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Development of *In-Vitro* cell based assay for the determination of biological activity of FSH using a CHO based recombinant cell line

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The In-Vitro bioassay was developed for the determination of biological activity of Follicle Stimulating Hormone (FSH) using recombinant Chinese hamster ovary (CHO) based cell line that expresses the human FSH receptor (CHOSI FSHR CRELuc B-5) as well as luciferase reporter construct. The signal measured here comes from the luciferase activity that is dependent on the FSH stimulation. The present work focuses on an In-Vitro receptor-binding assay in which binding of FSH to its receptors (expressed on CHO cells) gives a dose and/or activity-dependent production of cAMP quantified by luciferase assay system. This kind of In-Vitro bioassay is used to determine the biological potency of FSH molecule. The assay is simple, has high throughput (performed in 96 half well area plates), and shows reproducible dose-response curves in the concentration range of 10.5-0.02 mIU/mL. With overall repeatability 6.5% and spike recovery of 100.7% and the assay has high precision and accuracy. Specificity was demonstrated by the lake of activity of assaying a variety of proteins manufactured by Intas. The In-Vitro cell based reporter gene assay has the preferred characteristics which makes it suitable to determine the biological activity of Recombinant Human Follicle Stimulating Hormone rHuFSH.

Keywords: Isohormones, recombinant glycoprotein hormone, bioactivity, sialylation

Abbreviations: Drug Substance (DS), Drug Product (DP), Chinese hamster ovary (CHO), National Institute for Biological Standards and Control (NIBSC), International Unit (IU), International System of Units (SI), Parallel Line Analysis (PLA)

The Glycoprotein hormone, follicle stimulating hormone (FSH), plays a vital role in reproductive function as it stimulates the development of the ovarian follicle and spermatogenesis. FSH has considerable potential for treatment of both male and female infertility (Bishop et al., 1995). Gonadotrophic cells of the anterior pituitary are responsible for the production of Follicle stimulating hormone (FSH) and released into the circulation and plays a vital role in the regulation and maintenance of essential reproductive processes such as gametogenesis, follicular development and

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ovulation. The mode of communication of cells is through chemical signals. These chemical signals in the form of signaling molecules bind to specific receptors in the target cell. This molecule receptor union activates receptor protein and results in transduction of the signal carried by specific extra cellular messenger. A highly cell specific and sensitive response is obtained by the activation of receptor proteins. These activation triggers a cascade of reactions which is amplified and the active response is initiated. The structure of signaling molecule plays an essential role in receptor binding, activation and to initiate the sensitive signal transduction pathway. FSH is a glycoprotein hormone which is responsible for the reproductive function such as development of the ovarian follicle and spermatogenesis. FSH is 34 kDa glycolhormone consist two non-covalently linked polypeptide the α- and β- subunits.

Assessment of biological activity is very important aspect in the quality control of therapeutic proteins. FSH binding to its receptor on gonadal cells increases intracellular levels of cAMP by activating adenylyl cyclase (Richards, 1994). cAMP is the primary second messenger for the trophic effects of FSH (Means et al., 1980).

It was shown earlier that rHuFSH elicited a dose–dependant increase in luciferase activity (Albanese et al., 1994). Granulosa aromatase bioassay (GAB) (Jia et al. 1986a, 1986b) and Sertoli cell bioassay (SCB) (Padmanabhan etal. 1987) for FSH has limited sensitivity involving non human cells for assays. Prior treatment of serum with polyethylene (Jia et al. 1986a, 1986b) is must in case of rate granulose cell assay whereas the immature rat Sertoli cell assay is valid over only a limited range of serum volumes (Padmanabhan et al. 1987).These assays are highly heterogeneous and not produced consistent results as they both are dependent upon the induction of aromatase and with this dissimilarity it is difficult to interpret the physiological significance of bioactivity (Christin-Maitre et al. 1996). To address these issues, we have demonstrated a unique bioassay to study the In-Vitro binding affinity and intrinsic bioactivity of rHuFSH using CHOSI-FSHR-Luc assay (Albanese et al., 1994).

