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R&D SYSTEMS

Saliva Sensitive

Quantikine™ ELISA

Mouse/Rat/Porcine/Canine IGF-II/IGF2 Immunoassay

Catalog Number MG200

For the quantitative determination of activated mouse/rat/porcine/canine Insulin-like Growth Factor 2 (IGF2) concentrations in serum-free cell culture supernates and serum.

This package insert must be read in its entirety before using this product.
For research use only. Not for use in diagnostic procedures.

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INTRODUCTION

Insulin-like growth factor-2 (IGF2) is a charter member of the insulin family of polypeptides (1, 2). As such, it has a family fold composed of three helices with three conserved intra-chain disulfide bonds. IGF2 is synthesized as a preproprotein that contains a signal sequence, a mature region, and a C-terminal prosegment, commonly referred to as the E domain (3, 4). Proteolytic processing generates the mature molecule that contains a N-terminal B domain, followed by a C domain, a A domain, and a D domain. The B-C-A sequence is reminescent of proinsulin, which is processed further to remove the C domain (1). Mature IGF2 is highly conserved among mammals, sharing 97%, 90%, 93% and 90% amino acid (aa) identity with rat, human, porcine, and canine IGF2, respectively (1). Although the mature molecule circulates as a 7.5 kDa, non-glycosylated monomer, it is not the only isoform of IGF2 found in blood. Depending upon the cell type, some or all of the E-domain, with or without glycosylation, may remain associated with secreted IGF2. This is likely due to the presence of multiple E-domain sites for proteolytic cleavage, and the level of activation of site-specific proteases. Two rodent (rat) extended forms are known to exist. One includes aa #25-111 (based on preproprecursor aa numbering), is 11-17 kDa in size, and is termed "Big-IGF2". A second constitutes the entire proprecursor sequence (aa #25-180), is 20-26 kDa in size, and is termed Pro-IGF2 (5). This situation parallels that in human where a third high molecular weight (HMW) form is also present (6-9).

The contribution of HMW forms to total circulating IGF2 varies, depending upon the *in vivo* source and measurement methodology (5, 10). In human, HMW IGF2 is reported to constitute 10-25% of measurable IGF2; in rodent, it may be as high as 90% of measurable IGF2 (5-9, 11). Total serum IGF2 levels are reported to be anywhere from 300-1100 ng/mL (5, 8). In the blood, mature IGF2 is assumed to bind to either IGFBP-1, 2, 4 or 6, or to form a ternary complex with either IGFBP-3 or 5 plus ALS, or to bind to soluble IGF2/M6P receptor (which may carry 20% of total circulating IGF2) (12, 13, 14). Once dissociated from its carrier protein, mature IGF2 has mitogenic, antiapoptotic, and insulin-like activities on a wide variety of cell types (1, 2). HMW IGF2 also has physiologic significance and shares many properties with mature IGF-II. Relative to mature IGF2, HMW IGF2 binds with equal affinity to the IGF2/M6P and Igf-I receptor (which it activates), and based on its hypoglycemic activity, likely activates the A isoform of the insulin receptor (9, 12, 13, 15). It also forms circulating complexes with the IGFBPs. However, when bound to IGFBP-3, it does not complex with ALS. This is believed to allow for rapid diffusion and subsequent activation of the insulin receptor (11). Finally, it has effects distinct from mature IGF2 as a promoter of cell survival (16). Thus, HMW IGF2 appears to have both overlapping, and distinct properties relative to the mature (or short) form of IGF2.

The Quantikine™ Mouse/Rat/Porcine/Canine IGF-II/IGF2 Immunoassay is a 3.5 hour solid-phase ELISA designed to measure IGF2 in serum-free cell culture supernates and serum. It contains *E. coli*-expressed recombinant IGF2 and antibodies raised against the recombinant factor. This immunoassay has been shown to accurately quantitate the recombinant factor. Results obtained using natural IGF2 showed linear curves that were parallel to the standard curves obtained using the Quantikine kit standards. These results indicate that this kit can be used to determine relative mass values for natural IGF2.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for IGF2 has been pre-coated onto a microplate. Standards, control and samples are pipetted into the wells and any IGF2 present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for IGF2 is added to the wells to sandwich the IGF2 immobilized during the first incubation. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of IGF2 bound in the initial step. The color development is stopped and the intensity of the color is measured.

