Determination of IGF-I in fish species by radioimmunoassay (RIA)

Introduction

The following procedure and materials are required to measure IGF-I in a range of species of fish. The procedure follows the basic principle of radioimmunoassay in which 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 bound to the IGF-I antibody is inversely proportional to the concentration of unlabelled IGF-I present in the sample. The separation of free and bound IGF-I 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 is removed from the assay tube and the tube counted in a gamma counter.

Materials and Reagents

A. RIA Buffer
B. Tuna IGF-I reference standard (GroPep Bioreagents Cat # AEU020).
C. [^{125}I]-labelled Tuna IGF-I (prepared 'in house').
D. Samples prepared for testing
E. Primary antibody (Barramundi IGF-I polyclonal antiserum (Rabbit) (GroPep Bioreagents
F. Cat # PAF1).
G. Secondary Antibody (Ant-rabbit IgG)
H. Rabbit gamma globulin
I. 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 Aldrich)

B. IGF-I or IGF-II Reference Standards

1. Reconstitute a 20 µg vial of Barramundi IGF-I (*Lates calcarifer*) reference standard (GroPep Bioreagents product # YU020) in 10 mM HCl to a final concentration of 0.1 mg/ml. Ensure complete reconstitution before proceeding.
2. Place 20 µl aliquots of the 0.1 mg/ml IGF-I 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. Ensure that each standard is mixed thoroughly before using it to produce the following standard in the sequence.
4. To produce the series of barramundi IGF-I reference standards add an equal volume of a standard and buffer to produce the next standard in the dilution set. To a series of 10 x 2 ml Eppendorf tubes add the following solutions:

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

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C. Preparation of [125I]-labelled barramundi IGF-I

[^{125}I]-labelled barramundi IGF-I (40 - 80 Ci/g) can be prepared using Pierce® Iodination Beads (Thermo Fisher Scientific) according to the manufacturers' protocol.

The half-life of [^{125}I]-labelled barramundi IGF-I is 60 days so do not use beyond 60 days from the date of preparation.

Preparation:

1. Determine the number of counts per minute (cpm) per µl of undiluted [^{125}I]-labelled barramundi IGF-I
2. Place 2 ml of RIA buffer (or convenient volume) in a 5 ml (or suitably sized) polypropylene tube.
3. Add the appropriate volume of [^{125}I]-labelled barramundi IGF-I into the RIA buffer so that 50 µl of this working solution gives 20,000 cpm
4. Mix thoroughly
5. Place a 50 µl test sample in a gamma counter tube and verify that approximately 20,000 cpm is contained in the 50 µl sample.

D. Preparation of samples for testing using acid/ethanol extraction to separate IGF-I from IGF binding proteins

Acid/Ethanol Solution:

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.

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 (Molecular Weight. 121.14) in 500 ml sterile distilled or Milli-Q water) and vortex
4. Spin in a microfuge at 10,000g 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 is GroPep Bioreagents Barramundi IGF-I antiserum (Catalogue # PAF1). The final concentration of the antiserum should be approximately 1:30,000. The assay requires 50 µl of the working solution per assay tube (approximately 2 ml of the working solution of antibody are required for completing a standard curve)

Preparation:

1. Add 500 µl of RIA buffer to one vial of GroPep Bioreagents Barramundi IGF-I antiserum (Catalogue # PAF1) 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). The dilution to obtain a working solution will be 1/5,000 for anti-fish IGF-I, 1/10,000 for anti-rat IGF-I and 1/1,250 for anti-human IGF-II antibody. Note that working solutions are stable for 5 days if stored at 4°C.

F. Preparation of Secondary Antibody

The secondary antibody should be an anti-Rabbit gamma globulin (IgG). There are a wide range of commercial suppliers and each laboratory should determine the optimum amount required. We have used a number of different reagents and generally use 50 µl of a 1:20 dilution in RIA buffer per assay tube (approximately 2 ml of the working solution of antibody are required for completing one standard curve performed in triplicate).

G. Preparation of Rabbit gamma globulin

The Rabbit gamma globulin used in preparing this method was obtained from Dako Laboratories. Alternative sources of this reagent may be used, but it should be noted that the amount required for optimum precipitation should be determined. A volume of 25 µl of a 1:200 working solution is required for each assay tube (approximately 1 ml of the working solution of antibody (1:200 dilution) are required for completing a standard curve)

Preparation of a 1:200 working solution of Rabbit gamma globulin:

1. Place 5 µl of Rabbit gamma globulin 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

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

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Preparation:

1. Add 438 mg NaCl 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.

Note that this solution will not be effective unless it is used at 4°C.

Procedure for Radioimmunoassay

1. Number all the tubes required for completing the assay. The recommended tubes are polypropylene tubes of dimensions 12 x 75 mm.
2. Add the assay reagents in the order as shown in the table 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).

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

3. Once all necessary reagent additions have been made as outlined, mix the assay tubes thoroughly by vortexing.
4. Cover the tubes to prevent contamination 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 gamma globulin 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 supernatant from each tube as soon as 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. Use a suitable statistical program to calculate results.

	Total	Blank	Zero standard	Reference standards	Samples
IGF-I Reference standards (1 - 10)	-	-	-	**200 µl**	-
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**

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