IGF-II RIA

Radioimmunoassay for Quantitative Measurement of

Insulin-like Growth Factor-II

(IGFBP blocked)

Product Code: IGF-R30

DE/CA40/00809/8

For In-Vitro Use Only!

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PRECAUTIONS

General

All reagents are for in vitro use only!

In conducting the assay, follow strictly the test protocol. The acquisition, possession and use of the kit is subject to the regulations of the national nuclear regulatory authorities.

Reagents with different lot numbers should not be mixed.

Reagents contain Sodium-Azide as preservative, however, highly diluted (0.02%). Sodium-Azide is very toxic, R-Phrases: 28, 32, 50/53 and S-Phrases 28, 45, 60, 61 must be considered.

<u>First aid procedures</u>:

Scin contact: Wash affected area thoroughly with water at least 15 minutes. Discard contaminated cloths and shoes. See a physician.

Eye contact: In case of contact with eyes, rinse immediately with plenty of water at least 15 minutes. In order to assure an effectual rinsing spread the eyelids. See a physician.

Ingestion: If swallowed, wash out mouth thoroughly with water, provided that the person is conscious. Immediately see a physician.

The handling of radioactive and potentially infectious material must comply with the following guidelines:

The material should be stored and used in a special designated area.

Do not eat, drink or smoke in these areas.

Never pipette the materials with the mouth.

Avoid direct contact with these materials by wearing laboratory coats and disposable gloves.

Spilled material must be wiped off immediately. Clean contaminated areas and equipment with a suitable detergent.

Unused radioactive material and radioactive waste should be disposed according to the recommendations of the national regulatory authorities.

Radioactivity

Before ordering or using radioactive materials, it is necessary to take the appropriate actions to ensure compliance with national regulations governing their use. Local rules in each establishment, which define actions and behaviour in the radioactivity working areas, should also be adhered to. The advice given here does not replace any local rules, instructions or training in the establishment, or advice from the radiation protection advisers. It is important to follow the code of good laboratory practice in addition to the specific precautions relating to the radionuclide I-125 used.

Iodine-125 has a radioactive half-life T1/2 of 60 days and emits 35,5 keV gamma radiation, 27 – 32 keV x-rays and no beta radiation. Shielding is effective done by lead, first half value layer is 0.02 mm lead, reduction to 10 % is made by 0.2 mm.

To reduce the radiation dose time spent handling radioactivity should be minimized (plan ahead), and distance from source of radiation should be maximized (doubling the distance from the source quarters the radiation dose).

Formation of aerosols, e.g. by improper opening and mixing of vials or pipetting of solutions which may cause minute droplets of radioactivity become airborn, is a hazard and should be avoided.

Solutions containing iodine should not be made acidic, because this might lead to the formation of volatile elemental iodine.

As some iodo-compounds can penetrate rubber gloves, it is advisable to wear two pairs, or polyethylene gloves over rubber.

For cleaning of contaminated areas or equipment, the Iodine-125 should be rendered chemically stable by using alkaline sodium thiosulphate solution together with paper or cellulose tissue.

MATERIALS

Material provided

The reagents listed below are sufficient for 100 tubes including the standard curve.

AB) Acidification Buffer
(1 bottle, 12.5 ml, ready for use)

DB) Dilution Buffer
(1 bottle, 125 ml, ready for use)

A) Assay Buffer
(1 bottle, 30 ml, ready for use)

B) 1st Antibody (anti-hIGF-II) containing rabbit IgG and recomb. hIGF-I
(1 bottle, 11 ml, lyophilized)

C) Tracer (^{125}I-IGF-II) (< 3 µCi or < 110 kBq)
(1 bottle, 11 ml lyophilized)

D) Rabbit immunoglobulin for non-specific binding (NSB)
(1 vial, 500 µl, lyophilized)

E - L) Standards. Concentrations are given on vial labels
(8 vials, 500 µl each, lyophilized)

M + N) Controls (human plasma). Concentrations are given on vial labels

(2 vials, 100 µl each, lyophilized)

O) 2nd Antibody (anti-rabbit immunogobulin)
(1 vial, 1 ml, lyophilized)

P) Precipitation Reagent
(1 bottle, 55 ml, ready for use after adding O)

Required Materials Not Provided

1) Ice-cold deionized water

2) Pipettes: 10 ml, 1 ml, 500 µl, 250 µl, 100 µl, 10 µl;
100 µl, 500 µl and 1 ml repeating pipettes are recommended.