LIMITATIONS OF THE PROCEDURE

- FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.
- The kit should not be used beyond the expiration date on the kit label.
- Do not mix or substitute reagents with those from other lots or sources.
- If samples generate values higher than the highest standard, further dilute the activated samples with calibrator diluent and repeat the assay.
- Any variation in diluent, operator, pipetting technique, washing technique, incubation time or temperature, and kit age can cause variation in binding.
- Variations in sample collection, processing, and storage may cause sample value differences.
- This assay is designed to eliminate interference by other factors present in biological samples. Until all factors have been tested in this Quantikine™ Immunoassay, the possibility of interference cannot be excluded.

TECHNICAL HINTS

- When mixing or reconstituting protein solutions, always avoid foaming.
- To avoid cross-contamination, change pipette tips between additions of each standard level, between sample additions, and between reagent additions. Also, use separate reservoirs for each reagent.
- For best results, pipette reagents and samples into the center of each well.
- It is recommended that the samples be pipetted within 15 minutes of pretreatment.
- To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary.
- When using an automated plate washer, adding a 30 second soak period following the addition of wash buffer, and/or rotating the plate 180 degrees between steps may improve assay precision.
- Substrate Solution should remain colorless until added to the plate. Keep Substrate Solution protected from light. Substrate Solution should change from colorless to gradations of blue.
- Stop Solution should be added to the plate in the same order as the Substrate Solution. The color developed in the wells will turn from blue to yellow upon addition of the Stop Solution.

MATERIALS PROVIDED & STORAGE CONDITIONS

Store the unopened kit at 2-8 °C. Do not use past kit expiration date.

PART	PART #	DESCRIPTION	STORAGE OF OPENED/ RECONSTITUTED MATERIAL
m/r/p/ca IGF2 Microplate	898751	96 well polystyrene microplate (12 strips of 8 wells) coated with a monoclonal antibody specific for IGF2.	Return unused wells to the foil pouch containing the desiccant pack. Reseal along entire edge of the zip-seal. May be stored for up to 1 month at 2-8 °C.*
m/r/p/ca IGF2 Standard	898753	2 vials of recombinant IGF2 in a buffered protein base with preservatives; lyophilized. *Refer to the vial label for reconstitution volume.*	Use a new standard and control for each assay. Discard after use.
m/r/p/ca IGF2 Control	898754	2 vials of recombinant IGF2 in a buffered protein base with preservatives; lyophilized. The assay value of the control should be within the range specified on the label.	
m/r/p/ca IGF2 Conjugate Concentrate	898752	300 µL of a concentrated polyclonal antibody specific for IGF2 conjugated to horseradish peroxidase with preservatives.	May be stored for up to 1 month at 2-8 °C after dilution.*
Conjugate Diluent 33	896071	21 mL of a buffered protein base with preservatives.	
Calibrator Diluent RD5-42	895565	2 vials (21 mL/vial) of a buffered protein base with blue dye and preservatives.	May be stored for up to 1 month at 2-8 °C.*
Pretreatment G	898503	6 mL of 15 mg/mL glycine, pH 2.0.	
Pretreatment H	898504	6 mL of buffer with preservatives.	
Wash Buffer Concentrate	895003	2 vials (21 mL/vial) of a 25-fold concentrated solution of buffered surfactant with preservative. *May turn yellow over time.*	
Color Reagent A	895000	12 mL of stabilized hydrogen peroxide.	
Color Reagent B	895001	12 mL of stabilized chromogen (tetramethylbenzidine).	
Stop Solution	895174	23 mL of a diluted hydrochloric acid solution.	
Plate Sealers	N/A	4 adhesive strips.	

* Provided this is within the expiration date of the kit.

