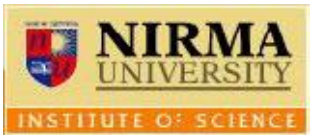
Development of *In-vitro* techniques which can measure changes in the tertiary structure of proteins and correlating these changes to *In-vivo* (animal-based) potency.

A Thesis

Submitted To Nirma University



For The Degree of

Doctor of PhilosoPhy

In

Science

By

Patel Ravish Becharbhai

(Registration Number: - 09EXTPHDS21)

UNDER THE GUIDEANCE

OF

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July 2013

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CERTIFICATE

This is certify that the thesis entitled Development of various In-vitro techniques which can measure changes in the tertiary structure of proteins and correlating these changes to In-vivo (animal-based) potency has been prepared by Mr. ravish Patel under my supervision and guidance. The thesis is his/her own work completed after careful research and investigation. The work of the thesis is of the standard expected of a candidate for Ph. D programme in 3Year Tenure and I recommended the it be sent for evaluation.

Date: 22nd July, 2013

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Declaration

I, Mr. Ravish Patel registered as research Scholar, bearing Registration No.09EXTPHDS21 for Doctoral Program under the Faculty of Science of Nirma University do hereby declare that I have completed the course work, Pre-synopsis, seminar and my research work as prescribed under R.Ph.D. 3.5.

I do, hereby, declare that the thesis submitted is original and is the outcome of the independent investigation/research carried out by me contains no plagiarism. The research is leading to the discovery of new correlation of scientific facts already known. This work has not been submitted to any other University or body in quest of degree, diploma or any other kind of academic award.

I do, hereby, further declare that the text, diagrams or any other material taken from other sources (including but not limited to books journals and websites) have been acknowledged, referred and cited to the best of my knowledge and understanding.

R. Patel

Date: 22/07/2013

Signature of Student

Affectionately Dedicated To,

My Family,
Whose love and affection are infinite.

My Friends,
Who are always with me in adversity and prosperity.

My Guide,
To whom I shall remain indebted for giving new shape and path to my life.

Acknowledgement

I extend my sincere thanks to.....

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"If friendship is your weakest point then you are the strongest person in the world"
-Abraham Linkcon

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"Success is getting what you want; happiness are wanting what you get"
-W.P.Kinsella

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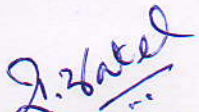
I might have missed few names, but I sincerely acknowledge all those who helped me directly or indirectly in materializing this research.

Thanks every one!



- Ravish Patel

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Abbreviations

AM	Assay Media
API	Active Pharmaceutical Ingredient
APL	Alkaline Phosphatase
ASGPR	Asialoglycoprotein Receptor
BCIP/NBT	5-Bromo-4-Chloro-3'-IndolyPhosphate and Nitro-blue Tetrazolium
BSA	Bovine Serum Albumin
BSC	Bio-Safety Cabinet
CHO	Chinese Hamster Ovary
CRS	Commercial Reference Standard
CZE	Capillary Zone Electrophoresis
Df	Degree of Freedom
DMSO	DiMethyl SulfOxide
DP	Drug Product
DS	Drug Substance
EC_{50}	Effective Concentration at 50
EPO	Erythropoietin
FSH	Follicle Stimulating Hormone
GCSF	Colony Stimulating Growth Factor
GH	Growth Hormone
GM	Growth Media
GMP	Good Manufacturing Practice
GRSD	Geometric Relative Standard Deviation
hCG	Human Chorionic Gonadotrophin
HEPA	High Efficiency Particulate Air
HPAEC-PAD	High Performance Anion Exchange Chromatography with Pulsed Amperometric Detection
HPLC	High-Performance Liquid Chromatography

ICH	International Conference on Harmonisation
IEF	Isoelectric Focusing
IFN	Interferon
IRS	Internal Reference Standard
IU	International Unit
LH	Luteinizing hormone
Ln	Natural Log
MAM	Mammalian Artificial Medium
NIBSC	National Institute of Biological Standard and Control
NA	Note Applicable
PBS	Phosphate Buffer Saline
PEG	PolyEthylene Glycol
pI	Isoelectric Point
PLA	Parallel-Line Assays
PTH	Parathyroid Hormone
PVDF	PolyVinylidene DiFluoride
RLU	Relative Luciferase Unit
RSD	Relative Standard Deviation
RT	Room Temperature
SD	Standard Deviation
SDS	Sodium Dodecyl Sulfate
SE-HPLC	Size Exclusion High Performance Liquid Chromatography
SOP	Standard Operating Procedure
SSE	Sum of Squares of Errors
STDEV	Standard Deviation of mean
$t_{1/2}$	Half-life
TBS	Tris Buffer Saline
WFI	Water for Injection

Abstract

Proteins are very large and complex molecules. Its three dimensional structure determines its functional characteristics. Only a small part of the structure participates in bioactivity and in some cases a few amino acids. Proteins also have many post-translational modifications which are quite heterogeneous even when produced by a single expression clone. These differences are even more magnified with minor variation in the process used for making them. In short, process determines the product characteristics, especially in terms of its functions. Therefore, merely showing that the primary structure is the same is not sufficient to prove that the protein is physiologically active and the activity is the same from batch to batch.

The best information on the tertiary structure is therefore provided by bioassays that truly reflect their functional properties. For example, human growth hormone (hGH) shows growth promotion in animals (*in-vivo*) as well as of cultured cells (*in-vitro*). The *in-vitro* assays for some proteins are able to pick up variation in the efficacy of the molecule and therefore closely reflect the *in-vivo* function. However, not always *in-vitro* assays can replace the *in-vivo* assays. This often leaves animal-based testing as the best option for looking into potency.

However, with better understanding of how the proteins bind to receptors on the target cells and how they transmit the signal, more cell-based assays are beginning to be accepted as study models for potency estimation. Often some proteins exert their *in-vivo* biological effect through complex interactions with receptors present on different cell-types often involving different epitopes, and therefore a single cell-based *in-vitro* assay is not enough to capture the overall biological effect of a therapeutic protein. Therefore, it is necessary to have orthogonal in-vitro assays that collectively can be used to evaluate the *"in-vivo"* potency without actually carrying out *in-vivo* animal testing.

Present invention focuses on a few *in-vitro* assays that are designed to reflect subtle variation in the three dimensional structure of proteins. These variations are then checked for trends in the biological activity of the molecule. A positive trend that is parallel and in proportion with the biological activity is the objective behind developing the *in-vitro* assay.

It is believe that the carbohydrate component and the degree of sialyation of glycoproteins are responsible for transmitting different physiologic signals to the target cells. The degree of sialylation of a recombinant glycosylated protein is often affected by the conditions used in expressing the recombinant protein, and therefore the heterogeneity of sialic acid content is dictated by culture conditions. Sialylation imparts a negative charge on the glycoprotein molecule giving rise to many different isoforms. All these isoforms have different half-lives and consequently different pharmacodynamic behavior. The heterogeneity in the degree of sialyation is assayed by the in-vivo bioassay in animal models. For example - the Steelman-Pohley bioassay which uses a rat model is applied for Follicle Stimulating Hormone. Similarly a mice assay is used for estimation of potency for Erythropoietin and Darbepoietin. Such in-vivo assays have limited precision, require large numbers of laboratory animals and involve cumbersome procedures for data generation and interpretation thereby lacking in high throughput.

On the other hand an in-vitro cell-based assays offer high sample throughput, higher accuracy and precision compared to any animal-based in-vivo assay. As a proof of concept, an in-vitro cell based report gene assay will be developed which will simulate the rat ovarian weight augmentation assay (Steelman-Pohley bioassay). 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 degrees of proliferation when the receptors are engaged by the target protein. The degree of proliferate will determine the degree of binding which in turn will be dependent on the degree of sialyation. The assay is supposed to mimic the in-vivo bioassay.

After establishing a correlation between the in- vivo animal based assay and the in-vitro cell-based proliferation assay, the animal assay can be substituted by the in-vitro method. Such assay can be use because of their simplicity, speed, high-throughput and cost, and will reduce the need of animals.

Synopsis

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1. Introduction

Sialic acid plays a very important role in the In-Vivo biological activity of Follicle Stimulating Hormone (FSH).The In-Vitro assays for some proteins are able to pick up variation in the efficacy of the molecule and therefore closely reflect the In-Vivo function. However, not always can In-Vitro assays replace the In-Vivo assays. This often leaves animal-based testing as the best option for looking into potency. The biopotency of human FSH is determined on the basis of In-Vivo (Steelman-Pohley) bioassay that is ovarian weight augmentation assay. 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 Cyclic adenosine monophosphate (cAMP) quantified by a luciferase assay system. This In-Vitro assay is used to detect subtle changes in the sialic-acid, which alters the binding of FSH to its receptor, thereby affecting luminescence intensity. The resulting signal is quantified in terms of In-Vitro specific activity using reference standard whose specific activity is known. This study shows an inverse liner correlation between the In-Vitro and In-Vivo activity indicating that the In-Vitro cell-based activity assay can allow substitution of In-Vivo biological potency assay that relies on the use of animals.

1.1 Molecule

Cells communicate with each other through chemical signals. These signaling molecules bind to specific receptors located in the target cell. Upon binding, the signaling molecule activates receptor proteins, which in turn transduce the signal carried by the specific extracellular messengers. The activation of such receptor proteins initiates a cascade of events that eventually culminate in highly structural features of a given signal determining not only its metabolic fate in the circulation but also its ability to bind and activate a specific target cell receptor, to generate a signal transduction and to induce a biological effect.

Follicle stimulating hormone (FSH), one of the master signals produced by the anterior pituitary gland, is involved in the regulation of several essential reproductive processes occurring at the gonadal level (Chapel et al., 1983a). This gonadotrophin belongs to a family

of closely related glycoproteins composed of FSH, Luteinizing hormone (LH), choriogonadotrophin hormone (CG) and thyroid stimulating hormones (TSH), which are synthesized in different cell types; TSH is synthesized in a distinct pituitary cell, LH and FSH are synthesized by gonadotropes and CG produced by the placental trophoblasts. All glycoprotein hormones are heterodimers consisting of a common alpha subunit non-covalently associated with a beta-subunit, which is structurally unique (Pierce and Parsons, 1981; Gharib et. Al., 1990b). The alpha subunit contains 92 amino acid residues, with five disulphide bonds contributing to its tertiary structure. The FSH beta subunit contains 111 amino acid residues, with six disulphide bonds. The specificity of each hormone resides in the beta-subunits (Lapthorn et al., 1994; Zeng et. Al., 1995; Liu and Dias, 1996; Remy et al., 1996; Grossman et al., 1997). The subunit of these glycoprotein hormones contain one or two Asn- linked (N-linked) heterogeneous oligosaccharides which have been shown to play an important role in both the In-Vivo and In-Vitro bioactivity of the hormone (Baenziger and Green, 1988; Stockell and Renwich, 1992; Smith et al., 1990). Of the four Asn-linked glycosylation sites in FSH, two are located on the alpha subunit (Asn 52 and 78) and two on the beta subunit (Asn 7 and 24). Receptors for all these trophic hormones belong to the superfamily of G-protein-coupled receptors and thus share several major structural features (Ulloa-Aguirre and Conn, 1998).