Here we are reporting reporter gene cell based assay for rHuFSH which binds to recombinantly expressed human FSH receptor (rHuFSHR) in CHOSI cells. rHuFSH activate G protein complex and the alfa subunit of G-protein activates adenylate cyclase that converts ATP to cAMP. cAMP activates the cAMP response elements (CRE) that in turn regulates the luciferase reporter gene expression. The biological potency of any FSH sample can be determined by the measurement of this cAMP level via luciferase expression. The study was undertaken to develop the In-Vitro bioassay method and to qualify its performance demonstrating its suitability for the intended purpose. This exercise will further confirm the suitability of the method and demonstrate the reliability of the method through an extensive investigation of various parameters based on a written protocol and defined acceptance criteria. A precise and accurate assessment of biological activity of FSH is necessary for consistent and controlled manufacture of a well-characterized protein product (Workshop on well characterized Biopharmaceutical Product, 1995). An In-Vitro bioassay based on the ability of FSH to bind its receptors when expressed on CHO cells gives the liner relationship between the relative luciferase unit (RLU) and number of bound rHuFSH to the receptors. This technique was developed to assess the potency of the therapeutic protein.

Materials and methods

Reagents

Cell culture reagents MAM PF-2 (Bioconcept, Catalog No. 10-2F24-I), Pluronic F-68 (Bioconcept, Catalog No. 5-75F02-H), Phenol

Red (Sigma, Catalog No. P3532 or any cell culture tested), Dialyzed FBS (Invitrogen. Catalog No. 26400-044), Gentamicin (Ranbaxy), L-Glutamine (Sigma, Catalog No.G7513 or Hyclone, Catalog No. SH30034), Luciferase assay system (Promega, Catalog # E1501), Cell culture lysis buffer 5X (Promega, Catalog # E153A), White 96 well half area Assay Plates (Corning, Catalog No. 3688), Dilution Plate, 0.5 mL Polypropylene Plates (Nunc, Catalog No. 260251), Luminometer (Turner BioSystems USA).

Growth and maintenance of CHOSI FSHR CRELuc B5-Cells

The transformed CHO cell line CHOSI FSHR CRELuc B5-Cells received by technology transfer from Eugenix Biotechnologies, Switzerland. The cell line was grown in MAM PF-2 medium supplemented with L Glutamine (100X), Plurronic F-68, Phenol Red Solution and Gentamicin were cultured at 37^0C in a humidified 5% CO_2 incubator.

Luminescence assay

Reporter gene assay was performed using assay medium supplemented with MAM PF-2, L Glutamine (100X), Plurronic F-68, FBS (Dialysed). Cells to be used should be minimum 24-36h from the last passaging. Cell count should be between $1x10^6$ to $1.5x10^6$cells/mL and with viability greater than or equal to 90 %. Take the required volume of the cell suspension into 50 mL sterile centrifuge tube(s) and centrifuge at 350 x g (equivalent to 1400 rpm) for three minutes at room temperature. Add 50 µL of the final cell suspension in a white 96 well half area Assay Plates (50,000 cell/well). Incubate plates at 37^0C for 30 min. After 30 min different concentrations of test component (dose number 1 to 8 in polypropylene "dilution" plate that is a concentration range of 10.5-0.02 mIU/mL) was added and incubated for ~4 h. At the end of FSH incubation, cells were lysed (use 5X lysis buffer), incubated for 15 min at room temperature before reading the plate in a luminometer (Turner BioSystems USA). After these add 50 µl of luferase assay reagents which provided in the Luciferase assay system (Promega, Catalog # E1501). Specific activity was calculated using PLA software (Stegmann System-beratung, Rodgau, Germany).