OTHER SUPPLIES REQUIRED

- Microplate reader capable of measuring absorbance at 450 nm, with the correction wavelength set at 540 nm or 570 nm
- Pipettes and pipette tips
- Deionized or distilled water
- Squirt bottle, manifold dispenser, or automated microplate washer
- 500 mL graduated cylinder
- Horizontal orbital microplate shaker (0.12" orbit) capable of maintaining a speed of 500 ± 50 rpm
- Test tubes for dilution of standards and samples

PRECAUTIONS

IGF2 is detectable in saliva. Take precautionary measures to prevent contamination of kit reagents while running this assay.

The Stop Solution provided with this kit is an acid solution.

Some components in this kit contain a preservative which may cause an allergic skin reaction. Avoid breathing mist.

Color Reagent B may cause skin, eye, and respiratory irritation. Avoid breathing fumes.

Wear protective gloves, clothing, eye, and face protection. Wash hands thoroughly after handling. Refer to the SDS on our website prior to use.

SAMPLE COLLECTION & STORAGE

The sample collection and storage conditions listed below are intended as general guidelines. Sample stability has not been evaluated.

Serum-Free Cell Culture Supernates - Remove particulates by centrifugation and assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Note: *Animal serum used in the preparation of cell culture media may contain high levels of animal IGF2 that shares a high sequence homology. For best results, use serum-free media for growth of cell cultures during the last 24 hours when assaying for IGF2 production.*

Mouse Serum - Allow blood samples to clot for 2 hours at room temperature before centrifuging for 20 minutes at 2000 x g. Remove serum and assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Rat/Porcine/Canine Serum - Allow blood samples to clot for 2 hours at room temperature before centrifugation for 20 minutes at 1000 x g. Remove serum and assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

SAMPLE PREPARATION

Follow the sample pretreatment and dilution procedure outlined below in order to dissociate IGF binding proteins from IGF2.

Serum-free Cell Culture Supernates	Serum
To 50 µL of cell culture supernate, add 50 µL of Pretreatment G.	To 10 µL serum, add 95 µL of Pretreatment G.
Mix well.	Mix well.
Incubate 10 minutes at room temperature.	Incubate 10 minutes at room temperature.
Neutralize the acidified sample by adding 50 µL of Pretreatment H.	Neutralize the acidified sample by adding 95 µL of Pretreatment H.
Mix well.	Mix well.
Prior to assay, cell culture supernate samples may require dilution. Assay samples within 15 minutes of pretreatment.	Prior to assay, serum samples require dilution. Mouse and rat samples require a 4-fold dilution. A suggested 4-fold dilution is 50 µL of pretreated sample + 150 µL of Calibrator Diluent RD5-42. Porcine and canine samples require a 20-fold dilution. A suggested 20-fold dilution is 10 µL of pretreated sample + 190 µL of Calibrator Diluent RD5-42. Assay samples within 15 minutes of pretreatment.
The concentration read off the standard curve must be multiplied by the appropriate dilution factor for cell culture supernates.	The concentration read off the standard curve must be multiplied by the dilution factor, 80 for mouse and rat and 400 for porcine and canine.

REAGENT PREPARATION

Bring all reagents to room temperature before use.

Note: *High concentrations of IGF2 are found in saliva. It is recommended that a face mask and gloves be used to protect kit reagents from contamination.*

m/r/p/ca IGF2 Control - Reconstitute the control with 1.0 mL of deionized or distilled water. Mix thoroughly. Assay the control undiluted.