3) Disposable polystyrene or polypropylene tubes. Conical tubes are highly recommended because of the small immunoprecipitates. The use of round-bottom tubes may cause formation of insufficiently compact pellets.

4) Vortex mixer

5) Centrifuge appropiate for precipitation of immunocomplexes.

6) Device for aspiration of liquid supernatant (e.g. connected to a water pump).

7) Gamma counter

ASSAY CHARACTERISTICS AND VALIDATION

The radioimmunoassay for IGF-II uses a specific, high-affinity polyclonal antibody. Its cross-reactivity with IGF-I is less than 0.05%.

The sensitivity of the assay is **0.1 ng/ml**.

The inter-assay variation coefficient has been found to be 7.9% , the intra-assay variation coefficient did not exeed 5.4% (three serum samples containing different concentrations of IGF-II were determined 6 times each in every assay).

REAGENT STORAGE AND PREPARATION

Upon receipt the kit should be stored at 2 - 8 °C until its expiry date. After reconstitution, the lyophilized reagents should be stored at –20°C. Avoid repeated thawing and freezing. The shelf-life of the components after opening is not affected, if used appropriately.

B + C) Reconstitute with 11 ml reagent A (Assay Buffer).

D) Reconstitute with 500 µl reagent A (Assay Buffer).

E - L) Reconstitute with 500 µl reagent DB (Dilution Buffer).

M + N) Reconstitute with 100 µl distilled water. Dilute according to samples with DB (Dilution Buffer) (e.g.1:101)

O) Reconstitute with 1 ml reagent A. Transfer dissolved material to reagent P immediately before use. The assay is unaffected by the possible occurrence of turbidity in the final reagent.

Ensure that the lyophilized materials are completely dissolved on reconstitution. It is recommended to keep reconstituted reagents at room temperature for half an hour and then to mix them vigorously with a Vortex mixer. This is important in particular for the controls M and N !

SAMPLE PREPARATION

The stability of IGFBP-bound IGF-II makes sample preparation simple. Serum and Heparin/EDTA plasma levels are comparable. Blood samples may be taken at any time of the day. Whole blood should be processed within a few hours and stored frozen at -20°C until measurement. IGF-II levels are usually not affected by improper handling or storage. They remain stable over several days in normal and in various clinical situations even under conditions of high temperature (37°C). Avoid repeated freezing and thawing cycles, although IGF-levels in normal sera remained unchanged after 10 cyles. Frozen samples are stable over many years. Samples may also be freeze-dried without suffering any loss of activity.

Serum or plasma samples should be diluted 1 to 30 - 400-fold (or even more) depending on the expected values with the Dilution Buffer. Usually, a dilution of 1 : 100 - 150 is appropiate.

Example: Add 10 µl serum to 1 ml Dilution Buffer (dilution 1: 101).

If very low levels are expected (e.g. in extreme GH deficiency or in GH receptor deficiency), serum or plasma samples may be diluted 1: 20 or less with the Dilution Buffer. Sufficient acidification can be achieved by adding 1/10th of the diluted sample volume Acidification Buffer.

Example: Dilute 10 µl serum with 200 µl Dilution Buffer (1: 21). Add 20 µl Acidification Buffer (total dilution 1: 23).

In body fluids other than serum or plasma (e.g. cerebrospinal fluid, ocular vitrous fluid, or urine), or in conditioned cell culture media, IGF-II concentrations may be extremely low. These samples can be directly measured without dilution after adding 1/10th of their volume Acidification Buffer.

Example: add 20 µl Acidification Buffer to 200 µl conditioned cell culture medium (dilution 10:11, dilution factor 1.1).

According to the common dilution of serum or plasma samples the dilution of the controls (M and N) should be about 1:100 with Dilution Buffer.

ASSAY PROCEDURE

Samples (standards and patient specimens) should be assayed in duplicate. For optimal results, accurate pipetting and adherence to the protocol are recommended.

