

DEVELOPMENT OF AN IN-HOUSE RADIOIMMUNOASSAY FOR HUMAN GROWTH HORMONE

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Summary

A simple and sensitive double-antibody radioimmunoassay for human growth hormone (hGH) was developed, optimised and validated. The anti-hGH sera raised in 2 rabbits were highly specific with low cross-reactions of 0.19% and 0.3% with human placental lactogen and 0.21% and 0.13% with human prolactin. The mean sensitivity of the assay determined from 28 assays was found to be 0.4 ± 0.2 mIU/L. Mean recovery of added exogenous hGH was $98.8 \pm 6.8\%$. Linearity studies of samples diluted at 1:2, 1:4 and 1:8 gave values of $101.3 \pm 5.3\%$, $109.6 \pm 13.4\%$ and $97.3 \pm 13\%$ respectively of those expected. The reproducibility of the assay was good; within assay coefficient of variation for serum samples with GH concentrations of 2.7, 13.6 and 28.2 mU/l ranged from 5.1 to 8.3% while the inter-assay precision varied from 4.9 to 10.3%. The in-house assay showed good correlation ($r = 0.96$, $p < 0.001$) with a commercial hGH RIA kit (Dainabot, Japan). A reference normal adult fasting GH level of <7 mIU/l was established from 95 samples assayed by this method.

Keywords: Human growth hormone, radioimmunoassay, double-antibody.

INTRODUCTION

The measurement of circulating levels of growth hormone (GH) is of clinical importance in the diagnosis and treatment of GH secretory disorders. Determination of a random basal level of this hormone, however, has very limited clinical value since normal serum GH levels are often low or undetectable during most of the day. Physiological factors such as exercise, meals and even the stress of venepuncture can stimulate GH secretion, thus resulting in raised GH levels. In order to assess the pituitary function of patients suspected of GH hyposecretion, it is therefore necessary to measure GH after appropriate stimulation with insulin,² arginine³ or L-Dopa⁴ or suppression with a glucose load as used in the investigation of suspected acromegaly.⁵

The routine method for measurement of GH is radioimmunoassay (RIA) although there is now a trend towards the use of more sensitive immunoradiometric assays (IRMA). Most laboratories are dependent on commercial kits which are costly, less readily available and have short shelf lives. The problems faced in interpreting or comparing results from assays using a variety of commercial reagents have been highlighted in a number of studies.⁶⁻⁸ These studies have shown that GH results are variable and dependent on the specificity of the antibody (polyclonal vs

monoclonal), immunopotency and matrix of the reference standard, diluent, purity of tracers and the basic method of measurement, be it competitive binding or sandwich assay. As diagnosis of GH deficiency is largely dependent on the GH results measured after provocative testing, it is important for the clinician to be fully aware of the expected 'normal' GH levels by a particular assay system and to interpret the results accordingly. Since analysis of multiple blood samples will invariably increase the cost of investigations, it is therefore more practical and cost-effective when an in-house assay is available. This paper describes the experiments and results of optimisation and validation of an in-house assay for GH.

MATERIALS AND METHODS

ASSAY BUFFER

The buffer used was 0.01 M phosphate buffered saline (PBS), pH 7.4. All chemicals used in the buffer preparation were purchased from the Sigma Chemical Company, St. Louis, U.S.A. A litre of buffer consisted of 0.13 g KH_2PO_4 , 1.28 g Na_2HPO_4 , 9.0 g NaCl, 4.1 g EDTA (disodium, $2\text{H}_2\text{O}$) and 5.0 g bovine serum albumin (BSA). The polyethylene glycol solution (PEG) contained 80.0 g of

PEG 8000 (Sigma Chemical Company, St. Louis, U.S.A.) and 0.1% (v/v) Tween 20 in PBS. Both solutions were stored at 4°C and used within one month.