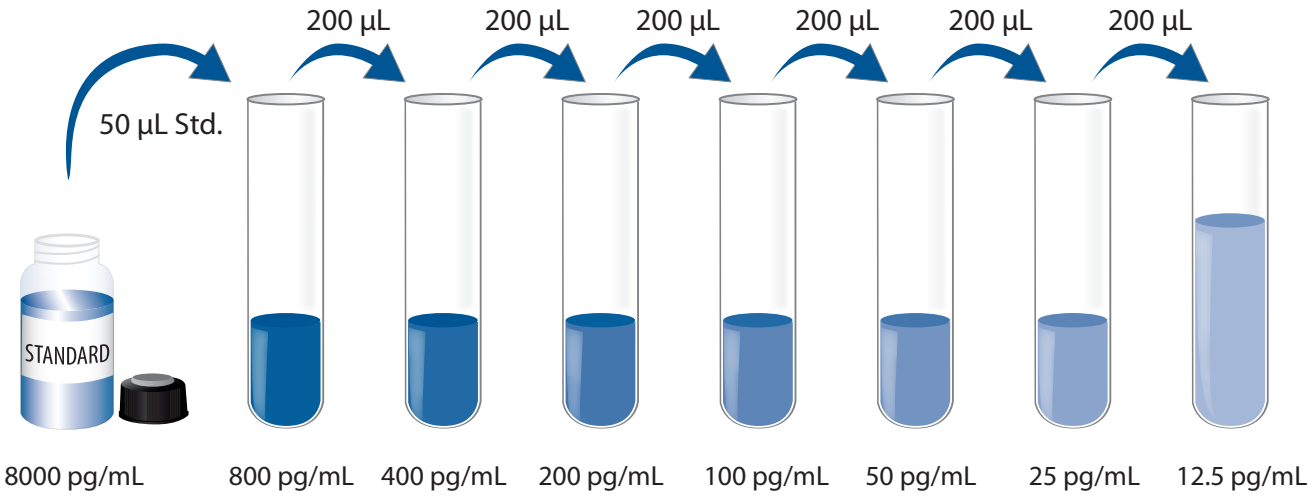
Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Add 20 mL of Wash Buffer Concentrate to 480 mL of deionized or distilled water to prepare 500 mL of Wash Buffer.

Substrate Solution - Color Reagents A and B should be mixed together in equal volumes within 15 minutes of use. Protect from light. 100 µL of the resultant mixture is required per well.

m/r/p/ca IGF2 Conjugate (1X) - Add 0.215 mL of m/r/p/ca IGF2 Conjugate Concentrate directly to the Conjugate Diluent 33 vial. Mix well.

m/r/p/ca IGF2 Standard - **Refer to the vial label for reconstitution volume.** Reconstitute the m/r/p/ca IGF2 Standard with deionized or distilled water. This reconstitution produces a stock solution of 8000 pg/mL. Mix the standard to ensure complete reconstitution and allow the standard to sit for a minimum of 5 minutes with gentle agitation prior to making dilutions.

Pipette 450 µL of Calibrator Diluent RD5-42 into the 800 pg/mL tube. Pipette 200 µL into the remaining tubes. Use the stock solution to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The 800 pg/mL standard serves as the high standard. Calibrator Diluent RD5-42 serves as the zero standard (0 pg/mL).



ASSAY PROCEDURE

Bring all reagents and samples to room temperature before use. It is recommended that all standards, control and samples be assayed in duplicate.

Note: *High concentrations of IGF2 are found in saliva. Wear a face mask and gloves to protect kit reagents from contamination.*

1. Prepare all reagents, standard dilutions, control, and activated samples as directed in the previous sections.
2. Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, and reseal.
3. **Wash plate two times with Wash Buffer immediately prior to use.** Wash by filling each well with Wash Buffer (400 µL) using a squirt bottle, manifold dispenser, or autowasher. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.
4. Add 50 µL of Calibrator Diluent RD5-42 to each well.
5. Add 50 µL of standard, control, or pretreated sample* per well. Cover with the adhesive strip provided. Incubate for **1 hour** at room temperature on a horizontal orbital microplate shaker (0.12" orbit) set at 500 ± 50 rpm. A plate layout is provided to record standards and samples assayed.
6. Aspirate each well and wash, repeating the process three times for a total of four washes. Wash by filling each well with Wash Buffer (400 µL) using a squirt bottle, manifold dispenser, or autowasher. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.
7. Add 100 µL of m/r/p/ca IGF2 Conjugate to each well. Cover with a new adhesive strip. Incubate for **2 hours** at room temperature on the shaker.
8. Repeat the aspiration/wash as in step 6.
9. Add 100 µL of Substrate Solution to each well. Incubate for 30 minutes at room temperature. **Protect from light.**
10. Add 100 µL of Stop Solution to each well. Gently tap the plate to ensure thorough mixing.
11. Determine the optical density of each well within 30 minutes, using a microplate reader set to 450 nm. If wavelength correction is available, set to 540 nm or 570 nm. If wavelength correction is not available, subtract readings at 540 nm or 570 nm from the readings at 450 nm. This subtraction will correct for optical imperfections in the plate. Readings made directly at 450 nm without correction may be higher and less accurate.