Granulosa cells and Sertoli cells are the target cells for FSH action and they are the only cell types which express the FSH receptor (Simoni et al., 1997). In the ovary, FSH regulates granulose cell function, including the development and selection of ovarian follicules and oocyte maturation. In testes, FSH regulates the function of Sertoli cells, which in turn provide physical and biochemical support for proper development and maturation of germ cells. Binding of the FSH molecule to and activation of its cognate receptor involves the participation of several structural determinants of the ligand present in both the primary sequence and the carbohydrate residues of the molecule (Comarnous, 1992). Characterization of the molecular basis of FSH-FSH receptor interaction and signal transduction is of paramount importance from a clinical point of view since it may allow the

development and production of a verity of analogues potentially useful to exogenously regulate gonadal function.

1.2 Scientific Principle of the In-Vitro Cell Based Method

(C Albanese et. al. 1994)

FSH binding to its receptor on gonadal cells increases intracellular levels of cAMP by activating adenylyl cyclase. cAMP is the primary second messenger for the trophic effects of FSH. As many promoters respond to changes in cAMP levels through discrete cAMP response elements (CREs), this bioassay was developed utilizing the FSH receptor dependent regulation of cAMP production coupled to a sensitive reporter gene, such as luciferase.

In this cell based assay FSH binds to recombinantly expressed human FSH receptor (rhFSHR) in CHOSI cells. hFSHR activates G protein complex and the alpha subunit of G-protein activates adenylate cyclase that converts ATP to cAMP. cAMP activates the cAMP response elements (CRE) that in turn regulate the luciferase reporter gene expression. Due to the measurement of the cAMP level via luciferase expression, the biological potency of any FSH sample can be determined.

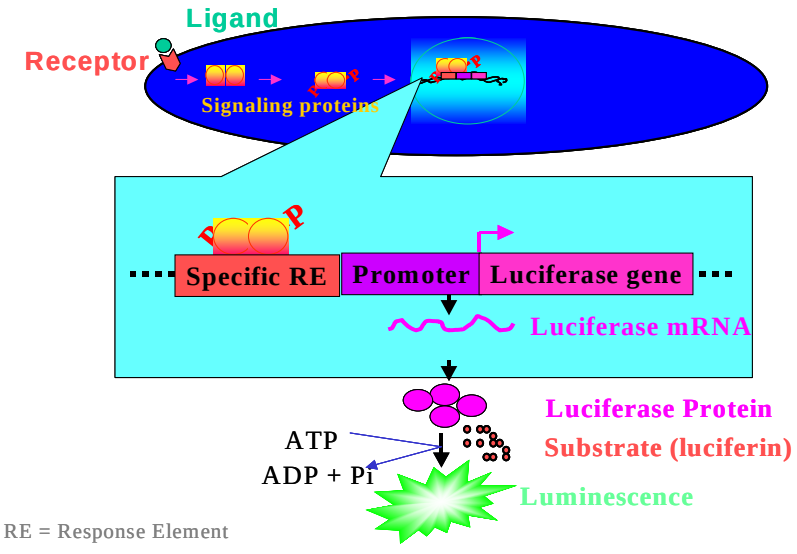


Figure 1 Model of signal transductional pathways of the FSH receptor.

1.3 Scientific principle of the Rat Ovarian Weight Augmentation Method:

This bioassay for the determination of potency of FSH preparation measures ovarian hypertrophy resulting from exogenous FSH treatments of immature female rats when administered in conjunction with HCG. Briefly for the FSH bioassay, 23 days old SD rats are injected with FSH test solutions twice daily for 3 days. Doses are diluted in diluents containing hCG and administered subcutaneously. Animals are euthanized 74 hrs after the initial injection; ovaries removed and excised free of fat and connective tissues and promptly weighed using a balance.

2 Motivations, Objective and Scope

Assessment of more than 100 batches of rhFSH drug substance (follitropin alpha) demonstrated that the specific activity, isoforms pattern by isoelectric focusing (IEF), and sialylation pattern by glycan mapping were consistent (RM Bassett and R Driebergen 2005). This observation allowed the development of a physicochemical analytical method for measuring the FSH content (by mass of protein) for the product, which was traditionally assessed in bioactivity content.

Quantification of follicle-stimulating hormone (FSH) for clinical use has traditionally involved the use of In-Vivo bioassays, particularly the rat ovarian weight augmentation assay (Steelman-Pohley bioassay). This assay has limited precision, requires large numbers of laboratory animals and involves cumbersome procedures for data generation and interpretation. Recent advances in manufacturing procedures for recombinant human FSH (r-hFSH) have resulted in a preparation (follitropin alpha; Gonal-F) that is highly consistent in both isoforms profile and glycan species distribution. As a result, follitropin alpha can be reliably quantified using an optimized size exclusion high-performance liquid chromatography (SE-HPLC) method, and vials can be filled by mass. Preliminary clinical studies suggest that the fill-by-mass process results in a product that delivers a more consistent clinical response and is more effective than follitropin alpha vials filled by bioassay in women undergoing controlled ovarian stimulation. Non-bioassay methods such as

SE-HPLC are likely to become increasingly important for quality testing and regulatory purposes, provided that the manufacturing process is well controlled and produces a protein of highly consistent physico-chemical properties (Driebergen R and Baer G 2003).

2.1 Background

Potency determination of rHu FSH Drug substance and Drug product is done by In-Vivo bioassay as per FSH draft monograph at Intas bio-pharmaceuticals (Monograph no. 2285, Pharm Europa, Vol.21, No. 3, July 2009). So far, all the release and stability samples (DS and DP) were analyzed using In-Vivo bioassay. However, stability studies require a large number of samples to be analyzed by In-Vivo bioassays which will incur very high cost and also need to be outsourced. Thus, an equivalent In-Vitro Bioassay alternative method is being proposed for the potency determination. The In-Vivo bioassay & In-Vitro bioassay method equivalency has been established by potency determination of 90 samples (DS & DP) with both the methods. It is, therefore, decided that only at selected time-points in the stability studies the analysis shall be made by the In-Vivo bioassay method while the In-Vitro bioassay will be used for sample analysis of the entire stability studies. The selection of time-points is made in such a way so as to ensure an appropriate check in the overall quality of DS and DP batches.

2.2 Chronological events leading to this situation

For potency and specific activity determination of rHu-FSH molecule by animal based In-Vivo method there are important concerns;

1. Technical: (method variability and non robustness),
2. Ethical: (using large number of animals per test),
3. Logistic issues (to maintain cold chain during shipment for In-Vivo study, dependence on third party for proper transfer of samples to Contract laboratory etc.) and
4. Commercial concerns such as very high cost of analysis.

In order to overcome all these concerns, an alternate FSH mediated cell based reporter gene In-Vitro bioassay method has been developed, qualified and validated, and found to be suitable for its intended use in batch release and stability studies.

2.3 Proposed plan & objective of this Study

Proposed plan is to switch over from animal based In-Vivo bioassay to cell based reporter gene In-Vitro bioassay for determination of potency of rHu-FSH.

Objective of this study is to provide justification and analysis of risk associated with this change.

For better clarity and understanding, justification for this change control has been categorized in following three categories:

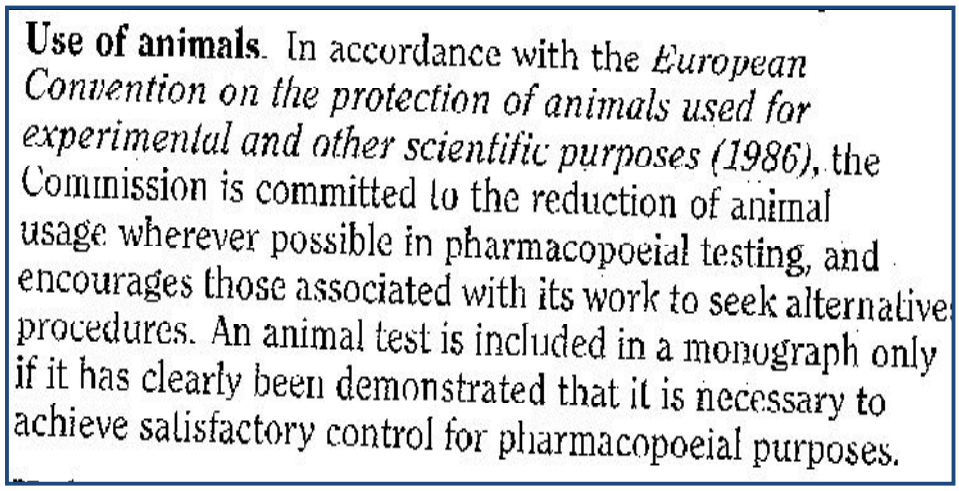
2.3.1 Regulatory perspectives

2.3.2 Technical and quality perspectives

2.3.3 Logistics, administrative and commercial perspectives

2.3.1 Justification from Regulatory perspectives

2.3.1.1 According to European Pharmacopoeia 6.0 (Introduction part; page no vi),



Use of animals. In accordance with the *European Convention on the protection of animals used for experimental and other scientific purposes (1986)*, the Commission is committed to the reduction of animal usage wherever possible in pharmacopoeial testing, and encourages those associated with its work to seek alternative procedures. An animal test is included in a monograph only if it has clearly been demonstrated that it is necessary to achieve satisfactory control for pharmacopoeial purposes.

From above mentioned pharmacopeial guidance, it has been interpreted that, on ethical ground regulatory authorities encourage minimizing use of the laboratory animal during analysis of product. Both EDQM (European Directorate for the Quality of Medicines and Healthcare) as well as US FDA have recommended to follow the 3R concept to establish alternative methods to Reduce, Refine and Replace the use of animals in the quality control of Biologicals. In the existing animal based In-Vivo bioassay method for FSH, large numbers of

animals are sacrificed for each assay (approximately 80 female immature wistar rats for each test). Proposed In-Vitro bioassay method is based on FSH-binding to recombinantly expressed human FSH receptor (FSHR) in CHOSI cells.

2.3.1.2 According to Indian Pharmacopoeia 2007 (General chapters; page no 9)',

> **Alternative Methods.** The tests and assays described are the official methods upon which the standards of the Pharmacopoeia are based. Alternative methods of analysis may be used for control purposes, provided that the methods used are shown to give results of equivalent accuracy and enable an unequivocal decision to be made as to whether compliance with the standards of the monographs would be achieved if the official methods were used. Automated procedures utilising the same basic chemistry as the test procedures given in the monograph may also be used to determine compliance. Such alternative or automated procedures must be validated.
>
> In the event of doubt or dispute, the methods of analysis of the Pharmacopoeia are alone authoritative and only the result obtained by the procedure given in this Pharmacopoeia is conclusive.

