GroPep Bioreagents Pty Ltd

Determination of IGF-I or IGF-II by Radioimmunoassay (RIA)

Introduction

The following procedures and materials are required to perform a specific IGF-I or IGF-II radioimmunoassay. The procedure follows the basic principle of radioimmunoassay where there is competition between a radioactive and a non-radioactive antigen for a fixed number of specific antibody binding sites.

The amount of [^{125}I]-labelled IGF-I or IGF-II bound to the specific species IGF-I or IGF-II antibody is inversely proportional to the concentration of unlabelled IGF-I or IGF-II of that species present. The separation of free and bound IGF-I or IGF-II is achieved by using a second antibody and a precipitating reagent. The mixture is centrifuged so that the precipitated antigen-antibody complex forms a semi-solid pellet; the supernatant containing the unbound labelled and unlabelled IGF-I or IGF-II is removed from the assay tube and the tube counted in a gamma counter.

Materials and Reagents

A. RIA Buffer
B. IGF-I or IGF-II reference standards of the desired species
C. [^{125}I]-labelled IGF-I or IGF-II
D. Samples for testing
E. Matched Primary Antibody for the species of IGF chosen
F. Secondary Antibody
G. Rabbit gamma globulin (IgG)
H. Polyethylene glycol (PEG) solution

A. RIA Buffer
(30 mM NaH_2PO_4, 0.02% protamine sulphate, 10mM EDTA, 0.025% NaN_3, 0.05% (v/v) Tween-20, pH 7.5)

Preparation of 1 litre of buffer:

1. In a 1 litre volumetric flask, add 500 ml distilled or Milli-Q water.

2. Add 4.68 g $NaH_2PO_4.2H_2O$ (Analytical Grade)

3. Add 200 mg protamine sulphate (Sigma)

4. Add 3.72 g EDTA di-sodium salt (Analytical Grade)

5. Add 250 mg sodium azide (NaN_3), (Analytical Grade)

6. Mix to completely dissolve solids; adjust to pH 7.5.

7. Adjust volume to 1 litre with distilled or Milli-Q water.

8. Add 0.5 ml Tween-20 (# P1379, Sigma)

B. IGF-I or IGF-II Reference Standards

1. Reconstitute a 20 µg vial of the desired GroPep IGF-I or IGF-II in 10 mM HCl to a final concentration of 0.1 µg/µl. Ensure complete reconstitution before proceeding.

2. Place 20 µl aliquots of the 0.1 µg/µl peptide solution into 5 ml tubes and add 1,980 µl of RIA buffer to each tube. Mix thoroughly. This 1 µg/ml stock solution is stable for at least 12 months at -20°C.

3. Place 20 µl of the 1 µg/ml stock solution into a 5 ml tube and add 1,980 µl of RIA buffer to give a working standard solution of 2 ng/200 µl or 2,000 pg/200 µl. Mix thoroughly.
4. In order to produce the series of IGF-I reference standards, add an equal volume of a standard and buffer to produce the next standard in the dilution set. i.e., to a series of 10 x 2 ml eppendorf tubes, add the following solutions:

Ensure that each standard is mixed thoroughly before using it to produce the following standard in the sequence

Ref. Std.#		Conc. of standard
Std 1	800 µl of working standard solution	2,000 pg/200 µl
Std 2	800 µl of working standard solution	1,000 pg/200 µl
Std 3	800 µl of working standard solution	500 pg/200 µl
Std 4	800 µl of working standard solution	250 pg/200 µl
Std 5	800 µl of working standard solution	125 pg/200 µl
Std 6	800 µl of working standard solution	62.5 pg/200 µl
Std 7	800 µl of working standard solution	31.2 pg/200 µl
Std 8	800 µl of working standard solution	15.6 pg/200 µl
Std 9	800 µl of working standard solution	7.8 pg/200 µl
Std 10	800 µl of working standard solution	3.9 pg/200 µl

C. Preparation of the [^{125}I]-labelled IGF-I or IGF-II working solution

[^{125}I]-labelled IGF-I or IGF-II (40 - 80 Ci/g) is prepared using the Chloramine - T method of iodination.
Given that the half-life of [^{125}I] is 60 days, it is not advisable to use tracer beyond 60 days from the date of preparation.