REFERENCE STANDARD

The reference standard used was the 1st I.R.P. Human Growth Hormone (HGH) for Immunoassay 661217 obtained from the National Institute for Biological Standards and Control, Holly Hill, Hampstead, London. The working standard was prepared by diluting the reference standard to 200 mIU/l with assay buffer, snap-frozen into smaller aliquots and kept at -20°C. The top assay standard of (60 mIU/l) was prepared by diluting 300 ul of the working standard with 700 ul of buffer while the other 6 standards were prepared by serial double-dilution until 0.94 mIU/l

PRODUCTION OF ANTISERA

Preparation of Immunogens. All preparation, purification and concentration procedures were carried out at 4°C. 10 mg of human growth hormone preparation, HGHP30-HAc,⁹ was repurified by chromatography on DEAE cellulose DE52 using stepwise ionic strength gradients of ammonium acetate buffer 0.05 M to 0.30 M, pH 8.5. The purity of the preparation was checked by polyacrylamide gel electrophoresis (PAGE). The required fractions were pooled and diluted with 0.01 M (PBS) pH 7.4 (100 ug/ml) and kept at -20°C in 1 ml aliquots of 100 ug.

The second antibody was goat anti-rabbit IgG. Rabbit IgG was prepared from normal rabbit serum by ammonium sulphate precipitation followed by DEAE-Sephacel (Pharmacia AB Sweden) chromatography using a salt gradient of 0.015 M - 0.300 M potassium phosphate buffer. The IgG fractions were pooled and then concentrated by pressure filtration at 2 kg/cm² in a 47 mm Ultrafiltration Cell using PTGC Pellicon membrane, NMWL 10000 (Millipore Corporation, Bedford, Massachusetts). The rabbit IgG preparation was aliquoted and stored at -20°C. Purity of the IgG solution was confirmed by immunodiffusion and immunoelectrophoresis.

Immunisation Protocol. Two New Zealand white rabbits about 3 months old were used to raise the primary antibody and a 1 year old male goat of about 40 kg was used to raise the second antibody. The protocol was as described by Vaitukaitis *et al*¹⁰ with some modifications. For primary injection, 100 ug of the immunogen emulsified in 1:3 ratio

with Freund's Complete Adjuvant was injected intramuscularly into 2 sites of the hind legs of each animal. This was followed by two booster injections at biweekly intervals administered subcutaneously at 4 sites on the back of the animals. Subsequent monthly boosters were given in saline and test bleeds were obtained 7-10 days after each injection. Antisera were appropriately aliquoted, and either kept at -20°C for immediate use, or lyophilised for longer storage. Due to the initial unavailability of the in-house tracer, antisera titres were assessed using commercially purchased ¹²⁵I-labelled GH from Diagnostic Products Corporation, U.S.A.

Antiserum Specificity. To assess the specificity of the primary antiserum produced, a series of standard curves were set up with increasing concentrations of up to 20 ug/ml of several potential cross-reactants, namely human FSH, LH, TSH, BHCG, HPL and prolactin. Except for HPL which was purchased from Sigma Chemical Company, U.S.A. all the other hormones were donated by Dr. S S Lynch of the Birmingham and Midland Hospital for Women, Birmingham, England. The percentage cross-reaction was calculated from the amount of cross-reactant required to reduce antibody binding by 50%.

RADIOIODINATION

Labelled GH tracer was prepared at 6 weekly intervals using 4 ug of iodination grade growth hormone obtained from the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK), U.S.A. and the Chloramine-T method as described by Hunter and Greenwood¹¹ with some modifications. Purification of the iodinated GH tracer was carried out by Sephadex G100 column (1 x 40 cm) chromatography (Pharmacia Fine Chemicals, Uppsala, Sweden), with 0.05 M sodium phosphate eluting buffer containing 0.5% (w/v) BSA. The peak protein fractions were diluted appropriately with assay buffer, aliquoted and kept at -20°C.

RIA PROTOCOL

Duplicate RIA tubes containing 100 ul of sample or standard were incubated with 100 ul of diluted antiserum in a final volume of 0.4 ml in assay buffer for 4 hours at room temperature and another 20 hours after the addition of 100 ul of about 10000 c.p.m. tracer containing 0.5% (v/v) normal rabbit serum (NRS). 100 ul of diluted goat anti-rabbit serum was then added to all tubes except the total count tubes and after another

2 hours at room temperature and addition of 300 ul of 8% PEG 8000, separation was done by centrifugation at 2000 x g for 30 mins. The supernatant was decanted and discarded and the bound fraction in the pellets counted in a gamma counter (LKB-Wallac 80000).

ASSAY OPTIMISATION

Primary Antiserum Concentration. The effect of varying the amount of antiserum was studied by incubating a series of tubes consisting of 0, 0.78 and 200 mIU/l of GH standards with antiserum of different dilutions (1:55000, 1:60000, 1:65000 and 1:75000).

