since LR^3IGF-I has a low affinity for insulin- like growth factor binding proteins, there is no requirement for an extraction step.

5. Preparation of Reagents

Note: Assay plates should be coated the day before the assay is performed; therefore not all reagents are prepared at the same time.

6. Assay Procedure

Day 1

Reconstitute the 100µg vial of lyophilized **Coating Antibody (MAN100)** in 100µl of PBS(1mg/ml). Gently mix at room temperature to allow complete solution of freeze-dried product. Then further dilute by adding 22µl of this 1mg/ml solution to 11ml of PBS(2µg/ml). Add 100µl of the 2µg/ml Coating Antibody solution to the empty wells of the ELISA plate. Cover plate with parafilm, place in ziplock plastic bag and incubate overnight at 4°C.

Day 2

Efficient removal of the liquid at each step is essential to good performance.

1. Aspirate or decant contents of the 'coated' plate. Blot against paper towelling.
2. Add 250µl of PBS-Tween to each well. Incubate for 30 minutes at roomtemperature (not shaking).
3. Prepare the **LR^{3}IGF-I Standard** as follows:
   - **Solution A**. Reconstitute the 1mg vial of **LR^{3}IGF-I (AM001)** with 1ml 100mM Acetic Acid. Gently mix at room temperature to allow complete solution of freeze- dried product.
   - **Solution B**. Take 50 µl of **Solution A** and add 4.95 ml PBS-Tween. Mix thoroughly.
   - **Solution C**. Take 500 µl of **Solution B** and add 9.5 ml PBS-Tween. Mix thoroughly. Follow the Table below to prepare the Assay Standards:

Concentration [ng/mL]	Volume of Stock Solution	Volume of PBS-Tween
200	2 ml Solution C	3 mL
100	1 ml Solution C	4 mL
50	500 µl Solution C	4.5 mL
20	200 µl Solution C	4.8 mL
10	100 µl Solution C	4.9 mL
5	50 µl Solution C	4.95 mL
2	20 µl Solution C	4.98 mL
0	0	5 mL

Note that standard solutions should not be stored. The assay must be performed on the same day the standards are prepared.

4. Samples of culture media or from chromatography steps in a downstream processing protocol may be tested undiluted or a dilution series may be prepared in PBS-Tween or in the appropriate culture media to bring samples into the assay range.
5. Aspirate or decant the contents of the 'blocked' plate. Blot against paper towelling and wash once with PBS-Tween. Blot plate dry.
6. Add 100µl of standards and samples into the coated wells. Cover and incubate plate at room temperature on a plate shaker for 60 minutes. Make sure that the position of each standard and sample is recorded accurately.
7. Reconstitute the 100µg vial of lyophilized **Detection Antibody (MAO100)** in 100µl of PBS(1mg/ml). Gently mix at room temperature to allow complete solution of freeze-dried product. Then further dilute by adding 11µl of this 1mg/ml solution to 11ml of PBS (1µg/ml).

8. Aspirate or decant the contents of the plate and wash 3 times with PBS-Tween. Blot against paper towelling after each wash.
9. Add 100µl of the 1µg/ml Detection Antibody solution to the coated wells. Cover plate and incubate on a plate shaker for 60 minutes at room temperature.
10. Dilute the **Streptavidin-HRP** 1:200 in PBS-Tween. Prepare 11 ml for one 96 well plate.
11. Aspirate or decant the contents of the plate and wash 3 times with PBS-Tween. Blot against paper towelling after each wash.
12. Add 100µl Streptavidin-HRP to each well of the assay plate. Cover plate and incubate on a plate shaker for 30 minutes at room temperature.
13. Dispense 11ml of **TMB Substrate** into a centrifuge tube to equilibrate to room temperature. **This reagent should be prepared no more than 30 minutes before use and wrapped in aluminium foil to protect from light.**
14. Aspirate or decant the contents of the plate and wash 3 times with PBS-Tween. Blot against paper towelling after each wash.
15. Add 100µl of the TMB Substrate to each well. Cover plate with aluminium foil and incubate on a plate shaker for 20 minutes at room temperature.
16. Stop the enzyme reaction by adding 50µl of 2M H_2SO_4 to each well. Tap the plate gently to mix and ensure air bubbles have been eliminated.
17. Determine the absorbance of each well within 30 minutes of stopping the reaction, using a microplate reader set to 450 nm. If possible, use dual wavelength readings at 450 nm and 650 nm. Subtract the absorbance values obtained at 650 nm from those obtained at 450 nm

Interfering substances

No interference was seen in culture media (DMEM) or DMEM containing 1% or 10% foetal bovine serum. Recovery of LR^3IGF-I spiked into these media was 100 %.

Each laboratory should determine whether the matrix in which LR^3IGF-I is present interferes with this assay. It may be necessary to dilute standards and test samples in the same matrix.

Technical Hints

- No difference was found using PBS containing bovine serum albumin for blocking or dilution of samples or reagents. PBS-Tween alone is an effective “blocking”solution.
- No 'soak' is required between washes.
- Substrate solution should remain colourless until added to the plate.
- Substrate solution incubated in the wells should change from colourless to gradations of blue. After stopping the reaction with acid, the substrate will turn yellow.
- Add the stop solution to the plate in the same order as the substrate solution was added.
- Avoid excessive foaming of solutions during mixing.
- To avoid cross-contamination, change pipette tips between addition of each level of standard, sample additions and reagents.
- Use unique reservoirs for each reagent.