*Samples may require dilution. See Sample Preparation section.

CALCULATION OF RESULTS

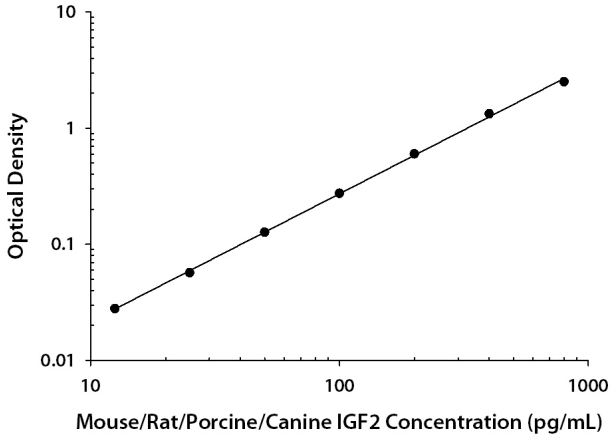
Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density (O.D.).

Create a standard curve by reducing the data using computer software capable of generating a log/log curve-fit. As an alternative, construct a standard curve by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis and draw a best fit curve through the points on a log/log graph. The data may be linearized by plotting the log of the m/r/p/ca IGF2 concentrations versus the log of the O.D. on a linear scale, and the best fit line can be determined by regression analysis.

Because samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

TYPICAL DATA

This standard curve is provided for demonstration only. A standard curve should be generated for each set of samples assayed.



(pg/mL)	O.D.	Average	Corrected
0	0.020 0.020	0.020	—
12.5	0.047 0.049	0.048	0.028
25	0.075 0.078	0.077	0.057
50	0.146 0.147	0.147	0.127
100	0.294 0.296	0.295	0.275
200	0.620 0.621	0.621	0.601
400	1.335 1.370	1.353	1.333
800	2.525 2.538	2.532	2.512

PRECISION

Intra-Assay Precision (Precision within an assay)
Three samples of known concentration were tested twenty times on one plate to assess intra-assay precision.

Inter-Assay Precision (Precision between assays)
Three samples of known concentration were tested in twenty separate assays to assess inter-assay precision. Assays were performed by at least three technicians using two lots of components.

	Intra-Assay Precision			Inter-Assay Precision		
Sample	1	2	3	1	2	3
n	20	20	20	20	20	20
Mean (pg/mL)	46.1	81.7	285	43.5	80.7	275
Standard deviation	2.14	3.41	10.8	2.58	4.97	16.8
CV (%)	4.6	4.2	3.8	5.9	6.2	6.1

RECOVERY

The recovery of IGF2 spiked to levels throughout the range of the assay in various matrices was evaluated.

Sample Type	Average % Recovery	Range
Serum-free cell culture media* (n=4)	92	84-101%
Mouse serum** (n=4)	105	86-124%
Rat serum** (n=4)	84	78-89%

LINEARITY

To assess the linearity of the assay, samples containing and/or spiked with high concentrations of IGF2 were diluted with calibrator diluent to produce samples with values within the dynamic range of the assay.

		Serum-free cell culture media** (n=4)	Mouse serum** (n=4)	Rat serum* (n=4)	Porcine serum** (n=4)	Canine serum** (n=4)
1:2	Average % of Expected	103	102	95	115	107
	Range (%)	98-108	99-106	90-101	110-119	101-110
1:4	Average % of Expected	100	104	92	116	107
	Range (%)	97-104	100-106	88-94	112-120	103-115
1:8	Average % of Expected	91	108	106	114	100
	Range (%)	86-96	105-111	96-121	111-116	94-111
1:16	Average % of Expected	91	113	113	108	98
	Range (%)	87-94	109-118	106-121	105-110	89-112

*Samples were pretreated prior to assay.

**Samples were pretreated and diluted prior to assay.

SENSITIVITY

Twenty-two assays were evaluated and the minimum detectable dose (MDD) of IGF2 ranged from 0.591-1.87 pg/mL. The mean MDD was 1.08 pg/mL.