Matrix Effect. In order to assess the matrix effect on the assay system, a series of GH standard curves were set up in different media containing phosphate buffer alone or 100 ul of either charcoal-stripped human serum or normal horse serum.

Tracer Concentration. 3 sets of GH standard curves were set up with different concentrations of the tracer (5000, 10000 and 20000 c.p.m. per assay tube), added to each set.

Sequence of Tracer Addition. To study the effect of delayed tracer addition, 2 GH standard curves were set up. In one, the tracer was added immediately prior to incubation and in the second, the tracer was added only after 4 h preincubation with the antiserum.

ASSAY VALIDATION

Linearity Study. This was assessed by measuring the GH concentrations of several human sera as neat samples, and after dilution with buffer at 1:2, 1:4 and 1:8.

Recovery Study. This was carried out by adding known quantities of antigen GH to normal serum samples and then assaying them. Recovery was calculated as the percentage of hormone recovered over the actual amount added.

Reproducibility. In this study, 114 serum samples from normal subjects (n = 65) and patients (n = 49) were analysed both by the in-house assay and with a commercial double-antibody radioimmunoassay kit (Dainabot, Japan).

NORMAL REFERENCE VALUE

Fasting serum samples obtained from 95 adults (students and staff of the University and the Institute) were assayed by the in-house method in order to determine the normal reference range.

DATA ANALYSIS

All RIA results were analysed using the 1224 RIACALC, RIA Lab. and Data Management Program (LKB – Wallac) and the Minitab Data Analysis Software.

RESULTS

The two rabbits immunised with GH responded well and gave the best titres (expressed as the final dilution at which the antisera bounded 50% of the tracer) after the fourth booster (Fig. 1). The titres decreased from 20 weeks onwards. Due to the similarity of the titres, antisera from bleeds at weeks 12 and 16 were pooled from rabbit 1A. A higher titre was obtained when in-house tracer was used. The most suitable initial dilution for antiserum 1A which gave 30% binding was 1 in 65000. Antiserum 1A was used at a final dilution of 1 in 325000 using in-house tracer while antiserum 2A was used at a final dilution of 1 in 300000. The association constants of antisera 1A and 2A were found to be 6.56×10^{10} and 3.8×10^{10} L/M respectively. Antisera 1A and 2A showed cross-reaction of 0.19% and 0.3% respectively with HPL and 0.21% and 0.13% respectively with prolactin. Both antisera gave virtually no cross-reaction with FSH, LH, HCG and TSH up to a concentration of 20 ug/ml (Table 1). Antiserum 1A was used for the rest of the studies due to its higher titre as compared to 2A. As for the goat anti-rabbit serum, the optimum titre obtained with 8% PEG was at a final dilution of 1 in 120.

TABLE 1
CROSS-REACTIONS OF
RABBIT ANTI-GH SERA

Hormone	% Cross-reaction	
	1A	2A
HGH	100.0	100.0
Prolactin	0.21	0.13
HPL	0.19	0.30
FSH	<0.001	<0.001
LH	<0.001	<0.001
TSH	<0.001	<0.001
HCG	<0.001	<0.001

The specific activity of the iodinated GH ranged from 50 to 100 uCi/ug and was stable for up to 5 weeks. Since the sensitivity of the