Results

In-Vitro Cell based reporter gene assay for rHuFSH

This *In-Vitro* assay requires generation of a stable cell line that will express the receptors for the target protein (FSH) on the surface of cells. These cells will then respond to varying degree of receptors which are engaged by the target protein i.e. as demonstrated in figure 1 the concentration of FSH increased the corresponding RLU is also linearly increase. In this cell based assay rHuFSH binds to recombinantly expressed human FSH receptor (FSHR) in CHOSI cells. FSHR activates G protein complex and the alfa subunit of G-protein activates adenylate cyclase that converts ATP to cAMP. cAMP activates the cAMP response elements (CRE) that in turn regulates the luciferase reporter gene expression. The biological potency of any FSH sample can be determined by the measurement of this cAMP level via luciferase expression.

The sigmoidal nature of the dose-response curve is typically found along with a 4.5 fold increase in relative luciferase unit. The EC_{50} is around 0.698 mIU/mL depending on the cultural condition and harvest density of cells. The lower limit of detection was found to be about 0.02 mIU/mL. Initial experiments were directed towards the development and optimization of assays for the potency determination of FSH. These include the growth curve analysis of the cell line, sub culturing optimization, cell density, dose range optimization, Stimulation time with drug, Substrate and lysis buffer volume and effect of passage number.

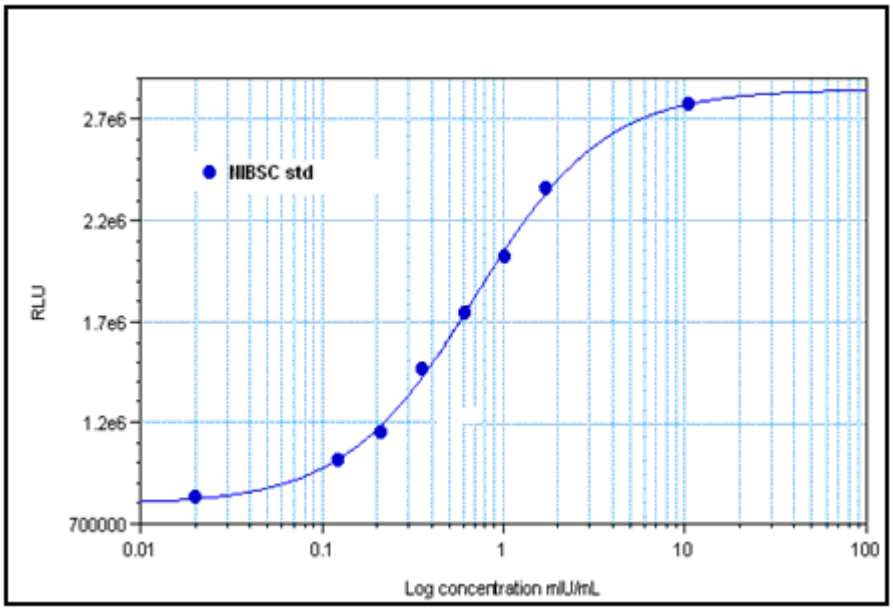

Figure 1 Responsiveness of the CHO FSH-R cell line to increasing amounts of rHuFSH (Gonal-F, Serono). Experiments were carried out in 96 well culture plates in 50µl of cell suspension at a density of 50,000 cells/well for 4 h. Data shows that the rHuFSH preparations were able to induce a significant dose dependent luciferase Production.

