

## Protocol for Long<sup>TMR<sup>3</sup></sup> IGF-I ELISA

### PRINCIPLE OF THE ASSAY

This assay employs the quantitative 'sandwich' enzyme linked immunoassay technique (ELISA) to measure LongTMR3IGF-I. Purified murine monoclonal antibody specifically against LongTMR3IGF-I is coated onto a microtiter plate. Standards and samples are pipetted into the wells and any LongTMR3IGF-I present is bound by the immobilized antibody. After washing away unbound proteins, a purified rabbit polyclonal antibody specific for LongTMR3IGF-I is added to the wells to form a complex with the immobilized antibody and LongTMR3IGF-I. Following a wash to remove unbound polyclonal antibody, a peroxidase conjugated goat anti-rabbit immunoglobulin is added to each well to allow detection of the complex. After a final wash, the substrate solution, ortho-phenylene diamine (o-PD), is added to the wells and color develops in proportion to the amount of LongTMR3IGF-I bound to the immobilized monoclonal antibody. The reaction is stopped and color intensity is measured by absorbance of the substrate at 490 nm in a microplate reader.

A standard curve is prepared, plotting the optical density versus the concentration of LongTMR3IGF-I in the standard wells. By comparing the optical density of the samples to this standard curve, the concentration of LongTMR3IGF-I in the unknown samples is then determined.

### GROPEP REAGENTS USED:

#### Coating Antibody

(Product Code: MAA1)

Protein G purified mouse monoclonal anti LongTMR3IGF-I antibody. The vial contains sufficient reagent to coat 1 x 96 well microtiter plate.

#### Capture Antibody

(Product Code: PAE1)

Protein G purified rabbit anti LongTMR3IGF-I polyclonal antibody. The vial contains sufficient reagent to detect samples in 1 x 96 well microtiter plate.

#### LongTMR3IGF-I Media grade

(Product Code: AU100; 100 µg)

**Store reagents refrigerated at 2 - 8°C.**



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## Additional Reagents Needed:

Suppliers indicated are given as examples. Each laboratory should obtain their own reagents and validate the assay

- **96 well vinyl assay plates**  
(Costar, Cambridge, MA; Cat No: 2595)
- **Anti-rabbit Ig, horseradish peroxidase linked whole antibody (from donkey)**  
(Jackson Laboratories; Cat No: 711-035-152)
- **o-phenylene diamine dihydrochloride**  
(o-PD; Sigma Chemical Company, St Louis, MO; Cat No: P1526)
- **Hydrogen peroxide**  
(30 % (v/v); Sigma Chemical Company, St Louis, MO; Cat No: H0904)
- **Bovine Serum Albumin (BSA)**  
(RIA grade; Sigma Chemical Company, St Louis, MO; Cat No: A7888)
- **Coating Buffer**  
(0.05 M carbonate buffer pH 9.6)  
Na<sub>2</sub>CO<sub>3</sub>            0.795 g    NaHCO<sub>3</sub>            1.465 g  
Add distilled/deionised water to 500 ml. Store at 4°C for a maximum of 14 days
- **Wash Buffer**  
(phosphate buffered saline containing 0.05% (v/v) Tween 20)  
  
NaCl    146.1 g  
KH<sub>2</sub>PO<sub>4</sub>    1.0 g    Na<sub>2</sub>HPO<sub>4</sub> 4.6 g  
Tween 20    2.5 ml  
Add distilled/deionised water to 5,000 ml  
Store at 4°C for a maximum of 14 days
- **Substrate Buffer**  
(citric acid in phosphate buffer, pH 5.0)  
Citric acid    3.65 g  
Na<sub>2</sub>HPO<sub>4</sub>    4.76 g    Add distilled/deionised water to 500 ml.  
Store at 4°C for a maximum of 14 days



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- Stop Solution Sulphuric Acid (1 M)
- Blocking Buffer Wash Buffer containing 2% (w/v) BSA (RIA grade)
- Assay diluent Wash Buffer containing 0.5% (w/v) BSA (RIA grade)
- Plate reader Capable of measurement at 490 nm, configured for microtiter plates
- Pipettes 50 µl, 100 µl, and 200 µl adjustable for running the assay; 1 ml, 5 ml, 10 ml, and 25 ml for reagent preparation
- Plate washer Manifold dispenser, squirt bottle or automated microtiter plate washer
- Incubator Capable of maintaining a constant temperature of 37°C
- Water Distilled / deionised water is required
- Laboratory items Tubes, racks, reagent reservoirs and other general laboratory items are required for preparation steps in the assay

## SAMPLE COLLECTION and STORAGE

The reagents have been developed as a support product for companies using LongTMR3IGF-I in reduced serum and serum-free media for cell culture. It is likely to be used to measure LongTMR3IGF-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 LongTMR3IGF-I has a low affinity for insulin-like growth factor binding proteins, there is no requirement for an extraction step, even from media containing fetal bovine serum (FBS).

## PREPARATION OF REAGENTS

**Note: that assay plates should be coated the day before the assay is performed. Therefore not all reagents are prepared at the same time**

Prepare buffers as indicated in the list of reagents required. The following is a method to prepare reagents for 1 x 96 well microtiter plate.