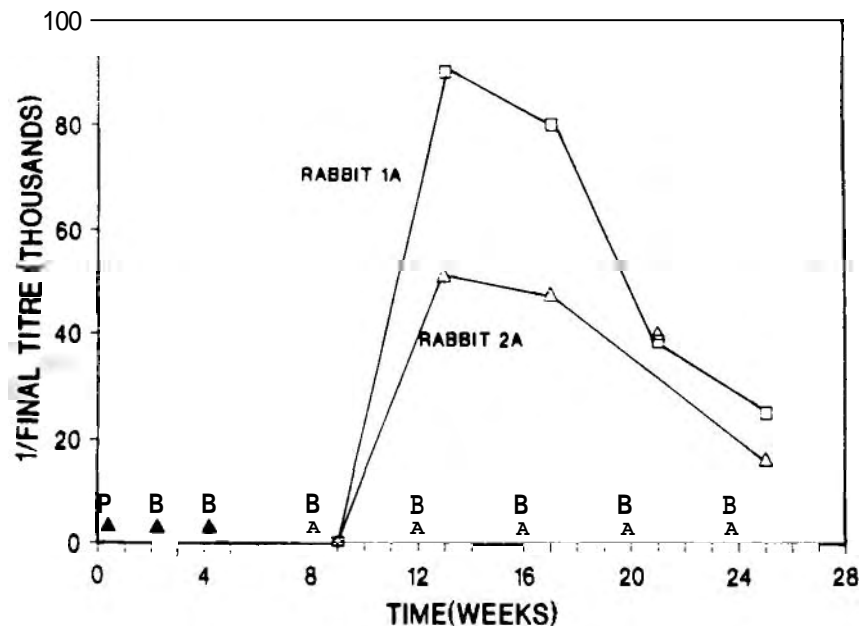


FIG. 1: Immunisation and antisera production schedule for rabbit anti-human growth hormone. P = Primary injection, B = Booster injection.

standard curve increased when lower concentrations of tracer were used, the optimum tracer concentration was kept constant at about 10000 c.p.m. per assay tube. There was no adverse matrix effect observed on the assay system. The displacement of the standards despite the different serum proteins of each medium, showed no appreciable change. All later studies used horse serum in the standards.

Results from optimisation of incubation time for the in-house assay showed that the 20h and 24h primary incubation periods at ambient room temperature gave similar acceptable bindings of about 35–38%. A longer incubation time of 48h increased the total binding to 45%. When the incubation temperature was reduced to 4°C, the assay system required a total of 72h incubation time in order to achieve a similar total binding.

The results from recovery and linearity studies are summarised in Tables 2 and 3 respectively. The recoveries (mean \pm SD) of added GHG at concentrations of 3.4, 19.6 and 56.7 mIU/l were 94.1 \pm 5.9%, 98.2 \pm 5.6% and 103.1 \pm 6.6% respectively. Diluting serum samples at 1:2, 1:4 and 1:8 gave estimates of 101.3 \pm 5.3%, 109.6 \pm 13.4% and 97.3 \pm 13% respectively of original values of undiluted sera. Table 4 summarises the overall precision of the in-house assay. The

intra-assay coefficients of variation (CVs) at doses 2.7, 7.5, 13.6 and 28.2 mIU/l calculated from assays using different lots of labelled GH were 6.5%, 3.8%, 4.3% and 3.7% respectively and the corresponding inter-assay CVs were 8.9%, 6.5%, 5.6% and 4.9% respectively. The most suitable working range, defined as the spread of analyte concentration with a CV of less than 10% from the precision profile of a standard curve, ranged from 0.94 mIU/l to 60 mIU/l.

The in-house assay showed very good correlation with RIA from the Dainabot kit ($r = 0.95$, $p < 0.001$, $y = 0.56x + 1.15$) (Fig. 2). The GH levels as measured by our in-house assay were, however, negatively biased when compared to values with the Dainabot kit. Table 5 summarises the reproducibility and the sensitivity of our in-house assay as compared to the Dainabot assay. The mean minimum detectable concentration defined as the dose at 3 standard deviations (SD) from zero binding calculated from 28 assays was found to be 0.4 mIU/l. Our in-house assay showed better precision and higher sensitivity when compared to the Dainabot kit.

The histogram plot of fasting GH measured in the 95 'normal' individuals is shown in figure 3. Since the values were not normally distributed, the normal reference fasting

TABLE 2
RECOVERY STUDY

Initial GH mIU/l	Added GH mIU/l	Measured GH mIU/l	% Recovery
2.4	3.4	5.6	94.1
	19.6	21.9	99.5
	56.9	61.9	104.5
3.4	3.4	6.4	88.2
	19.6	22.2	95.9
	56.9	60.0	99.5
2.7	3.4	5.7	88.2
	19.6	24.0	108.7
	56.9	58.4	97.9
4.2	3.4	7.6	100.0
	19.6	23.2	96.9
	56.9	60.9	99.6
8.8	3.4	12.2	100.0
	19.6	27.4	94.9
	56.9	73.7	
Mean % Recovery			98.8 ± 6.8

value was set at <7 mIU/l based on the fact that GH levels of 97% of the subjects were below this value.