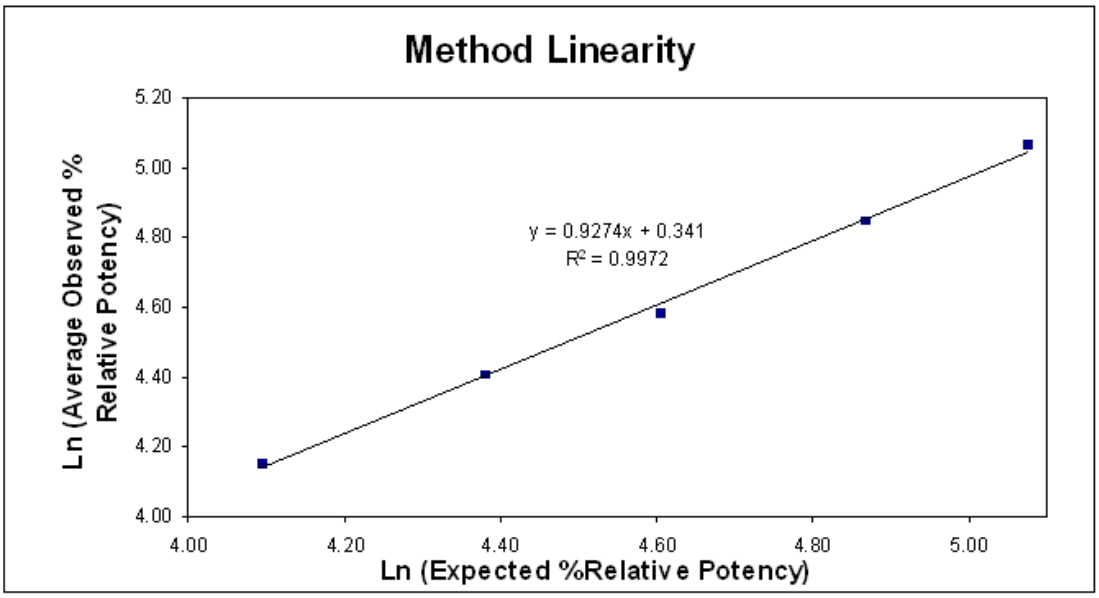

Figure 2 Linearity of method. Test bulk dilutions were prepared to get potencies, 160 % (8.80µg/mL), 140 % (7.15 µg/mL), 100 % (5.5 µg/mL), 80 % (4.4 µg/mL) and 60 % (3.3µg/mL). The samples were analyzed in In-Vitro Cell based reporter gene assay as if they were 5.5 µg/mL. Nine independent assays were performed by three analysts.

Linearity

The linearity of an analytical procedure is its ability (within a given range) to obtain a test results which are directly proportional to the concentration (amount) of analyte in the sample. Five potencies approximately evenly spaced over a log transformed potency scale, centered on 100% potency were selected. These were 60%, 80%, 100%, 130% and 160% potency level. Data from linearity experiments were analyzed as described below:

Observed relative potency converted into observed % relative potency. Percent relative potency values transformed into natural-log values. Percent expected relative potency & values transformed into natural-log values. Calculated the mean of natural-log transformed data at each potency level.

Table 1 Regression (R^2), Intercept and Slope output for method linearity

R^2	**0.997**
Intercept	0.341
Slope	0.927

A linear relationship between the expected natural log (Ln) relative potencies and observed natural log (Ln) relative potencies was demonstrated in figure 2.Linear regression analysis of the data yielded regression (r^2) 0.997, slope 0.92 and Intercept 0.341 (table 1).

Specificity

The Specificity of the reporter gene assay was determined by assaying a variety of Hormones growth factors, glycoprotein and IgG like molecules manufactured by intas. Fig 3 shows that while rHuFSH displays a typical dose-response curve, none of the other protein molecules, like Peg IFN 2b, rHuPTH, rHuGCSF, rHu Peg GCSF, rHuEPO, Rituximab were active in the assay. Results from these experiments are represented in figure 3 and a table 2 shows the assay is specific to rHuFSH and other protein does not show responsiveness to the CHOSI FSHR CRELuc B5-Cells.

Table 2 Results of Method Specificity

Sr.No.	Sample	Dose-response
1	rHu FSH(NIBSC standard)	Observed
2	rHu IFN alfa 2b	Not observed
3	rHu PTH	Not observed
4	rHu Peg GCSF	Not observed
5	rHu EPO	Not observed
6	rHu Rituximab	Not observed

Accuracy and precision

Precision and accuracy are important parameters for judging the usefulness of an assay. The Precision of the Reporter gene assay was assessed by determining repeatability (intra-assay variation) and intermediate precision (inter-assay variation). Accuracy of the method should be assessed over the specified range of the method (Validation of Analytical Procedures: Text and Methodology, ICH Guideline: Q2 (R1)). Data obtained from testing of the five different simulated potency samples (spiked with assay media) from the method linearity assessment were utilized to assess the accuracy. rHuFSH was spiked with assay media to give expected activities of 160, 130, 100, 80, and 60%. The average % recovery must be 100 % ± 15 % per potency levels which were observed (Table 3).