From above mentioned text it has been interpreted that, there is a provision of using alternate method provided:

- Suitability of the method for intended purpose is established- for this method qualification and method validation activity has been carried out.
- Ability of proposed method for unequivocal decision- for this method comparability between current In-Vivo method and proposed In-Vitro method has been carried out and both methods found to be comparable (details regarding comparability exercise has been provided in section 3.2).

Justification for using alternate method is same as provided in above 2.3.1.2 section of this document.

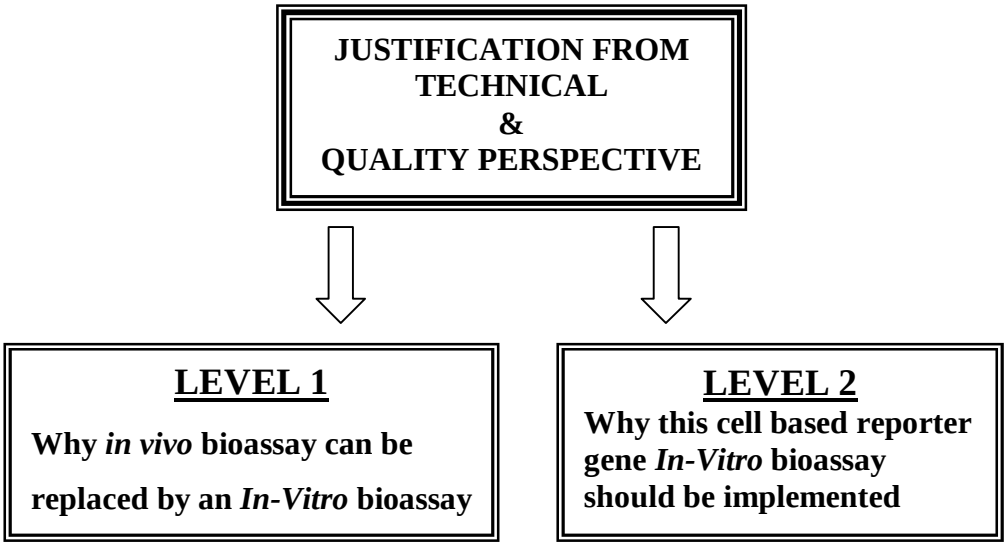
Manufacturing process of rHu-FSH was validated in 2012 and proved to be capable enough to deliver product with predefined and approved specifications consistently (approximately 200 batches of drug substance and drug product have been manufactured since 2008). Innovator Company of rHu-FSH (Merck Serono) has also switched over from In-Vivo

bioassay to In-Vitro bioassay (cell based reporter gene assay). Based on orthogonal tests of structure and glycosylation and consistent manufacturing history, several regulatory and technical consultants also suggested and encouraged to move to In-Vitro bioassay. For example, Adrian Bristow (Head, Technology Development and Infrastructure, NIBSC and Member EDQM Group 6) has commented that "for any product (Follitropin) exhibiting a closely controlled range of properties, any In-Vitro bioassay is likely to offer an acceptable level of control of the potency"

Moreover, In future if there is any deviation, OOT (out of trend) or OOS (Out of specification) during manufacturing process, quality of product can be further confirmed by In-Vivo assay in addition to In-Vitro results (should the need arise in the investigation).

2.3.2 Justification from technical & quality perspective:

This part of justification can be further categorized in following two categories:



2.3.2.1 Why In-Vivo bioassay can be replaced by an In-Vitro bioassay:

FSH preparations are heterogeneous and contain multiple isoforms, which differ in their carbohydrate structure. Carbohydrates are important to prevent degradation and to delay clearance of FSH from the circulation.

Carbohydrate plays an essential role for the In-Vivo biological activity of FSH.

Activity of FSH is completely abolished when it is completely deglycosylated but in case of In-Vitro, higher activity is observed. This is because when FSH molecule is desilylated, due to decrease in stearic hindrance, the interaction between FSH receptor and FSH is facilitated resulting in elevated In-Vitro activity.

It indicated that there is direct correlation between degree of Sialylation and In-Vivo activity and inverse relationship in case of In-Vitro bioassay.

There are several complementary methods during either manufacturing of rHu-FSH DS or in final DS e.g. CZE, IEF, Sialic acid estimation etc. that can ensure proper control of molecular heterogeneity of FSH. If results of these analyses are in accordance with predefined acceptance criteria then it can be technically concluded that during In-Vitro activity obtained is a function of FSH molecule itself, not degree of desialylation.

Assessment of more than 200 batches of rhFSH indeed demonstrated that the specific activity by In-Vivo bioassay, isoforms patterns, dissociation of subunits by iso electric focusing as well as degree of sialylation were highly consistent. This fact is a strong evidence of a controlled manufacturing process that complies to predefined acceptance criteria consistently. Thus the validated In-Vitro alternative of the In-Vivo bioassay should be acceptable by the regulatory agencies.

As a historical case in a similar fashion (that is of by demonstration of controlled manufacturing history) the Pharm Europa monograph based In-Vivo bioassay of rhFSH molecule has been replaced by the innovator with a physicochemical test.

During development of In-Vitro method, special rHu FSH samples were generated forcefully in order to find the capability of the method to detect such samples.

During development of In-Vitro method, special rHu FSH samples were generated forcefully in order to find the capability of the method to detect such samples.

The list of Samples generated were,

1) Assay response on Thermally Aggregated Samples with respect to kinetics
2) Assay response on partial Enzymatic desialylation using Prozyme kit
3) Assay response on Different Iso-forms of rHu FSH
4) Assay response on Samples which failed In-Vivo

It was clearly demonstrated that there are consistent and reproducible relationships between assay response and different types of sample that are equivalent or not inferior in comparison with In-Vivo bioassay.

2.3.2.2 Why this cell based reporter gene In-Vitro bioassay should be implemented:

2.3.2.2.1 This method was developed in Bio-characterization department, Intas Bio-pharmaceuticals, qualified and validated in Quality Control department in collaboration with Bio-characterization department and method found to be precise, accurate and specific for the determination of potency and specific activity of rHu FSH.

2.3.2.2.2 Method comparability exercise between existing In-Vivo method and proposed In-Vitro method has been carried out and on the basis of which it was concluded that:

• Sample which is out of specification in potency (P7 CB D03 DES 36hr) analyzed by In-Vivo assay, due to improper isoform distribution pattern (desialylation of the molecule), showed comparable results in the In-Vitro assay. When analyzed by In-Vitro bioassay the batch showed enhanced activity beyond the upper specification limit (due to inverse relationship between In-Vivo and In-Vitro bioassay).

• For low potent sample (sample with 80 % potency), results obtained by In-Vitro method are similar (with respect to potency) to the results obtained at Contract laboratories (Bioneeds) by In-Vivo method. This shows the low potent samples can be accurately estimated by proposed In-Vitro method.

• For super potent sample (sample with 125% potency) results obtained by In-Vitro method are similar (with respect to potency) to the results obtained at Contract laboratories (Bioneeds) by In-Vivo method. This shows, like low potent sample, super potent sample also can be accurately estimated by proposed In-Vitro method.

• All Drug substance and drug product samples, analyzed by In-Vitro bioassay method, resulted in similar potency value as obtained by In-Vivo method at Contract laboratories (Bioneeds).

From the above conclusions it can be summarized that the proposed In-Vitro bioassay method is comparable for potency estimation with In-Vivo bioassay which is being carried out at

Contract laboratories (Bioneeds). In addition, the In-Vitro assay is reliable and results obtained are reproducible confirming the non-inferiority of this assay over In-Vivo bioassay. Therefore, proposed In-Vitro bioassay can be used to analyze routine lot release samples of DS & DP as well as stability samples.

For details refer Summary of the Research Work.

2.3.2.2.3 As a result of this proposed change over, there is no impact on release specification of Drug Substance and Drug Product of rHu-FSH, it shall remain the same i.e. 80-125% of stated potency as recommended in the Pharm Europa.

2.3.3 Justification from logistic & administrative perspectives:

2.3.3.1 In accordance with European Pharmacopeia and Indian Pharmacopeia, female wistar rats should be used for assay. The strain is very expensive to procure from overseas vendors and has limitations of breeding sessions to maintain a stock of approximately 100 rats per assay. Implementation of In-Vitro bioassay shall overcome this concern also.

2.3.3.2 In proposed method, all activities pertaining to analysis such as sample preparation, Cell Seeding, incubation, data interpretation etc shall be carried out by QC itself, while in existing In-Vivo method either we are completely or partially dependent on contract laboratories. After this switch over it will be more feasible to evaluate and investigate any OOT (out of trend), deviation and OOS (out of specification) results, which is otherwise very difficult in case of contract laboratories.

2.3.3.3 In current scenario we have to send samples to contract laboratories to Bangalore for analysis, to maintain proper cold chain during transition period is in itself a significant quality concern, though we have minimized that risk by availing courier services from Blue Dart Courier, one of the renounce courier services across India but after implementation of this In-Vitro bioassay this risk will be almost nil.

2.3.3.4 From commercial perspectives also, this change is required as cost of existing method is approximately Rs.50, 000/- for Bioneeds (Bangalore), while for In-Vitro bioassay it would be approximately Rs.4500/- per assay.

2.3.3.5 For Quality control department it is very critical for timely release of batches in order to fulfill market commitment. In current scenario there is huge dependency on contract

laboratory for release of batches. Many times due to several factors e.g. unavailability of animals or other resources, this is not possible. After implementation of this assay this issue also shall be resolved.

3 Summary of the Research Work

3.1 Executive summary

The objective of this study was to show that the In-Vitro bioassay of rHu FSH is equivalent in terms of potency recovery to that of the In-Vivo bioassay described in the monograph. This was necessary to replace the In-Vivo bioassay with the In-Vitro alternative. Different DS (Drug Substance) & DP (Drug Product) samples of rHu FSH were analyzed by both methods and the estimated potency was compared. The results were tested for statistical significance between the differences of mean potency values obtained from both the methods. This analysis established the validity of the In-Vitro alternative method and showed how close the agreement is between the results obtained with the two methods. From the analysis it is concluded that both the methods are equivalent for potency recovery (with a precision of <11% of 90% Confidence Interval of Difference between Mean Potency Values) and the In-Vivo assay can be replaced selectively with that of the In-Vitro alternative (Figure 2, Table 1).