Quantity required:
50 µl of a working solution of [^{125}I]-labelled IGF-I is required per assay tube.

Preparation:
1. Determine the number of counts per minute (cpm) per µl of undiluted [^{125}I]-labelled IGF-I .

2. Place 2 mls of RIA buffer (or convenient volume) in a 5 ml (or suitably sized) polypropylene tube.

3. Add the appropriate volume of [^{125}I]-labelled IGF-I into the RIA buffer such that 50 µl of this working solution of radiolabelled IGF-I reagent gives 20,000 cpm.

4. Mix thoroughly.

5. Place a 50 µl test sample in a suitable gamma counter tube and verify that approximately 20,000 cpm is contained in the 50 µl sample.

D. Acid / Ethanol Extraction of Serum samples to release IGFs from Binding Proteins

Acid / Ethanol Extraction Solution:
1. Carefully add 62.5 ml of 2M HCl to 437.5 ml of 100 % ethanol. Mix gently and when cool transfer to a 500 ml sterile glass bottle and store at -20°C.

Method for Acid / Ethanol Extraction of Serum Samples:
1. To 40 µl of plasma or serum add 160 µl of the acid / ethanol extraction solution.
2. Vortex and incubate at room temperature for 30 min.
3. Add 80 µl 0.885 M Tris (51.8 g Tris base (M. Wt. 121.14) in 500 ml sterile distilled or Milli-Q water) and vortex.
4. Spin in a microfuge at 10,000 g (~13.000 rpm) or maximum speed) for 10 min at 4°C
5. Collect supernatant and assay 50 µl in triplicate.

Acid / Ethanol Blank Solution:
1 ml RIA buffer
4 ml Acid / Ethanol Extraction Solution
2 ml 0.885 M Tris
Make up fresh for each standard curve.

E. Preparation of Primary Antibody

The primary antibody to be used in this method is anti-human IGF-I polyclonal antiserum (Cat# PAA2. It is recommended that the final assay concentration of the anti-human IGF-I polyclonal antiserum be approximately 1/80,000.

Quantity required:
50 µl of the working solution per assay

Preparation:
1. Add 250 µl of RIA buffer to one vial of anti-human IGF-I polyclonal antiserum (rabbit) to give an antiserum stock solution of 1/50 dilution; ensure that the lyophilized pellet is completely dissolved before proceeding.

2. Place 10 µl of the 1/50 stock solution into a 5 ml polypropylene tube. *The remaining 240 µl of the 1/50 stock solution may then be aliquoted and stored at - 20°C.*

3. Add 2,657 µl RIA buffer to the 5 ml polypropylene tube and mix thoroughly. This antibody solution is the working antibody solution (1/13,333 dilution). *Note that working solutions are stable for 5 days if stored at 4°C.*

F. Preparation of Secondary Antibody

The secondary antibody used was GroPep Goat anti-Rabbit gamma globulin (IgG) (Ca# PSA1). *Please note that alternative sources of this reagent may be used but the amount required for optimum precipitation will have to be determined before use.*

(Alternative: Millipore sheep anti-rabbit IgG (IgG Fraction) (Catalog No AB7130). Use 50 ul of a 1:20 dilution in RIA buffer per assay tube)

Quantity required:
50 µl of a working solution is required for each assay tube (approximately 2 ml of the working solution of antibody are required for completing one standard curve performed in triplicate).

Preparation of a working solution of secondary antibody:

1. To a vial of GroPep Goat anti-Rabbit antiserum add 2.5 ml RIA buffer. Mix thoroughly to dissolve. Add vial contents to a further 10 ml RIA buffer. Mix thoroughly.