Precision can be defined as the closeness of agreement; here precision includes repeatability and intermediate precision. Repeatability of an analytical method describes the closeness of individual measures of an analyte when the procedure is applied repeatedly and under the same operating conditions.

Repeatability should be assessed using a minimum of nine determinations that cover the specified range (e.g. three concentrations/three replicate each) or by using a minimum of six determinations at the nominal potency level of 100% per assay. It is recommended that six assays to be performed to capture the overall repeatability. As per method capability, up to nine independent dilutions of the sample of the nominal potency level of 100% may be tested per assay. An assay included total 3 plates. In each plate the test sample was analyzed twice at 100 % potency such that 6 determinations of relative potency were obtained. Intra-plate, intra-assay precision and average repeatability were expressed in % GRSD. Table 4 represents Intra plate variation ranged from 2.5 % to 10.7 % GRSD and repeatability per assay ranged from 5.1 % to 7.2 % GRSD. Overall intra assay variation (average repeatability) observed was 6.5 % GRSD.

Intermediate precision is the degree of reproducibility of results obtained under different operating conditions like different days, different analysts etc. Three different analysts performed experiments over multiple days in order to assess the intermediate precision. Data obtained from the testing of five different simulated potency samples from the method linearity assessment were utilized to calculate intermediate precision.

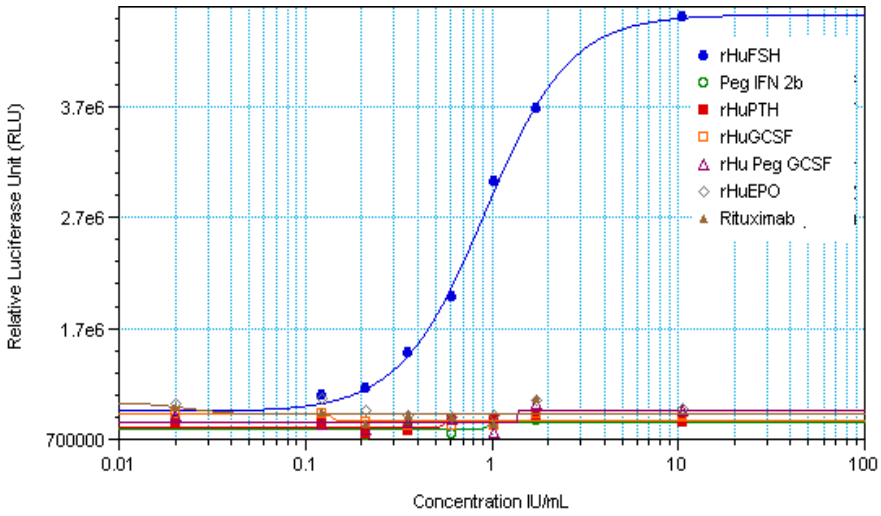

Figure 3 Specificity of Reporter Gene Assay. Indicated recombinant protein molecules were tested in the CHO FSH-R cell based reporter gene assay.