The MDD was determined by adding two standard deviations to the mean O.D. value of twenty zero standard replicates and calculating the corresponding concentration.

CALIBRATION

This immunoassay is calibrated against a highly purified *E. coli*-expressed recombinant mouse IGF2 produced at R&D Systems®.

SAMPLE VALUES

Serum - Samples were evaluated for the presence of IGF2 in this assay.

Sample Type	Mean (pg/mL)	Range (pg/mL)	Standard Deviation (pg/mL)
Mouse serum (n=10)	17,985	13,440-21,180	2430
Rat serum (n=10)	9605	5340-12,625	2071
Porcine serum (n=10)	63,456	43,160-87,529	13,959
Canine serum (n=10)	70,297	50,200-116,705	20,437

Serum-Free Cell Culture Supernates:

Mouse embryonic fibroblast conditioned media (R&D Systems®, Catalog # AR005) was prepared according to the protocol published by Xu, C. *et al.* (17). Serum-free media [80% Knockout™ D-MEM, 20% Knockout™ serum replacement, 1% MEM non-essential amino acids, 2 mM GlutaMAX™, 0.1 mM β-mercaptoethanol, 4 ng/mL basic fibroblast growth factor (FGF basic)] was conditioned by γ-irradiated CF-1 fibroblasts at 37 °C for 24 hours. An aliquot of the cell culture supernate was removed, assayed for mouse IGF2, and measured 5580 pg/mL.

CMT-93 mouse rectal carcinoma cells were cultured in DMEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin sulfate, until confluent. The cells were then switched to serum-free DMEM for an additional 24 hours. An aliquot of the cell culture supernate was removed, assayed for mouse IGF2, and measured 2856 pg/mL.

SPECIFICITY

This assay recognizes natural and recombinant IGF2.

The factors listed below were prepared at 100 ng/mL in calibrator diluent and assayed for cross-reactivity. Preparations of the following factors at 100 ng/mL in a mid-range recombinant mouse IGF2 control were assayed for interference. No significant cross-reactivity or interference was observed.

Recombinant mouse:

Cyr61
Endocan
IGFBP-1
IGFBP-2
IGFBP-3
IGFBP-4
IGFBP-5
IGFBP-6
IGFBP-L1
IGF-1
IGF1R
INSR
WISP-1

Recombinant human:

IGF-1
IGF1R
IGF2R

Recombinant mouse IGFBP-7 interferes at concentrations > 50 ng/mL.