Flow Chart of Assay Protocol

#	Tube	DB	E-L M, N Patients	D	B	C	P
1,2	**Total**	–	–	–	–	100	–
3,4	**NSB**	100	–	100	–	100	500
5,6	**B_0**	–	100 **E**	–	100	100	500
7-20	**Standards**	–	100 **F- L**	–	100	100	500
21,22	**Control 1**	–	100 **M**	–	100	100	500
23,24	**Control 2**	–	100 **N**	–	100	100	500
25,26	**Sample 1**	–	100	–	100	100	500
27,28	**Sample 2**	–	100	–	100	100	500
etc.							

All volumes are given as µl.

1) Labelling of the assay tubes should be done in the following order:

1, 2	total counts, **TC**
3, 4	non-specific binding, **NSB**
5, 6	zero standard **E** (**B_0**)
7, 8	standard **F**
9, 10	standard **G** etc.
21, 22	high control **M**
23, 24	low control **N**
25, 26	etc.duplicates of samples.

2) Add **100 µl** of Dilution Buffer **DB** to tubes 3 and 4.

3) Add **100 µl** of reagents **E - L** (**standards**) to tubes 5 to 20, (zero standard (**E**) to tubes 5 and 6, standard **F** (0.4 ng/ml) to tubes 7 and 8, etc).

4) Add **100 µl** of diluted reagent **M** (high control) to tubes 21 and 22 and diluted reagent **N** (low control) to tubes 23 and 24.

5) Add **100 µl** of diluted (or only acidified) samples to tubes 25 and 26, etc.

6) Add **100 µl** reagent **D** (NSB) to tubes 3 and 4.

7) Add **100 µl** reagent **B** (1st Antibody) beginning with tube 5.

8) Add **100 µl** reagent **C** (tracer) to all tubes.

9) Remove tubes 1 and 2 (total counts) or mark or seal with a stopper.

10) Mix tubes with a vortex mixer.

11) Incubate tubes at **2 - 8 °C** for **2 days**. Incubation of 1 day or more than 2 days will also be appropriate.

12) Add **500 µl** reagent **P** (after addition of reagent **O** !), beginning with tube 3. The reagent should be cold (**2 - 8 °C**).

13) Mix tubes with a vortex mixer.

14) Incubate tubes at **2 - 8 °C** for **1 hour**

15) Add **1 ml** ice-cold **distilled water**.

16) Centrifuge all tubes except tubes 1 and 2 at least at **3000 x g** for **30 min** at a temperature of **2 - 8 °C**.

17) Aspirate the supernatant (except tubes 1 and 2!). The remaining supernatant should not be higher than **2 mm** above the precipitate. Take care that the precipitate remains intact.

    Depending on local conditions and procedures, the supernatant may also be decanted instead of aspirated.

18) Count the activity of all tubes (including tubes 1 and 2) for 1 to 3 min.

Extended washing procedure for increased precision

The second incubation step (step 14) is directly followed by step 16 (centrifugation) and step 17 (aspiration). Proceed then with step 15 and add 1 ml of ice-cold water. This should not be done too vigorously in order to keep the precipitate intact. Do not mix again! Centrifuge the tubes at **2-8°C** at **3000 x g for 5 min**., aspirate the supernatant, and count the radioactivity of all tubes in the gamma-counter (step 18).

This extended procedure results in a somewhat higher precision and reduces the non-specific binding NSB. This is also bound up with a higher work expenditure. The higher precision may be irrelevant for most measurements and should therefore be used only in special cases.

EVALUATION OF RESULTS

Establishing the Standard Curve

The standards provided contain the following concentrations of IGF-II :

Standard	E	F	G	H	I	J	K	L
ng/ml	0.0	0.4	0.9	2.0	4.5	10.0	22.5	50.0

1. Calculate the average counts (AC) of each pair of tubes. This gives the values for B
2. Subtract the average counts (AC) of tubes 3 and 4 (non-specific binding NSB) from the mean counts of the standards, controls and patient samples. This gives the corrected values for B.
3. The corrected value from the zero standard E(tubes 5 and 6) is B_0.
4. Calculate the percent bound (% B/B_0):

   % $B/B_0 = B/B_0 \times 100\%$.
5. Plot % B/B_0 versus the standard concentrations on either semi-logarithmic or logit-log paper. For convenience, it is recommended to use computer assisted data reduction programs.
6. Quality control 1, calculate the non-specific binding NSB in %:
   NSB / Total Counts TC x 100%
   = (AC tubes 3 + 4 / AC tubes 1 + 2) x 100%.
   It should be < 5% (%NSB/TC< 5).