Table 3 Percent recovery per potency level and average percent recovery

% Potency level						Average
Simulated Potency	160	130	100	80	60	
% Recovery	99.1	98.4	97.7	102.5	105.9	100.7
Lower 95% Confidence Limit for the % Recovery	94.4	90.9	90.4	96.6	96.4	97.8
Upper 95% Confidence Limit for the % Recovery	104.0	106.4	105.6	108.9	116.4	103.6

Table 4 Repeatability of reporter gene assay

	Analyst 1	Analyst 2	Analyst 3
Variation	Assay 1	Assay 2	Assay 3
Intra-plate: Plate A	2.5%	2.6%	4.0%
Intra-plate: Plate B	5.7%	10.7%	9.0%
Intra-plate: Plate C	5.7%	0.8%	8.0%
Inter-assay	5.1%	7.2%	6.9%
Overall Repeatability	6.5%		

Table 5 Intermediate precision of reporter gene assay

% Potency Level					
160	130	100	80	60	Analyst
Intermediate Precision 'per Analyst per Potency level'					
11.0	5.1	7.3	5.0	13.7	Analyst 1
3.4	16.5	17.6	12.1	5.0	Analyst 2
5.3	10.5	0.7	5.7	5.4	Analyst 3
Intermediate Precision per Potency level					
6.4	10.7	10.5	8.1	13.0	NA
Average Intermediate Precision					
9.4					NA

The % GRSD per an analyst per potency ranged from 5.0 % to 13.7 % for an analyst 1, 3.4 % to 17.6 % for an analyst 2, 0.7 % to 10.5 % for an analyst 3 and the % GRSD per potency level ranged from 6.4 % to 13.0 %. The variation with average intermediate precision is 9.4 % has been observed.

Discussion

Our was to develop a potency assay for quality control testing of recombinant human follicle stimulating hormone which is convenient, non-radioactive, accurate and precise. We have adopted cell based reporter gene assay to measure FSH bioactivity with a high degree of sensitivity and specificity. We have also demonstrated assay suitability, reproducibility, precision and accuracy for routine quality control testing. The most significant characteristic of the CHOSI cells (recombinant receptor cell line), is that the bioassay retains species specificity; dose not relies on primary tissue culture, no use of radioisotopes and can be completed within a 5hr after hormone addition.

This homologous bioassay which shows its specificity to rHuFSH only uses a luciferase gene (reporter gene) which is linked to the promoter of the glycoprotein hormone that is highly responsive to cAMP (S. Christin-Maitre et.al. 1996). Reporter gene cell based bioassay is simple and highly reproducible when multiple plates analyzed on different days, thus enabling its high throughput screening. The assay shows dose-response curves in the concentration range of 10.5 to 0.02 mIU/mL/well, with EC_{50} of 0.2-0.45 mIU/mL. With intra assay variability 6.5%, inter assay variability 9.4% and spike recoveries are 100.7%, the assay has the precision and accuracy requires for a potency

assay. It has a high degree of specificity since variety other protein molecules dose not show any specificity. The main advantage of the reporter gene assay is the speed with which samples can be processed and the absence of long incubation time which helps to minimize the variability between samples. We also have checked certain robustness parameters like number of cells per well, Cell incubation, the time of incubation with drug, Reading at two different systems, which shows assay has the capacity to remain unaffected by small, but deliberate variations in the optimized parameters and provides an indication of its reliability during normal usage (data not shown). The recombinant follicle stimulating hormone is highly heterogeneous and the In-Vivo biological activity has depended on its being adequately sialylated (sialic acid content). rHuFSH is glycosyalated protein and produced from genetically modified organism not from chemical synthesis which may encounter process related and product related modification/changes. Analysis of desialylated rHuFSH sample shows high activities in In-Vitro (R.Patel, Unpublished data) as the molecules were shown full receptor binding in In-Vitro as molecules were shows full receptor binding because of the less steric hindrance (Galway, A.B. et.al. 1990). This bioassay is allowing correlation of silaic acid content and biological activity In-Vitro. This bioassay should provide new strategies for studies of rHuFSH structure-function and efficient testing of rHuFSH produced recombinantly.

We conclude stating that the cell based reporter gene assay is a reliable and sensitive assay for the biological characterization of the glycohormone and efficient testing of routine samples for batch release. Finally, the cell based In-Vitro reporter gene bioassay is simple, time-saving and useful for replicating assays with a large number of data points.

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