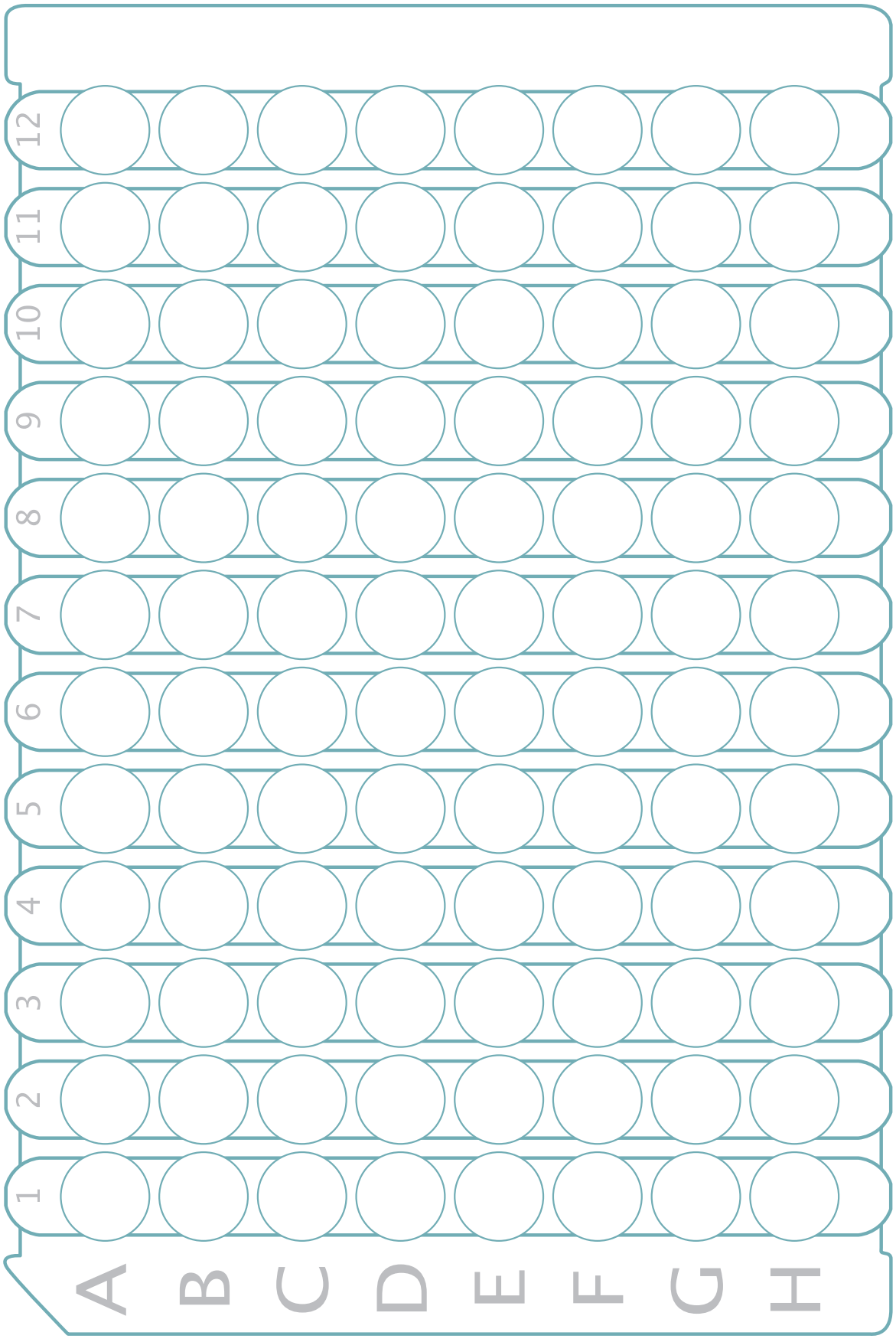
Recombinant human IGF2 does not interfere but does cross-react approximately 1.4% in this assay.

REFERENCES

1. Rotwein, P. (1999) In: Rosenfeld, R. & C.J. Roberts (Eds) Molecular Biology of IGF-I and IGF-II. Humana Press, Totowa, N.J., P. 19.
2. Denley, A. *et al.* (2005) Cytokine Growth Factor Rev. **16**:421.
3. Rotwein, P. & L.J. Hall (1990) DNA Cell Biol. **9**:725.
4. Stempien, M.M. *et al.* (1986) DNA **5**:357.
5. Qiu, Q. *et al.* (2007) Endocrinology **148**:4803.
6. Hudgins, W.R. *et al.* (1992) J. Biol. Chem. **267**:8153.
7. Hill, D.J. (1990) Early. Hum. Dev. **21**:49.
8. Espelund, U. *et al.* (2005) Eur. J. Endocrinol. **153**:861.
9. Zapf, J. *et al.* (1992) J. Clin. Invest. **90**:2574.
10. Duguay, S.J. *et al.* (1998) J. Biol. Chem. **273**:18443.
11. van Doorn, J. *et al.* (2002) Clin. Chem. **48**:1739.
12. Jones, J.I. & D.R. Clemmons (1995) Endocr. Rev. **16**:3.
13. Valenzano, K.J. *et al.* (1995) J. Biol. Chem. **270**:16441.
14. Bond, J.J. *et al.* (2000) J. Endocrinol. **165**:253.
15. Perdue, J.F. *et al.* (1991) Endocrinology **129**:3101.
16. Singh, S.K. *et al.* (2008) Growth Factors **26**:92.
17. Xu, C. *et al.* (2001) Nat. Biotechnol. **19**:971.

PLATE LAYOUT

Use this plate layout to record standards and samples assayed.



NOTES