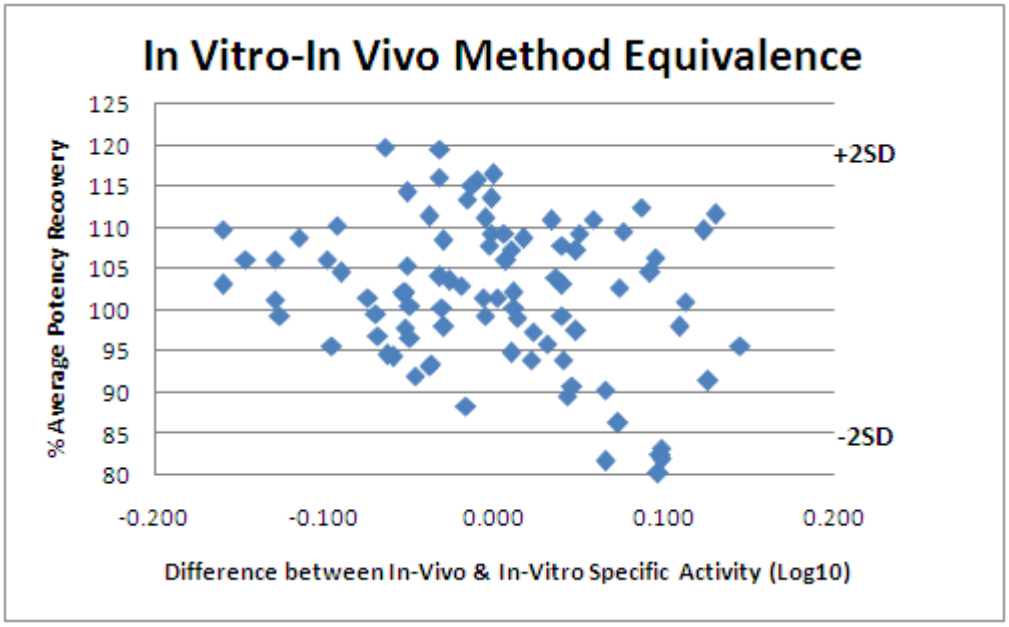


Figure 02: Residual plot of Difference between means of potency of the methods against average percentage potency recovery for each sample tested.

Lower acceptance limit	80.00
Upper acceptance limit	125.00
Diff between Means (DM)	-0.002
MSE	0.003
Harmonic mean of df	90.00
SE of difference	0.008
α	0.050
tcrit	**1.653**
Lower 90% CI of DM	96.6
Upper 90% CI of DM	102.5
Equivalence	**Equivalent**

Table 1 Analysis of statistical validity for equivalence

3.2 Data Analysis

To establish the validity of the alternative method it should be shown how close the agreement is between the results obtained with the two methods, instead of how well the in-vitro and in-vivo bioassays are correlated. Hence the following approach was applied to establish statistical significance of equivalence between the methods:

1. Calculate the average of the estimated potency values for each sample and estimate the recovery in percentage. This is the closest approximation to the true value for each sample.
2. Calculate log transformed (Log10) potency values for each sample by the two methods.
3. Calculate for each sample the difference in potency values (Log10 transformed) obtained by the two methods (for example the difference D= Log10 transformed potency value by in-vivo bioassay – Log10 transformed potency value by in-vitro bioassay)
4. Plot the values of D against percentage recovery average calculated in step 1. This is a residual plot and ideally, the points will be scattered around a mean value of 0.

5. Calculate the standard deviation (SD) of D and the 95% confidence limits as D+2SD and D-2SD, and draw horizontal lines on the plot through these values. This range D+2SD and D-2SD indicates the range between which 95% of all the calculated D values are expected to lie.

6. Calculate the standard error of D (i.e. the standard error of difference) using the following formula: √ Variance/Harmonic Mean.

7. Calculate the Lower and Upper 90% Confidence interval of Difference between Means (DM) to indicate the precision with which the DM has been estimated.

For bioassays a difference of ±20% (80-125%) indicates a good precision and results obtained by two different methods within this range considered to be equivalent.

3.3 Acceptance criteria to establish equivalence.

1. The in-vivo bioassay & in-vitro bioassay method for the testing of mean specific activities of rHu FSH should result in a 90% Confidence Interval limits of Difference between Means (as defined above) to be within 80-125% to be considered as Equivalent.

2. The residual plot of D value (Potency obtained by in-vivo bioassay – Potency obtained by in-vitro bioassay, Log10 transformed for each sample tested) and percentage recovery against the average potency value should show random distribution around a mean value of D. The range of D+2SD and D-2SD of these D values should contain 95% of all the estimated D values.

3.4 Results

Analysis of statistical validity for equivalence is shown in Table 01. Figure 02 show the plot of residuals for Difference between means of potency of the methods against average percent potency recovery for each sample tested.

3.5 Observation & Discussion

The observed range of 90% confidence interval of difference in means was 96.6 to 102.5 (Table 01). The residual plot show random distribution around mean and thus rules out any bias. Moreover 95% of the residuals as expected lies within ± 2 SD (Standard Deviation).

4 Conclusions

Based on the excellent correlation between In-Vitro and In-Vivo bioactivity assay as shown in the present study, it is clear that In-Vitro receptor-binding assay using CHO-hFSHR-Luc assay can substitute for animal based-ovarian weight gain assay. The former can therefore be used to determine the potency of the FSH for different manufacturing lots and therefore can be used for batch release of drug substance and drug product. The In-Vitro assay is sensitive and high throughput, making it easier and reliable to study variations in the manufacturing process. The study confirms that both the methods are equivalent in terms of potency recovery and thus the In-Vitro bioassay can be used wherever suitable. The sample size [n=90] and its quality of this study allows the replacement of the In-Vivo assay. It is recommended that a few commercial batches should be analyzed by both the methods as per the availability, for potency determination and statistical validity tests similar to the one described here to be done. This will ensure the complete replacement of the monograph based In-Vivo bioassay with the In-Vitro bioassay.

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1. Introduction

1. Introduction

1.1 Potency Assay for Bio-similar

Bioassay is a term used for biological assay and is of two types: *in-vitro* and *in-vivo* assays. *In-vivo* bioassays are carried out in live organisms, such as, mice, rat, hamster, etc.; where as *in-vitro* bioassay is carried out using a part of cells/ tissue derived from living organisms, such as, human, rat, monkey, hamster, etc. Bioassays are typically conducted to measure the effects of any effecter on living organism. Bioassays may be qualitative or quantitative; the latter often involves an estimation of the concentration or potency of a substance by measurement of the biological response that it produces. Quantitative bioassays are typically analyzed using the methods of biostatistics. Bioassays are essential in the development of every drug.

1. A bioassay or biological assay is a biological testing procedure for estimating the concentration of an active pharmaceutical ingredient (API) either in the form of bulk (Drug Substance – DS) or formulated product (Drug Product – DP). In contrast to the common physical or chemical methods detailed information on the biological activity of the active substance is achieved. Over the last decade biological assays (bioassays) have become more important for effective quality control program in biopharmaceutical development and manufacturing.
2. Bioassay is a test which is used to evaluate the relative potency of a chemical or mixture of chemicals by comparing its effect on a living organism with the effect of a standard preparation on the same organism. Bioassays are frequently used in the pharmaceutical, biopharmaceutical and vaccine industries to evaluate the potency.

It is essentially a technique to assess the activity of a given ligand by measuring the response of a living cell, and measuring this response against that generated by increasing concentration of a reference ligand.

1.2 The use of bioassays includes:

1. Measurement of the pharmacological activity of an unknown active pharmaceutical ingredient.
2. Investigation of the function of endogenous mediators (cell signaling).
3. Determination of the side-effect profile, including the degree of drug toxicity.
4. Measurement of the concentration of an unknown intermediate, drug substance or drug product.

1.3 Types of Bioassays:

There are various types of bioassays, such as -

1. Cytokine induced proliferation (Proliferative Bioassay).
2. Cytokine induced killing (Anti-proliferative Bioassay).
3. Functional Bioassays (like, measuring cAMP, calcium, protein expression, gene expression etc).
4. Protection against viral effects (Cytopathic effect reduction assay-CPER for anti-virals or Cytopathic assay for viruses)
5. Reporter gene assays.
6. Cytokine induced cytokine production (study of cytokine cascade).
7. Kinase receptor activated Assay (KIRA).

1.4 Historical Considerations

For more than 50 years, therapeutic preparations of gonadotrophins, including recombinant human FSH (Follicle stimulating Hormone), have been quantified by the Steelman-Pohely bioassay in International Units (Steelman and Pohley, 1953). However, this assay requires a large number of laboratory animals and has limited precision (Driebergen and Baer, 2003). Historically, follitropin alpha quantified using the Steelman-Pohley bioassay has variability up to 20% (Driebergen and Baer, 2003).

The rFSH follitropin alpha has a highly consistent isoform profile and distribution of glycans. Because of this property, it can be quantified in terms of mass units using the technique of size exclusion high performance liquid chromatography (SE-HPLC) (Lass and McVeigh,

2004). This technique enables rFSH follitropin alpha to be filled and released into the vial on the basis of mass (5.5µg equivalent to 75 IU bioactivity), with dose variability of only 2%. In the USA, follitropin alpha filled by mass (rFSH-fbm), was made commercially available in June 2004 and has progressively replaced follitropin alpha filled by bioassay (rFSH-bio).

1.5 Aims and Objectives

Potency determination of rFSH Drug Substance and Drug Product at Intas Pharmaceuticals Ltd. – a biopharmaceutical manufacture of rFSH was done using an *in-vivo* bioassay as per the FSH draft monograph in European Pharmacopoeia (Monograph no. 2285, Pharm Europa, Vol.21, No. 3, July 2009). The DS and DP release including the analysis of stability samples of DS and DP were analyzed using the *in-vivo* monograph method. Since stability studies require a large number of samples to be analyzed by *in-vivo* bioassay, it demands a large number of animal-based study which is not only costly but requires a large number of animals for test purpose, all of which are sacrificed at the end of the study.

The objective of this thesis work is to develop an equivalent *in-vitro* bioassay as an alternative method for potency determination. Furthermore, the study demonstrates equivalency between the *in-vivo* and *in-vitro* bioassays which is established by potency determination of 89 samples (of DS and DP) with both the methods. Having established equivalence between the two methods, it was possible to conduct stability studies of DS and DP using the *in-vitro* bioassay at all time points during the stability study, while only at a few selected time-points in the stability studies the analysis shall be made by the *in-vivo* bioassay method. The *in-vitro* bioassay will be used for sample analysis of the entire stability studies.

The study focuses on the development of a cell-based *in-vitro* reporter gene assay which can measure changes in the tertiary structure of proteins and further correlating these changes to *in-vivo* (animal-based) potency.

Experiments were designed to achieve the following objectives:

1. Design and development of *in-vitro* cell based assay.
2. Optimization of *in-vitro* cell based assay.
3. *In-vitro* cell based assay Validation.
4. *In-vitro* and *in-vivo* Method Correlation.