DISCUSSION

The in-house assay has been validated and optimised. The best GH antisera titres obtained at weeks 12 and 16 after the primary immunisation and the corresponding association constants of 3.8×10^{10} and 6.56×10^{10} L/M are comparable to those reported by Butt *et al.*¹² Our antisera titres are however appreciably higher, indicating that a higher yield of antisera was obtained. Both of our antisera showed minimal cross-reactions with other peptide hormones - antiserum 1A cross-reacting at only 0.19% to human placental lactogen as compared to 1.3% from the earlier report. The titre of our goat anti-rabbit IgG at a final dilution of 1 in 120 is also comparable to those produced commercially, such as the anti-sheep/goat IgG from the Scottish Antibody Production Unit.³ By using 8% PEG during separation as suggested by Edwards,¹⁴ the second antibody could be used at a higher dilution. The overall assay performance as assessed by precision profile using the second

TABLE 3
LINEARITY STUDY

Dilution	GH samples mU/l					% Mean recovery
	I	II	III	IV	V	
neat	27.4	39.3	13.3	32.1	28.2	
1:2	13.2	20.8	7.2	15.9	13.7	101.3 ± 5.3
1:4	7.1	12.9	3.7	8.7	6.7	109.6 ± 13.4
1:8	3.3	5.5	1.4	4.4	3.0	97.3 ± 13.9

Serum samples were assayed neat and after dilution with assay buffer to 1:2, 1:4 and 1:8.

TABLE 4
REPRODUCIBILITY OF IN-HOUSE ASSAY

	Intra-assay			Inter-assay		
	QC Low	QC Mid	QC High	QC Low	QC Mid	QC High
Number (n)	6	6	6	12	12	12
Mean GH (mIU/l)	2.7	13.6	28.2	2.9	13.8	30.7
SD	0.2	0.7	1.8	0.3	0.8	1.5
% CV	8.3	5.1	6.3	10.3	5.6	4.9

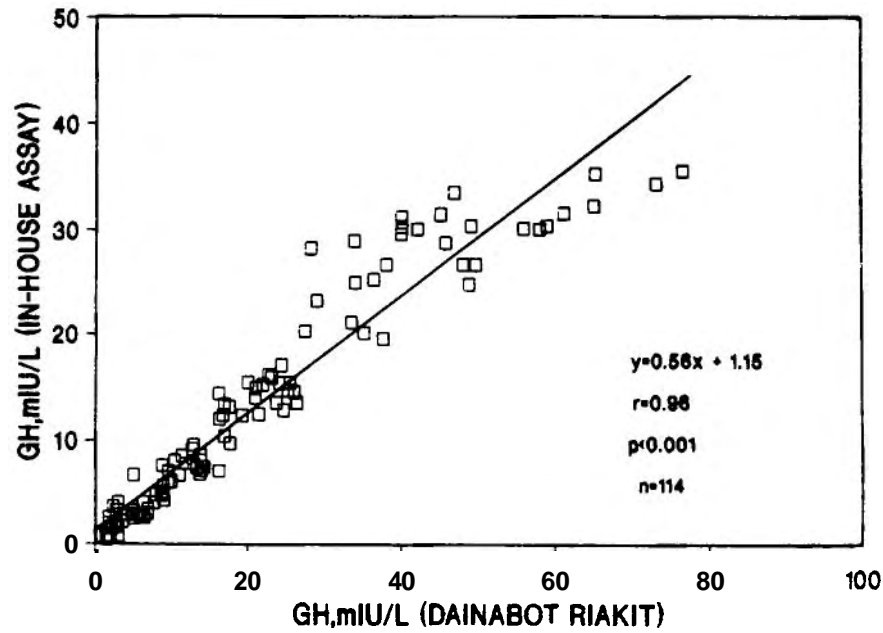


FIG. 2: Comparison of GH levels measured by in-house assay RIA and Dainabot kit.

TABLE 5
COMPARISON OF REPRODUCIBILITY AND SENSITIVITY OF
IN-HOUSE ASSAY AND DAINABOT KIT

	<u>Inter-assay</u>					
	<u>In-house (n = 12)</u>			<u>Dainabot (n = 4)</u>		
	<u>QC Low</u>	<u>QC Mid</u>	<u>QC High</u>	<u>QC Low</u>	<u>QC Mid</u>	<u>QC High</u>
Mean GH (mIU/l)	2.9	13.8	30.7	5.5	25.9	43.9
% CV	10.3	5.6	4.9	12.6	8.7	7.7

	<u>Minimal detectable concentration (mdc)</u>	
	<u>In-house (n = 4)</u>	<u>Dainabot (n = 5)</u>
	Mean GH (mIU/l)	0.4
SD	0.2	0.4