2. Place 200 µl in a 5 ml tube. Add 1,800 µl RIA buffer to the 5 ml tube. Mix thoroughly.

G. Preparation of the Rabbit gamma globulin (IgG)

The Rabbit gamma globulin used in preparing this method was obtained from Dako, CA, USA (Cat #X0903). *Alternative sources of this reagent may be used, but it should be noted that the amount required for optimum precipitation will have to be determined.*

Quantity required:
25 µl of a 1/200 working solution is required for each assay tube

Preparation of a 1/200 working solution of Rabbit gamma globulin:
1. Place 5 µl of Rabbit gamma globulin (IgG) into a 5 ml polypropylene tube
2. Add 995 µl RIA buffer to the 5 ml tube
3. Mix thoroughly

H. Preparation of Polyethylene glycol (PEG) solution

Quantity required:

1 ml PEG solution per assay tube (approximately 50 mls is sufficient to complete a standard curve)

Preparation:

1. Add 438 mg NaCl (Technical Grade) to a 100 ml beaker
2. Add 50 ml distilled or Milli-Q water and mix to dissolve
3. Add 3 g polyethylene glycol 6000 (Technical Grade)
4. Mix thoroughly until all the PEG has dissolved
5. Store at 4°C until required for assay. **Please note that this solution will not be an effective precipitating agent for the radioimmunoassay unless it is used at 4°C.**

Procedure for Radioimmunoassay

1. Number all the tubes required for completing the assay using a permanent marker pen. The tubes recommended for this application are polypropylene tubes of dimensions 12 x 75 mm.

2. Add the assay reagents in the order as depicted in the suggested assay schema (see Table 1 below). It is recommended that all reference standards, the zero reference standard, the unknown samples and the blank tubes be assayed in triplicate. **(Note that the blank tubes refer to a zero reference standard assayed in the absence of primary antibody - this gives the non-specific binding of the radiolabelled IGF-I in the assay).**

Table 1

Additions should be made in the order of reagents indicated. All assay treatments should be done in triplicate.

	Total	Blank	Zero std	Ref. stds	Samples
IGF-I Ref. stds. (1 - 10)	-	-	-	200 µl of std	-
Unknown samples	-	-	-	-	50 µl
[^{125}I]-labelled IGF-I	50 µl	50 µl	50 µl	50 µl	50 µl
RIA Buffer	-	250 µl	200 µl	-	150 µl
Primary antibody	-	-	50 µl	50 µl	50 µl
Total Volume:	**50 µl**	**300 µl**	**300 µl**	**300 µl**	**300 µl**

3. Once all necessary reagent additions have been made as outlined in Table 1, mix the assay tubes thoroughly by vortexing.

4. Cover the tubes to prevent contamination of the assay by dust and other particulate matter, and incubate at 4°C for 16 - 20 hours.

The following steps should be performed at 4°C for optimal results.

5. Add the following reagents to all RIA tubes (excepting the “Total” tubes) :

- 50 µl of anti-Rabbit gamma globulin working solution
- 25 µl of Rabbit IgG working solution

6. Vortex each tube and incubate for 30 minutes at 4°C.

7. Add 1 ml of PEG solution (4°C) to all RIA tubes (excepting the “Total” tubes).

8. Vortex each tube thoroughly.

9. Incubate for a further 10 minutes at 4°C.

10. Centrifuge all tubes (excepting the “Total” tubes) at 4000 g for 30 minutes at 4°C in a pre-cooled centrifuge.

11. Aspirate the supernatants from each tube as soon as the centrifugation is completed. Note that the pellet at the bottom of each tube may be difficult to see, and care should be taken when aspirating the supernatant.

12. Count the radioactivity contained in all tubes in a gamma counter. The details of the gamma counter and program used at Novozymes Ltd. to calculate the radioimmunoassay results are as follows:

Manufacturer and model number: LKB 1261 Multigamma Gamma Counter
Software: RIACALC LM by Wallac