### Coating Antibody

Reconstitute 1 vial of lyophilized coating antibody in 1 ml of coating buffer and add the entire contents of the vial to a further 10 ml coating buffer.

### Blocking Buffer

Prepare by the addition of 2% (w/v) bovine serum albumin (RIA grade) to the wash buffer. For example, add 0.5 g bovine serum albumin to 25 ml wash buffer. Allow it to dissolve.



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## Assay Diluent

Prepare by the addition of 0.5% (w/v) bovine serum albumin (RIA grade) to the wash buffer. For example, add 0.125 g bovine serum albumin to 25 ml of wash buffer and allow it to dissolve. Prepare sufficient diluent for the dilution of the capture antibody, the enzyme conjugate and for dilution of all the samples and standards.

## Capture Antibody

Reconstitute the vial of lyophilized capture antibody in 1 ml of assay diluent, then add the entire contents of the vial to a further 10 ml assay diluent.

## Antibody-Enzyme Conjugate

Dilute the stock reagent 1:2,000 in assay diluent. For example, to prepare enough for 1 x 96 well microtiter plate, add 5.5  $\mu$ l conjugate to 11 ml assay diluent.

**The appropriate concentration of this reagent (1:2000 - 1:10,000) should be established in each laboratory.**

## Substrate

Add 11  $\mu$ l of hydrogen peroxide solution and 11 mg o-PD to 11 ml substrate buffer. **This reagent should be prepared no more than 30 minutes before use and protected from light.**

## Standards

Prepare standards as follows:

### Solution A (1 $\mu$ g / $\mu$ l)

Reconstitute 1 vial of LongTMR3IGF-I Media grade (Lot No. JJE-A04b; 200  $\mu$ g) with 200  $\mu$ l 10 mM HCl.

### Solution B (10 ng / $\mu$ l)

Take 50  $\mu$ l of Solution A and make up to 5 ml with assay diluent or the appropriate cell culture media. Mix thoroughly.

### Solution C (0.1 ng / $\mu$ l)

Take 50  $\mu$ l of Solution B and make up to 5 ml with assay diluent. Mix thoroughly.

**Follow the Table below to prepare the Assay standards**

Concentration	Volume of stock solution	Volume of Assay Diluent
1.0 ng/100 $\mu$ l	100 $\mu$ l Solution C	900 $\mu$ l
0.8 ng/100 $\mu$ l	160 $\mu$ l Solution C	1840 $\mu$ l
0.6 ng/100 $\mu$ l	120 $\mu$ l Solution C	1880 $\mu$ l



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0.5 ng/100 µl	50 µl Solution C	950 µl
0.4 ng/100 µl	1000 µl (0.8ng/100 µl standard)	1000 µl
0.3 ng/100 µl	1000 µl (0.6ng/100 µl standard)	1000 µl
0.2 ng/100 µl	1000 µl (0.4ng/100 µl standard)	1000 µl
0.1 ng/100 µl	1000 µl (0.2ng/100 µl standard)	1000 µl
0	0	1000 µl

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

## Samples

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 assay diluent to bring samples into the detectable range.

## ASSAY PROCEDURE

### DAY 1

1. Reconstitute the vial of lyophilized **Coating Antibody** in 1 ml of coating buffer, then add the entire contents of the vial to a further 10 ml coating buffer. Pipette 100 µl of the solution into each well of 1 x 96 well vinyl assay plate. Cover plate and incubate overnight at 4°C.

### DAY 2

2. Aspirate and wash each well, repeating the process five times. The use of an automatic or semi-automatic plate washer is recommended but if this is not available, plates may be washed using a multi-channel pipette to fill each well with 250 µl wash buffer. Eject the wash buffer from the wells and tap the plate firmly on absorbent paper to remove excess moisture.

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

3. Pipette 200 µl of blocking buffer into each well. Cover and incubate for 2 hours at 37°C.

4. Prepare the standards as indicated above.

5. Prepare the samples as required. Dilute samples if required in assay diluent or in the appropriate culture media.



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6. Aspirate and wash the plate as described in step 2.
7. Pipette 100 µl standard or sample in at least duplicate (or other replicate set) into the wells. Cover and incubate plates at 37°C for 75 minutes. Make sure that the position of each standard and sample is recorded accurately.
8. Aspirate and wash the plate as described in step 2.
9. Reconstitute the vial of lyophilized **Capture Antibody** in 1 ml of assay diluent, then add the entire contents of the vial to a further 10 ml assay diluent. Pipette 100 µl of solution into each well of 1 x 96 well vinyl assay plate. Cover plate and incubate for 75 minutes at 37°C.
10. Aspirate and wash the plate as described in step 2.
11. Prepare the antibody-enzyme conjugate as described above. **The optimum concentration for use should be determined in each laboratory.** Pipette 100 µl antibody-enzyme conjugate solution into each well. Cover plate and incubate for 75 minutes at 37°C.
12. Aspirate and wash the plate as described in step 2.
13. Prepare the substrate solution as described above no more than 30 minutes before use and protect from light. Pipette 100 µl substrate solution into each well. Cover plate and incubate for 15 minutes at room temperature.
14. Stop the enzyme reaction by pipetting 50 µl H<sub>2</sub>SO<sub>4</sub>(1 mol/l) into each well.
15. Determine the optical density of each well within 30 minutes of stopping the reaction, using a microplate reader set to 490 nm.