2. Literature Review

2. Review of Literature

2.1 Literature Review

It is believed that the carbohydrate component and the degree of sialyation of glycoproteins play an important role in cell signaling and are responsible for transmitting different physiologic signals to the target cells upon binding of the glycoprotein to its cell-surface receptor. The degree of sialylation of a recombinant glycosylated protein is often affected by the conditions used in the expression of the recombinant protein; i.e. the heterogeneity of sialic acid content is dictated by culture conditions. Sialylation imparts a negative charge on the glycoprotein molecule and the different degrees of sialyation gives rise to many different isoforms of glycoproteins. All these isoforms have different half-lives and consequently different pharmacodynamic behavior (M.D'Antonio *et.al.,* 1999). The heterogeneity in the degree of sialylation is routinely assayed by the *in-vivo* bioassay in animal models. For example - the Steelman-Pohley bioassay which uses a rat model is applied for Follicle Stimulating Hormone. Similarly a mice assay is used for estimation of potency for Erythropoietin and Darbepoietin (J.W.M.Mulders *et. al.,* 1997). Such *in-vivo* assays have limited precision, require large numbers of laboratory animals, are low in throughput and involve cumbersome procedures for data generation and interpretation.

On the other hand an *in-vitro* cell-based assay offers high sample throughput, higher accuracy and precision compared to any animal-based *in-vivo* assay and avoids the use of animals.

The thesis work is aimed at developing a proof of concept, wherein an *in-vitro* cell based reporter gene assay will be developed which will simulate the rat ovarian weight augmentation assay (Steelman-Pohley bioassay). The first step in the development of the *in-vitro* assay is to generate a stable cell line that expresses the receptors for the target protein (FSH) on the surface of transformed CHO (Chinese hamster ovary) cells. These cells will then respond to varying degrees of dose dependent luciferase activation in term of Relative luciferase unit (RLU) when the receptors are engaged by the target protein. The degree of response will determine the degree of binding which in turn will be dependent on the degree of sialylation. The assay is supposed to mimic the *in-vivo* bioassay.

After establishing a correlation between the *in-vivo* animal based assay and the *in-vitro* cell-based proliferation assay, the animal assay can be substituted by the *in-vitro* method because of its simplicity, speed, high-throughput, lower cost, and elimination of the need for animals.

2.2 Molecule

Cells communicate with each other through chemical signals in the form of hormones. The signaling molecules bind to specific receptors located on the target cell. Upon binding, the signaling molecule activates receptor proteins, which in turn transducer the signal carried by the specific extracellular messengers. The activation of such receptor proteins initiates a cascade of events that eventually culminate in a highly structured manner inducing a biological effect.

Follicle stimulating hormone (FSH), one of the master signals produced by the anterior pituitary gland, is involved in the regulation of several essential reproductive processes occurring at the gonadal level (Chapel *et al.*, 1983). This gonadotrophin belongs to a family of closely related glycoproteins comprising of FSH, Luteinizing hormone (LH), Choriogonadotrophin (CG) hormone and thyroid stimulating hormone (TSH), which are synthesized in different cell types. TSH is synthesized in a distal pituitary cell. LH and FSH are synthesized by gonadotropes and CG is produced by the placental trophoblasts (Leonora A. *et al.*, 1995). All glycoprotein hormones are heterodimers promise consisting of a common alpha subunit which is non-covalently associated with a structurally unique beta-subunit (Pierce and Parsons, 1981; Gharib *et. al.*, 1990). As shown in Table 2.1 the alpha subunit contains 92 amino acid residues, with five disulphide bonds contributing to its tertiary structure. The FSH beta subunit contains 111 amino acid residues, with six disulphide bonds. The specificity of each hormone resides in the beta-subunits (Lapthorn *et. al.*, 1994; Zeng *et. al.*, 1995; Liu and Dias, 1996; Remy *et. al.*, 1996; Grossman *et. al.*, 1997). The subunit of these glycoprotein hormones contain one or two Asn- linked (N-linked) heterogeneous oligosaccharides which have been shown to play an important role in both the *in-vivo* and *in-vitro* bioactivity of the hormone (Baenziger and Green, 1988; Stockell and Renwich, 1992; Smith *et. al.*, 1990). Of the four Asn-linked glycosylation sites in FSH, two are located on the alpha subunit (Asn 52 and 78) and two on the beta subunit (Asn 7 and 24). Receptors for all

these trophic hormones belong to the super family of G-protein-coupled receptors and thus share several major structural features (Ulloa- Aguirre and Conn, 1998).

Table 2.1 Characteristics of human FSH.

Subunit	Molecular Size (kDa)	No. of carbohydrate structure	Disulphide Bridges	Properties
Alpha α	14	2	5	Subunit common to all the gonadotrophins
Beta β	17	2	6	Confers biological and immunological specificity

Granulosa cells and Sertoli cells are the target cells within the ovary and testis for FSH action and they are the only cell types which express the FSH receptor (Simoni *et. al.*, 1997). FSH regulates granulosa cell function, including the development and selection of ovarian follicles and oocyte maturation. In testes, FSH regulates the function of Sertoli cells, which in turn provide physical and biochemical support for proper development and maturation of germ cells. Binding of the FSH molecule and activation of its cognate receptor involves the participation of several structural determinants on the ligand, which are influenced by the primary sequence and the carbohydrate residues of the molecule (Comarnous, 1992). Understanding of the molecular interaction between FSH and the FSH receptor and the resulting signal transduction are of paramount importance from a clinical point of view as it allows the development of a variety of analogues which could potentially be used as agonist or antagonist to exogenously regulate gonadal function.

2.3 Scientific Principle of the *in-vitro* Cell Based Method

FSH binding to its receptor on gonadal cells increases intracellular levels of cAMP by activating adenylyl cyclase. cAMP is the major second messenger for the trophic effects of FSH (C Albanese *et. al.* 1994). As many gene promoters respond to changes in cAMP levels through discrete cAMP response elements (CREs), this bioassay utilizes the FSH receptor

dependent regulation of cAMP production coupled to a sensitive reporter gene such as luciferase (Jonathan *et.al.*, 2008).

In this cell based assay FSH binds to recombinant human FSH receptor (rhFSHR) expressed in CHOSI cells (figure 2.1). hFSHR activates G-protein complex and the alpha 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. Due to the measurement of the cAMP level via luciferase expression, the biological potency of any FSH sample can be determined.

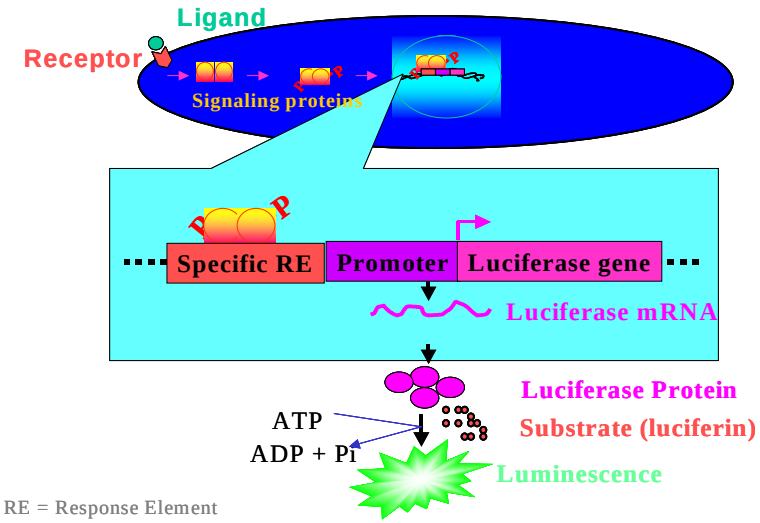


Figure 2.1 Model of signal transductional pathway of the FSH receptor.

2.4 Scientific principle behind the rat ovarian weight augmentation assay method:

The *in-vivo* bioassay to determine the potency of Follicle Stimulating Hormone (FSH) is an ovarian weight gain assay. Subcutaneous administration of rHu FSH with chorionic gonadotrophin (to ensure complete luteinisation) in immature female rats induces enlargement of ovaries and show dose dependent increase in the ovarian weight (ovarian enlargement). This dose dependent response is used in the determination of potency of rHu FSH against an accepted international reference standard or reference standard calibrated in International Units (IU) for rHu FSH (Steelman and Pohley, 1953).

2.5 Physicochemistry of FSH heterogeneity

FSH was first reported to exist as a family of molecules >25 years ago (Amir *et. al.,* 1966; Reichert, 1971; Suttajit *et.al.,* 1971; Vaitukaitis and Ross, 1971; Sherins *et. al.,* 1973).This heterogeneity was shown to be due to the content of sialic acid residues of the oligosaccharide chains on the molecule. As with other glycosylated molecules that contain N-linked oligosaccharides, terminal sialic acid residues cover galactose residues, thus preventing interaction with asialo-glycoprotein receptors present on the hepatic cells and thereby preventing its removal from the circulation (Morell *et. al.,* 1971; Ashwell and Harford, 1982). The greater contents of sialic acid on a glycoprotein results in less galactose residues available for interaction with hepatic cell receptors. An increased sialic acid content of the molecule gives it a more acidic isoelectric point which is no longer recognized by the hepatic receptors. Thus, sialic acid bearing forms of FSH would be expected to circulate for longer periods of time. The interaction between the sugar moieties of gonadotrophins and the receptors that recognize, bind and clear the hormones has been elucidated by the work of Baenziger and Green (1991).

2.6 FSH isoforms exhibit differences in plasma half-life

The suggestion that there was a physiological significance to sialic acid-dependent FSH heterogeneity was proposed with the observed difference in plasma half-life of the molecules depending upon the endocrine status of the pituitary donor (Bogdanove *et. al.,* 1973, 1974; Peckham and Knobil, 1976). Like all other glycoproteins, the more sialylated FSH will circulate for longer periods of time compared to the non-sialyated or less sialyated forms (Morell *et. al.,* 1971; Ashwell and Harford, 1982). However, with time in circulation, the more acidic forms will be desialylated by the ubiquitous enzyme - neuraminidase, and become progressively more basic. In fact, all basic forms can be produced from the acidic forms by treatment with neuraminidase (Sherins *et. al.*, 1973; Ulloa-Aguirre *et. al.,* 1984). This can confound any study that attempts to measure the half-life of FSH in the *in-vivo* situation. All *in-vivo* studies to date have demonstrated that the hormone's circulation time in serum is a major contributing factor to the overall biopotency of the glycoprotein hormone. Thus the, more sialylated molecules have a greater degree of bioactivity *in-vivo* than an