   Quality control 2, calculate the percent bound of zero standard E:
   B_0 / Total Counts TC x 100%
   = ((AC tubes 5 + 6 – AC tubes 3 + 4) / AC tubes 1 + 2) x 100%.
   It should be > 30% (%B_0/TC > 30).

Evaluation of sample concentrations

Read the concentration value (abscissa) corresponding to the % B/B_0 of the sample as in the example given below:

average counts of NSB: 1353 cpm

average counts of zero standard (B_0): 18183 cpm

average counts of sample: 10036 cpm

$\%B/B_0$ = (cpm sample - NSB) / (cpm B_0 - NSB) x 100%

= (10036-1353) / (18183 - 1353) x 100%

= 0.516 x 100%

= 51.6 %

For a 51.6 % value on the y-axis (ordinate) a value of 6.63 ng/ml on the x-axis (abscissa) was obtained. Multiply the concentration value determined graphically or by the aid of a computer programme with the dilution factor.

Example: 6.63 x 101 = 670 ng/ml.

If it is preferred to express the results as nmol/l, the values given as ng/ml should be divided by 7.469 to obtain nmol/l.

Example: 670 ng/ml / 7.469 = 89.7 nmol/l

Table 1: Serum levels of IGF-II in ng/ml in healthy subjects at various ages*

Percentile			
Age group	**5th**	**50th**	**95th**
Newborns	132	237	430
1-4 weeks	292	405	561
1-6 months	290	459	726
6-12 months	323	485	730
1-3 years	320	497	772
3-5 years	331	514	767
5-7 years	349	532	811
7-9 years	361	547	831
9-11 years	368	552	828
11-13 years	373	559	838
13-15 years	379	566	845
15-17 years	377	572	868
20-30 years	363	566	882
30-40 years	368	567	874
40-50 years	339	542	866
50-60 years	330	537	874
60-70 years	311	509	833

* Measurement was performed after acid-ethanol extraction, and values were not corrected for recovery. Blum W., Schweizer R.,: Insulin-like growth factors and their binding proteins; in Ranke MB (ed): Diagnostics of endocrine function in children and adolescents. Basel, Karger, 2003, pp 166-199.

Summary of the Assay

Reagent preparation:	Reconstitution	Dilution
1. Antibody (B)	in **11 ml** Assay Buffer **(A)**	
Tracer (C)	in **11 ml** Assay Buffer **(A)**	
NSB (D)	in **500 µl** Assay Buffer **(A)**	
Standards (E-L)	in **500 µl** Dilution Buffer **(DB)**	
Controls (M+N)	in **100 µl** distilled water	**1:101** with **DB**
2.Antibody (O)	in **1 ml** Assay Buffer **(A)** **Mix** immediately before use with **55 ml** Reagent **(P)**	
Dilute Sample with Dilution Buffer **(DB)** e.g. **1:101**		

Assay procedure in double determination

Addition of Reagent [µl]						
Nr. of Tubes	**Contents of Tubes**	**DB** (Dilution Buffer)	**E-L** (Standards) **M,N** (Controls) **Samples**	**D** (NSB)	**B** (1.Antibody)	**C** (Tracer)
1,2	Total	–	–	–	–	100
3,4	NSB	100	–	100	–	100
5,6	B_0	–	100 **E**	–	100	100
7-20	Standards	–	100 **F- L**	–	100	100
21,22	High Control	–	100 **M**	–	100	100
23,24	Low Control	–	100 **N**	–	100	100
25,26	Sample 1	–	100	–	100	100
27,28	Sample 2	–	100	–	100	100
etc.						

Nr.:1,2 remove until counting the activity.

Mix other tubes with a Vortex-Mixer.

Incubation at **2-8°C**, **2 days**

Add **500 µl P** (after addition of reagent **O**) **in all Tubes** (except 1,2).
The reagent-mix should be cold (2-8°C).

Mix with Vortex-Mixer.

Incubation at **2-8°C**, **1 h**

Add **1 ml cold A.dest.** carefully **in all tubes** (except 1,2)

Centrifugation at ≥ **3000 x g**, **30 min** at **2-8°C**

Aspirate the supernatant
(as a precaution e.g. ca. leave 2 mm as a remaining supernatant above the precipitate).

Count the **activity** of all tubes with a Gamma counter.