## CALCULATION OF RESULTS

Mean values of replicate standards should be calculated. For computer aided data analysis, an appropriate equation producing a dose-reponse fit (or similar) is recommended. Plot the concentration of LongTMR3IGF-I standard on the X axis and the optical density in absorbance units on the Y axis. Unknown values can be read from this curve and the LongTMR3IGF-I concentration in samples calculated by multiplying by the dilution factor and then by a factor of 10 to convert to ng LongTMR3IGF-I / ml.



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## TYPICAL DATA

Any variation in standard diluent, operator, pipetting and washing technique, incubation time and temperature can cause variation in the binding and affect the final results. The following examples contain data using the current methodology but standard curves, assay parameters and quality control data should be established for each individual laboratory.

The following data is typical for generation of a standard curve

Standards (ng/100 $\mu$ l)	Optical Density (Mean)	S.D.
0	0.193	0.006
0.1	0.488	0.018
0.2	0.802	0.012
0.3	1.039	0.033
0.4	1.308	0.023
0.5	1.411	0.009
0.6	1.583	0.045
0.8	1.960	0.069

## LongTMR3IGF-I standard curve

### ASSAY CHARACTERISTICS

#### Intra-assay precision

Three samples of known concentration were assayed in replicates of 20 to assess intra-assay precision

Sample	1	2	3
n	24	24	24
mean (ng/100 $\mu$ l)	0.115	0.391	0.494
Standard Deviation	0.007	0.025	0.034
% CV	6.28	6.32	6.97

#### Inter-assay precision

Three samples of known concentration were assayed five times to assess inter-assay precision

Sample	1	2	3
n	5	5	5
mean (ng/100 $\mu$ l)	0.145	0.410	0.550
Standard Deviation	0.029	0.033	0.036
% CV	19.94	8.22	6.66



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## RECOVERY

The recovery of LongTMR3IGF-I from cell culture media was measured by adding LongTMR3IGF-I to Dulbecco's Modified Eagles Medium (DMEM) containing 0.1% (w/v) bovine serum albumin or 10% (v/v) fetal bovine serum to a final concentration of 50 ng/ml. Samples of each medium were assayed using the ELISA.

The following data is typical for assay of LongTMR3IGF-I in cell culture medium.

DMEM 0.1% BSA	Optical Density
neat	>2
1:2	>2
1:4	>2
1:8	1.525
1:16	0.994
1:32	0.535
1:64	0.326

DMEM 10% FBS	Optical Density
neat	>2
1:2	>2
1:4	>2
1:8	1.521
1:16	0.986
1:32	0.548
1:64	0.342

Using this data the concentration of LongTMR3IGF-I in serum-free media or in media containing 10% (v/v) fetal bovine serum was determined as 45.12 ng/ml (90.2% recovery) and 44.46 ng/ml (88.9% recovery) respectively.

## PARALLELISM

To assess linearity of the assay, cell culture medium was spiked with 50 ng/ml LongTMR3IGF-I, diluted with assay buffer and then assayed.

Dilution	Observed (ng/100µl)	Expected (ng/100 µl)	% O/E
1:8	4.152	5.00	83
1:16	4.464	5.00	89
1:32	4.416	5.00	88



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## **SENSITIVITY**

The theoretical minimum detectable concentration of LongTMR3IGF-I was calculated from a standard curve performed in assay diluent. The minimum detectable concentration of LongTMR3IGF-I is 0.0058 ng/100 $\mu$ l. This was determined by adding two standard deviations to the mean optical density of 15 zero standard replicates and calculating the corresponding concentration from the standard curve. The assay is linear over the range 0.2 to 0.6 ng/100  $\mu$ l.

## **SPECIFICITY**

The assay detects only LongTMR3IGF-I. No significant cross reactivity to IGF-I or IGF-II was measured at concentrations up to 50 ng/ml. No interference was observed from components of fetal bovine serum.

## **CALIBRATION**

This ELISA is calibrated against Animal/Media grade recombinant cDNA derived LongTMR3IGF-I produced by GroPep Limited.

## **TECHNICAL HINTS**

- Substrate solution should remain colourless until added to the plate
- Substrate solution incubated in the wells should change from colorless to gradations of yellow/orange
- Add the stop solution to the plate in the same order as the substrate solution
- Avoid foaming of solutions during mixing
- To avoid cross-contamination, change pipette tips between addition of each level of standard, between sample additions and between reagent additions
- Use unique reservoirs for each reagent

**Long is a trade mark owned by GroPep Limited**

**LongTMR3IGF-I is covered by the following patents assigned to GroPep:**

US patent 5,330,971; European patent 429,586; Japanese patent 2,682,738; Australian patent 633,099; Canadian patent 2,033,176;



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