equimolar amount of a less heavily sialylated (and therefore shorter half-life) form of FSH (Table 2.2). Comparisons of different preparations of FSH in an *in-vivo* assay have shown that longer acting isoforms have increased biological activity. This has been demonstrated many times for FSH (Suttajit *et. al.*, 1971; Vaitukaitis and Ross, 1971) as well as other gonadotrophins, most notably, HCG (Van Hall *et. al.,* 1971). Progressive desialylation of FSH (Suttajit *et. al.,* 1971; Vaitukaitis and Ross, 1971; Pierce and Parsons, 1981) results in the loss of its biological activity *in-vivo*. Indeed, the production of recombinant FSH with no sialic acid residues results in FSH with an extremely basic isoelectric point, measurable receptor binding and *in-vitro* activity but no *in-vivo* bioactivity due to a great reduction in plasma half-life (Galway *et. al.,* 1990). This would be consistent with early observations in which desialylated FSH has little if any biological activity *in-vivo* due to its rapid removal from the circulation by the hepatic asialoglycoprotein receptors. Further support for the contribution of sialic acid and isoelectric point to plasma half-life is provided by the observation that FSH obtained from a young woman has an overall profile of more basic FSH forms and a more rapid disappearance rate from circulation than that of an elderly woman (Wide and Wide, 1984). As mentioned above, several groups have demonstrated a relationship between the isoelectric point of FSH isoforms and their circulating half-life (Blum and Gupta, 1985; Wide, 1986; Ulloa-Aguirre *et. al.*, 1992).While most believe that FSH heterogeneity affects plasma half-life, it should be mentioned that not all have been able to demonstrate this correlation. Robertson *et. al.*, (1991) could not demonstrate a significant difference between the circulating half-lives of FSH isoforms when injected into sheep. Further, the plasma kinetic parameters of isoforms with isoelectric point (pI) values between 5.5 and 4.1 showed very little difference when injected in to rodents (Wide, 1982). All gonadotrophin preparations exhibit fast and slow clearance times, which complicates the prediction of an overall plasma half-life (Table 2.2). Inter-animal variation also contributes to variability in data. A recent study (Harsch *et. al.,* 1993) demonstrated that there is intra-individual variation in relative abundance of different FSH isoforms due to metabolic deglycosylation of the molecule in circulation after secretion from the pituitary. This also lends support to the fact that acidic FSH forms are progressively broken down in circulation

and become more basic and shorter acting *in-vitro* and cleared by the liver. This study suggests that the presence of basic forms in circulation may be due to the fact that acidic forms are metabolically deglycosylated in circulation. The presence of basic forms in circulation does not necessarily prove that they were secreted in that form from the pituitary gland (S.C.Chappel 1995).

In summary, the *in-vivo* studies described above clearly demonstrate the presence of biochemically distinct forms of FSH, with varying degree of circulatory half-life and thereby different *in-vivo* potency. These forms differ in their content of sialic acid and plasma half-life. Most studies have demonstrated a relationship between sialic acid content of FSH isoforms, pI values and survival time in circulation (S.C.Chappel 1995). They support the view that FSH heterogeneity plays a role in regulating the amount of time that FSH is able to circulate and interact with its target cell receptor, thus affecting its *in-vivo* bioactivity (Table 2.3).

New research suggests that the carbohydrate structures also play a major role in determining the biological activity of FSH as has been seen for other glycoprotein hormones such as Erythropoietin. Experiments have shown that certain carbohydrate structures (two glycan chains on the beta-subunit) play an important role in determining the plasma half-life and thereby the *in-vivo* bioactivity of FSH (S.C.Chappel 1995).

Table 2.2. FSH isoforms.

Types of FSH isoforms	**Sialic Acid content**	***in-vivo* Half-life**
Acidic	High	Long
Basic	Low	Short

Table 2.3 Microheterogeneity of FSH (isoforms).

- Due to post-translation modifications.
- Specific differences in carbohydrate moieties added to protein backbone and in terminal sialic acid residues.
- These multiple forms differ in their plasma half-life and hence in their biological activity.
- Less acidic isoforms are removed more quickly from the circulation and therefore have lower potency *in-vivo*.
- More acidic isoforms have longer circulatory half-life and therefore have higher potency *in-vivo*.

2.7 Characterization of FSH isohormones

FSH, like the other gonadotrophins LH and human chorionic gonadotrophin (HCG), exists in different molecular forms (isohormones), which differ in their oligosaccharide structures, in particular the degree of terminal sialylation (Ulloa-Aguirreet.Al., 1988; Dahl and Stone, 1992). The α- and β-subunits contain both mono- or multi-antennary N-linked carbohydrate chains. The degree of sialylation determines the degree of charge differences which in turn determines receptor binding activity, biological activity and metabolic clearance rate (Ulloa-Aguirre *et. al.,* 1988). Multiple forms of gonadotrophins have been isolated and characterized from anterior pituitary glands, serum and urine of several species including man (Wide, 1982, 1985; Reader *et. al.,* 1983; Chappel *et. al.,* 1984; Ulloa-Aguirre *et. al.,* 1985, Chappel, 1995). Relatively acidic FSH isohormones, which are more heavily sialylated, exhibit lower receptor binding affinity and *in-vitro* biological activities than more basic isohormones. However, due to their lower clearance rate these more acidic forms have greater *in-vivo* biological activities (Ulloa-Aguirre *et. al.,* 1988). Thus, the isohormone composition of FSH preparations is likely to have important biological consequences. The protein is glycosyalated, and the degree of sialylation is affected by cell culture conditions. The sialylation imparts a negative charge on the FSH molecule, which gives rise to heterogeneous molecule consisting of many different isoforms (Olijve *et. al.,* 1996; Gervais *et. al.,* 2003). All isoforms are recognized by the *in-*

vivo rat bioassay, but have different pharmacokinetic half-lives in humans (Ulloa-Aguirre *et. al.*, 1988). Assessment of over 100 manufacturing lots (batches) of r-FSH drug substance (follitropin alpha) demonstrated that the specific activity, isoform pattern by isoelectric focusing (IEF) and sialylation pattern by glycan mapping were consistent (Driebergen and Baer, 2003). This observation allowed the development of a physicochemical analytical method for measuring the FSH content (by mass of protein) for the product, which was traditionally assessed by *in-vivo* bioactivity. The physicochemical method for assessing the FSH content can be successfully used to assess the r-FSH content in the active drug substance and the drug product (follitropin alpha). The final drug product is formulated based on the r-FSH protein content, thus ensuring more precise r-FSH content per vial.

2.8 The changing face of Biological assays

Biological activity is the specific ability or capacity of the product to achieve a defined biological effect. A "biological" analyte is considered by WHO as one of biological origin, which cannot be characterized adequately by chemical and/or physical means alone (WHO, Tech.Rep. Ser.800, 1990, 181-213). In the last century most biological medicines were first identified as having an activity in an experimental model rather than by their chemical structures. Very often, they were introduced into clinical practice before their structure was understood. In those cases, their potency in man was usually controlled by using the same biological model in a quantitative test (a bioassay).Therapeutic glycolproteins (e.g. MAbs, EPO, FSH) are complex with incompletely understood relationships between structure and biological activity. Larger molecules (e.g. IgM 750 kD, Clotting Factor VII 330kD) are beyond the current technical limits for routine structural analysis. The development of more complex biological therapies is continuing to increase the dependency on biological assays. The last 20 years have seen a progressive elimination of *in-vivo* animal based bioassays with the introduction of physicochemical assay *in-vivo* or an *in-vitro* cell based assay as an alternative. Table 2.4 summarizes the various physico-chemical tests that are employed for various products including hormones, vaccines and toxins. These physico-chemical methods have replaced the *in-vivo* bioassay (animal studies) as these methods have demonstrated good correlation with the *in-vivo* biological activity.

Table 2.4 Physico-chemical methods used for replacing animal based *in-vivo* assays for Therapeutics, Hormones, Vaccines and Toxins.

Product	**Then**	**Now**
Oxytocin	Depression of blood pressure in chickens Milk ejection in lactating rats Rat uterus muscle contraction	HPLC
Calcitonin	Hypocalcaemia in rats	HPLC
Gonadorelin	Ovarian ascorbic acid depletion in pseudo-pregnant rats	HPLC
Desmopressin	Anti-diuresis, rats	HPLC
Insulin	Reduction of blood sugar in mice Reduction of blood sugar in rabbits Hypoglycaemic convulsions in mice	RP-HPLC
Somatropin(recombinant growth hormone)	Weight gain or tibialwidth increase In hypophysectomised rats	SE-HPLC
Vaccines	*in-vivo* challenge protection assay	*in-vitro* immunochemical
Toxins	*in-vivo* toxicity assay	ELISA
Thrombolyticenzymes	Clot lysis	Chromogenicsubstrate assay
Cytokines -Interferon-alpha	*in-vitro* Cell proliferation assay	RP-HPLC
HormonesFollicle stimulating hormone	*in-vivo* Ovarian weight gain assay	IEF

In order to replace the bioassay, a strong body of data is needed which provides a strong correlation of product activity between existing bioassay and intended replacement is necessary. The alternative assay (either physicochemical or *in-vitro* cell based assay) should

be able to assess minor changes in the product(variants) which may arise from manufacturing process like oxidized, deamidated, cleaved and aggregated forms.

Animal-based bioassays are usually more physiologically relevant to the clinical endpoint. However, animal-based bioassay has number of distinct disadvantages which includes cost, time consuming, exhibit a high degree of variability, and are much more difficult to transfer between testing facilities. Correlating an animal-based bioassay with a cell culture-based bioassay is also equally challenging. This is not unexpected considering the high degree of variability displayed by animal-based bioassays because of the fact that samples tested in animal-based bioassays are subject to pharmacokinetic or pharmacodynamic variables. Developing an alternative cell culture-based bioassay that correlates with the animal-based bioassay can take several years and parallel analysis of many samples. Complete replacement of the animal-based bioassay with a cell culture-based bioassay may occur in several stages. Both the animal-based bioassay and alternative cell culture-based bioassay are performed in parallel in order to show a good correlation between the two test methods. The sponsor may also need to develop a specific physicochemical method along with the cell-based assay in order to ensure the consistency of the product analyzed by both methods. According to ICH guideline Q6B, a biological assay to measure the biological activity of the product may be replaced by physicochemical tests only in those instances where:

Sufficient physicochemical information about the drug, including higher-order structure should be well established by such physicochemical methods and relevant correlation to biologic activity. Biological activity depends on the integrity of certain physicochemical features of a molecule, including higher-order structure. Bioassays should be able to detect changes in biological activity resulting from physicochemical or structural changes in these physicochemical features. Analytical tests are designed to detect specific physicochemical and structural changes and correlating all product variants with potency. There have been cases where differences between products were identified by bioassays but not by physicochemical tests due to either lack of sensitive tests that can pick-up subtle changes in the structure or lack of correlation between modifications and potency. Physicochemical assays can be used for potency testing when such changes have a proportional effect on the

potency. For macromolecules, it is recommended that bioassays be used for comparability studies to confirm that product potency is unaffected whenever process is changed. When an appropriate potency assay is used for the drug product, an alternative method (physicochemical and/or biological) may suffice for quantitative assessment at the drug substance stage and vice-versa. However, the rationale for such a choice should be provided (ICH Guideline Q6B).

2.9 Replacement of *in-vivo* (animal based) assay for glycoprotein hormones

What features of the glycoprotein hormones have made the *in-vivo* bioassays so difficult to replace with *in-vitro* cell based assay/Physicochemical alternative?