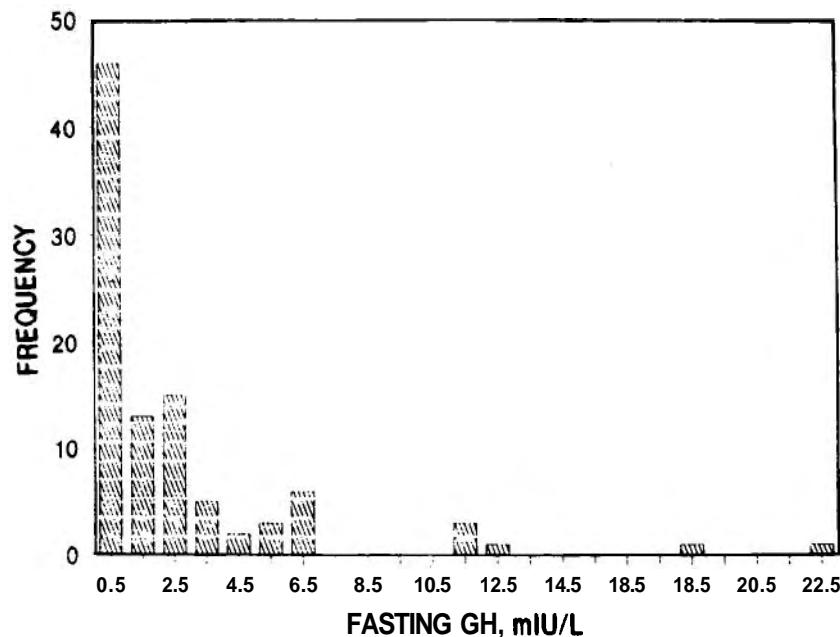


FIG. 3: Frequency distribution of fasting GH concentrations from normal adults (n = 95).

antibody - PEG for separation is good. The assay is robust and easy to set up since it only requires a total incubation time of about 26 hours and ambient room temperature. Recovery, linearity and reproducibility studies showed that the values of growth hormone measurement by this assay are accurate with an acceptable imprecision of less than 10% for the working range of standards from 0.94 to 60 mIU/l. Results from our in-house assay correlates well ($r = 0.96$, $p < 0.001$) with the commercial Dainabot RIA kit from undetectable values up to 35 mIU/L. The normal reference fasting GH value of < 7 mIU/l, based on 97% of the results from 95 serum samples, is comparable with that of < 10 mIU/l of an earlier report.⁵

The Chloramine-T method was chosen for tracer preparation since it is the most widely used method.¹⁶ Milder radioiodination procedures such as the use of lactoperoxidase and iodogen have been reported to be more suitable for radioiodination of peptide antigens.^{7,18} We however, have not investigated the use of other alternative radioiodination procedures since we found the Chloramine-T method to be highly suitable with consistent and reliable results. By limiting the amount of tracer used (10000 c.p.m. per assay tube) and delaying the addition of tracer, the sensitivity of the assay was improved in accordance with the theoretical guidelines reported by Hunter.¹⁹

With a minimum detectable concentration (MDC) of 0.4 mIU/l, the assay sensitivity was better than that of the Dainabot RIA kit.

There was no adverse matrix effect on the assay since the displacement of the standards containing either human-stripped serum or horse serum was similar to the standards in buffer alone. Although there was no overall adverse matrix effect, horse serum was routinely used in the preparation of standards since discrepancies may arise especially at low GH concentrations.⁸ Horse serum was used instead of charcoal-stripped human serum as the latter was more difficult to obtain and prepare.

The high cost, unreliable supply and short shelf lives of commercial kits can be avoided by the use of an in-house assay. The availability of large supplies of in-house RIA reagents confers many advantages. Reproducibility of assay technique and performance can be assured over the long term leading to obvious benefits in the interpretation of laboratory results and their comparability amongst the many users of the radioimmunoassay service.

ACKNOWLEDGEMENTS

This research project was supported in part by an IRPA grant from the Ministry of Science, Technology and Environment, Malaysia and IAEA. We wish to express our gratitude to

Dr. S Lynch (Birmingham) and NIDDK for generous donation of pure human pituitary peptides and to En. Ghazali Ahmad, Medical Laboratory Technologist, IMR, for technical assistance.

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