The discovery of recombinant DNA technology (Cohen *et.al.*, 1973) provided another approach to the pharmaceutical manufacture of complex proteins. In this case, the DNA that coded for the naturally occurring protein was introduced into the genome of a cell. The genetically constructed cell line became the immortal source of the recombinant gene product (expressed target protein). The DNA technology led to the development of many highly pure therapeutic proteins through cell fermentation and sophisticated purification procedures (Recombinant Insulin FDA Approval, 1982). Since then, pharmaceutical companies have rapidly employed DNA-derived proteins as therapeutics.

For the first time the pure form of r-FSH could be fully characterized (Loumaye *et. al.*, 1995). Additionally, developments in analytical methodology also allowed a more extensive assessment of the product throughout the production process. In 1995, the first r-FSH was made available, and was classified as follitropin alpha (r-hFSH, Howles, 1996), and was followed by second r-FSH (follitropin beta; Olijve *et. al.*, 1996), Analytical test could then be used to demonstrate purity, to quantify aggregation, dissociated subunits, oxidized alpha subunit, and to demonstrate the glycosylation and isoform pattern of all manufactured batches (Bagatti *et. al.*, 2001; Gervais *et. al.*, 2003).

Interestingly though, the FSH content of these recombinant protein was still assessed with an '*in-vivo*' rat ovary weight gain bioassay. As described above, this assay has been recognized as imprecise, costly, and remains under political and ethical pressures due to the use of

animals (Mulders *et. al.,* 1997). Furthermore; animal testing is widely discouraged when alternative solutions exist (European directive 86/609, art. 7.2; Castle, 1998; Artiges, 1999). The European Pharmacopoeia has continued to review the use of animals in drug product testing and has adopted the scientific principles described in the Technical Guideline for the elaboration of the "Monograph for Biologicals (4^{th} edition, 2003)". According to the guideline, *"when a battery of physic-chemical tests has been shown to adequately characterize a molecule, then physicochemical test methods should be employed"*. The International Conference of Harmonization (ICH, Guideline Q6B) also confirmed the need for a physic-chemical assay: '…..quantity, usually measured as protein content, is critical for a biotech product and should be determined by an appropriate assay, usually physicochemical in nature'.

In fact, review of the European Pharmacopeia (4^{th} edition, 2002) documents the use of *in-vivo* bioassay for only four protein hormones, erythropoietin (EPO), human chorionic gonadotrophin (HCG), human LH and human FSH. It is interesting to note that some of these protein hormones have been assessed using physicochemical analytical methods, such as high performance liquid chromatography (HPLC) assays for r-HCG, r-hLH, and N-glycan charge assay for EPO (Hermentin *et. al.,* 2002).

In contrast, manufactures of other recombinant proteins had developed physicochemical methods for quantifying the active component (Seamon, 1998). A physicochemical quantification approach is not immediately apparent for r-FSH and other glycosyalated proteins because glycosyalated proteins are highly heterogeneous and do not have a single active entity but a mixture of active forms varying in potency and circulatory half-life. Hence conceptually difficult for an HPLC potency assay (Bristow and Charton, 2002).In the case of glycosyalated proteins, the degree of sialylation is affected by cell culture conditions. The sialylation imparts a negative charge on the r-FSH molecule, which gives rise to a heterogeneous molecule consisting of many different isoforms (Olijve *et. al.,* 1996; Gervais *et. al.*, 2003). All of these isoforms are recognized by the '*in-vivo*' rat bioassay and also have differing pharmacokinetic half-lives in rats and humans (Ulloa-Aguirre *et. al.*, 1988). Therefore, the heterogeneous glycoprotein FSH did not immediately appear to be good

candidate for introducing alternative physicochemical analytical methods to assess the FSH content. Physicochemical approach to assess the r-FSH content (follitropin beta) is based upon the isoform pattern of the drug (Mulders *et. al.*, 1997; Storring *et. al.*, 2002). The analytical approach from Mulders *et. al.*, (1997) has demonstrated that isoform pattern of the drug substance can be directly linked to the level of bioactivity, and therefore can be used to formulate the drug product. Unfortunately, the physicochemical method of IEF has not been applied to the drug product, and the FSH content in the final product is verified till date with the traditional *in-vivo* (Animal based) assay (Follotropin Beta, European Product Monograph, 2003).

However, assessment of more than 100 batches of the r-FSH drug substance (follitropin alpha) did indeed demonstrate that the specific activity, isoform pattern by isoelectric focusing (IEF) and sialylation pattern by glycan mapping were consistent (Driebergen and Baer, 2003). This observation allowed the development of an alternative *in-vitro* cell based assay for measuring the FSH content for the product.

Necessary steps are described to validate replacement of an *in-vivo* bioassay with physico-chemical or *in-vitro* assay.

1. Satisfy the requirements of ICH Q6B

"Where the battery of physic-chemical test has been shown to adequately characterize the molecule, a physico-chemical assay alone may not be employed"

The Structural complexity and heterogeneity of the FSH molecule means that regulators are unlikely to be satisfied that this condition can be met.

2. What can we do?

Measure the ability of FSH to bind the receptor and transducer the single in an *in-vitro* assay system.

There are several complementary methods during either manufacturing of r-hFSH DS or final DP eg. CZE, IEF, Sialic acid estimation etc. If results of these analyses are in accordance with predefined acceptance criteria then it can be technically concluded that during *in-vitro* analysis activity obtained is a function of FSH molecule itself, not degree of desialylation.

3. Materials and Methods

3. Materials and Methods

3.1 Determination of potency of recombinant human follicle stimulating hormone by *in-vitro* bioassay

The objective of this study is to develop a method to determine the potency of recombinant human Follicle Stimulating Hormone (r-HuFSH) by *in-vitro* reporter gene assay.

3.1.2 Reagent and Materials

Note: Suggested vendor catalog numbers are listed for most items below. Alternate materials are acceptable when equivalent. Manufacturer's storage conditions and expiration dating apply to all materials unless otherwise stated.

1. MAM PF-2 (Bioconcept, Catalog No. 10-2F24-I).
2. Pluronic F-68 (Bioconcept, Catalog No. 5-75F02-H).
3. Phenol Red (Sigma, Catalog No. P3532 or any cell culture tested)
4. Dialyzed FBS (Invitrogen. Catalog No. 26400-044).
5. Gentamicin (Ranbaxy)

6.L-Glutamine (Sigma, Catalog No.G7513 or Hyclone, Catalog No. SH30034)

7. Luciferase assay Reagent (Promega, Catalog # E1501)
8. Cell culture lysis buffer 5X (Promega, Catalog # E153A)
9. DMSO (Sigma, Catalog No. D2650)
10. KH_2PO_4 (Sigma, Catalog No. P5655 or any cell culture tested)
11. Na_2HPO_4 (Sigma, Catalog No. S5136 or any cell culture tested)
12. NaCl (Sigma, Catalog No. S5886 or any cell culture tested)
13. KCl (Sigma, Catalog No. P5405 or any cell culture tested)
14. 0.5 % Trypan Blue (Hi-media, Catalog No. TCL 005-5)
15. 70 % Isopropyl Alcohol
16. Sodium Hypochlorite 0.2 %
17. Water for injection (WFI)

18. White 96 well half area Assay Plates (Corning, Catalog No. 3688)
19. Dilution Plate, 0.5 ml Polypropylene Plates (Nunc, Catalog No. 260251)
20. Microcentrifuge tubes, 1.5 ml, sterile
21. Serological pipettes, 2 ml to 50 ml, sterile
22. Filter System, pore size 0.22 µm, sterile (low protein binding)
23. Cell culture flasks, T-25 cm2 and T-75 cm2, sterile
24. Tubes 5 and 15ml Falcons (BD Falcon, Catalog no. 352096 and 352070)

3.1.3 Equipments

1. Calibrated pipettes and appropriate sterile tips recommended by the pipette manufacturer

- Single channel, able to pipette volumes between 5 µl and 1000 µl.
- Multi-channel, able to pipette volumes between 5 µl and 300 µl.

2. CO_2 incubator (37 ± 2)°C with 5 ±1 % CO_2 and equipped with water pan to provide humidity (Shell Lab, Catalog No. 3517-2)
3. Biological Safety Cabinet (BSC), HEPA filtered unidirectional airflow (ESCO, Catalog No. AC2-4E1)
4. Centrifuge (Eppendorf, Catalog No. AG 5811)
5. Pipette-Aid
6. Microscope, inverted light (Olympus, Catalog No. CKX41)
7. Haemocytometer with cover slip
8. Automatic cell counter (Beckman, Model Vi-CELL XR)
9. Water bath
10. Freezer, liquid nitrogen (MVE Cryogenics)
11. Refrigerator, 2 °C to 8 °C, for laboratory
12. Freezers, ≤-20 °C
13. Freezers, ≤-80 °C
14. Vortexer
15. Weighing balance
16. pH meter

17. Spinner (Integra Bioscience, Part No. 183001)

18. Spinner Flask with 2 Pendula (Integra Bioscience, Part No. 182026)

19. Plate Reader (Turner Biosystem, Modulus Microplate Multimode Reader) (Note: Use of different reader requires qualification prior to use)

20. SoftMax® Pro v5 software, Molecular Devices (Note: Use of different software requires qualification prior to use)

21. PLA 2.0 Software, Stegmann systems,Germany.(Note: Use of different software requires qualification prior to use)

3.1.4 Reagent preparation from kit

Thaw all reagents prior to use at RT (Room Temperature) before 1 hrs.

1. Luciferase Assay Substrate (lyophilized)

2. 10 ml Luciferase Assay Buffer

3. 30 ml Reporter Lysis 5X Buffer

Mix substrate buffer to lyophilized powder immediately before use in dark.

3.1.5 Storage of reagents

Luciferase Assay Reagent should be stored in aliquots and is stable at –20 °C for up to 1 month or at below -70 °C for up to 1 year after reconstitution or initial use. After preparation and freezing, the Luciferase Assay Reagent should be mixed well before use. Non reconstituted system components may be stored at –20 °C for 1 year. Store luciferase assay substrate in the dark.

Lysis Buffer may be stored at room temperature and should be stored away from direct sunlight. Cell Culture Lysis Reagent should be stored at –20 °C.

Note: In the assay substrate used should not be freeze-thawed for more than two cycles.

3.1.6 Procedure / Method detail

3.1.6.1General instructions

1. Perform all the work in accordance with general cell culture laboratory procedure.
2. Use WFI for preparation of all solutions and media.
3. Pre-warm the media to 37 ºC before adding to the cells, unless otherwise stated.

4. All work should be recorded in appropriate record formats.

3.1.6.2 Assay

General note for all pre-dilutions to be done for standard or samples:

1. Use assay media (AM) as diluent for dilution of standards and samples.
2. Dose preparation can be done outside the BSC (biosafty cabinate) but assay plate preparation must be done inside the BSC.
3. Arrange and label appropriately tubes, pre-dilution plates, assay plates etc.
4. Use fresh tip at each step of the dilution.
5. Preferably use multichannel pipette while transferring doses from 96 well polypropylene plate to the assay plate.

3.1.6.3 Dilution of NIBSC (Commercial Reference Standard):

1. Perform dilutions as per Table 5.
2. From an aliquot of 25 µl, (75000 mIU/ml), dilute it in microfuge tubes /pre plate to prepare Dose 1 i.e.36.75 mIU/ml that is 10.5 mIU/per well.
3. From 36.75 mIU/ml prepare 6.13 mIU/ml (Dose 2) and 3.60 mIU/ml (Dose 3). From 3.60 mIU/ml (Dose 3) prepare 2.12, 1.25 and 0.73 mIU/ml (Dose 4, 5 and 6, respectively). From 0.73 mIU/ml (Dose 7) prepare 0.43 mIU/ml (Dose 7) and from 0.43 mIU/ml (Dose 7) prepare 0.07 mIU/ml (Dose 8).
4. Prepare dose number 1 to 8 in polypropylene “dilution” plate.
5. Prepare minimum 80 µl of each dose. Schematic presentation of this dose preparation is shown in figure 3.2 and 3.3.

Table 3.1: Dilution of NIBSC (Reference standard) using manual or electronic pipette.

Step No.	Dose No.	Fold Dilution	Dilution Done In	Vol. of rHu-FSH solution (µl)	Vol. of Assay Media (µl)	Total vol. (µl)	Resulting Conc. mIU/ml	Final Conc. In wells after addition 50 µl cells to Plate (mIU/ml)*
1	NA	5	Tube	20	80	100	15000	-
2	NA	10	Tube	20	180	200	1500	-
3	NA	7.5	Tube	50	325	375	200	-
4	1	5.44	Plate	50	222	272	36.75	10.5
5	2	6	Plate	20	100	120	6.13	1.75
6	3	10.2	Plate	30	276	306	3.60	1.03
7	4	1.7	Plate	50	35	85	2.12	0.61
8	5	2.9	Plate	40	76	116	1.25	0.36
9	6	4.9	Plate	40	157	197	0.73	0.21
10	7	1.7	Plate	80	56	136	0.43	0.12
11	8	6.0	Plate	20	100	120	0.07	0.02

* Note:

- SoftMax® Pro v5 software plots resulting concentration mIU/ml on X axis.
- PLA 2.0 calculates potency using relative values of resulting concentration IU/ml.

3.1.6.4 Dilution of samples or Internal Reference Standard (IRS):

1. Perform dilutions as per Table 3.1.

2. Depending on the concentration of sample, perform first dilution ("X" fold) in micro centrifuge tube so as to bring down the concentration to 0.00543 mg/ml i.e. 5.43 µg/ml or 5434 ng/ml. This is equivalent to 75000 mIU/ml. (Note: Assumed specific activity for rHu-FSH is 1 mg =13800 IU/ml) Calculate value of "X" fold from Excel spreadsheet "Pre-dilution Template for Bioassay".

3. Do rest of the dilutions in microfuge tubes or polypropylene "dilution" plate as shown in Table 3.2.

4. Prepare minimum 80 µl of each dose. Schematic presentation of this dose preparation is shown in Figure 3.2 and 3.3.

Note: one dilution plate is sufficient for diluting 12 Samples or Reference Standards which means one dilution plate when fully utilized can be used to prepare samples and standards for 4 assay plates (4 Reference Standards and 8 Samples).

Table 3.2: Dilution of IRS or sample using manual or electronic pipette.

Step No.	Dose No.	Fold Dilution	Dilution done in	Vol. of rHu-FSH solution (µl)	Vol. of Media (µl)	Total vol. (µl)	Resulting Conc. mIU/ml	Final Conc. In wells after addition 50 µl cells to Plate (mIU/ml)*
1	NA	X	Tube	20	XX	YY	75000	-
2	NA	5	Tube	20	80	100	15000	-
3	NA	10	Tube	20	180	200	1500	-
4	NA	7.5	Tube	50	325	375	200	-
5	1	5.44	Plate	50	222	272	36.76	10.5
6	2	6	Plate	20	100	120	6.13	1.75
7	3	10.2	Plate	30	276	306	3.60	1.03
8	4	1.7	Plate	50	35	85	2.12	0.61
9	5	2.9	Plate	40	76	116	1.25	0.36
10	6	4.9	Plate	40	157	197	0.73	0.21
11	7	1.7	Plate	80	56	136	0.43	0.12
12	8	6.0	Plate	20	100	120	0.07	0.02

* Note:

- SoftMax® Pro v5 software plots resulting concentration mIU/ml on X axis.
- PLA 2.0 calculates potency using relative values of the resulting concentration IU/ml.

3.1.

6.5 Assay Plate Preparation:

Preparation of cell suspension:

1. Cells should be freshly cultured and their use should be within 24-36 hrs from the last passaging i.e. after 24 -36 hrs of sub culturing use the cells for bioassay.

2. Observe the cell suspension for gross morphology and growth behavior both visually and under a microscope.

3. Perform a cell count and check for viability. To be used in the assay, 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 the 96 well plate at 350 x g (equivalent to 1400 rpm) for three minutes at room temperature.

4. Aspirate the supernatant and discard.

5. Resuspend the cell pellet in AM and perform a cell count.

6. Dilute the cell suspension further in AM to get $1x10^6$ cells/ml.

7. 6 ml of above suspension is sufficient for one plate.

8. Take an aliquot from the cell suspension to reconfirm and record the final cell count.

Addition of cells to assay plate:

1. Add 50 µl of the final cell suspension to wells except media blank as shown in the figure 3.1.

2. Incubate plates in CO_2 incubator at 37^{o}C for 30 min with dummy plates placed on the top and bottom.

3. Add 70 µl of AM or PBS to the empty wells.

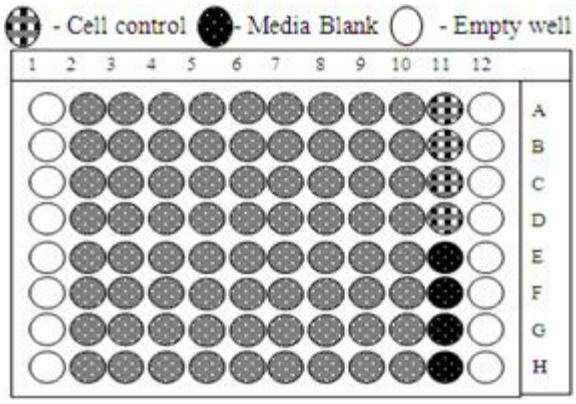


Figure 3.1: Picture of Assay plates to seed the cells.

Addition of doses to the assay plate:

1. After half an hour incubation of Assay plate add 20 µl of each dose of standard and sample from the dilution plate to the assay plate in triplicate as shown in schematic presentation in Figures 3.2 and 3.3.

2. Add 20 µl of assay media to cell control well and 70 µl in media blank well. Empty wells are kept to shield from the variation caused by the "edge effect".

3. One assay plate can accommodate two samples and one reference standard.

4. Incubate the plates at 37^0C immediately after protein addition for 5 hours in a CO_2 incubator.

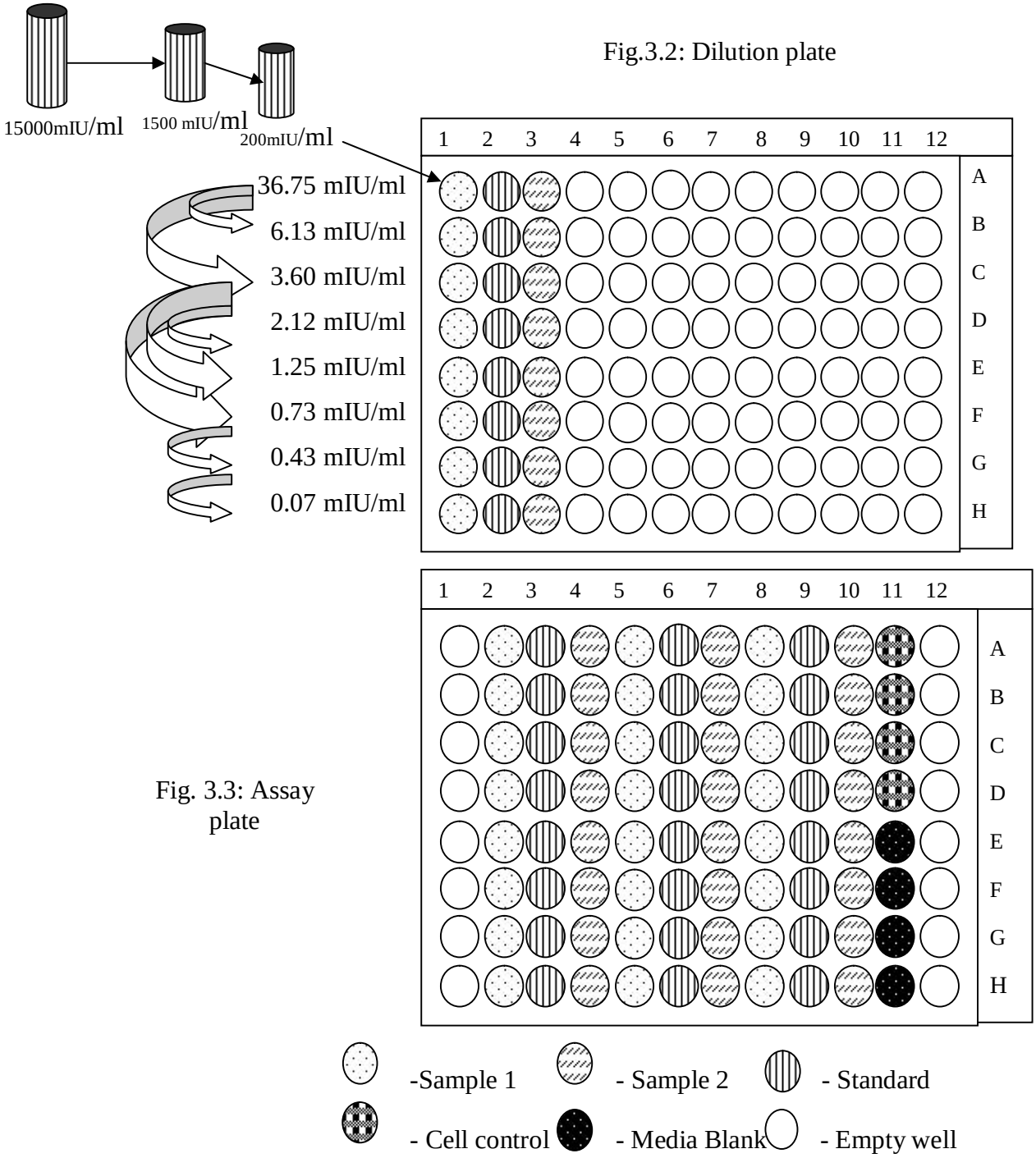


Fig.3.2: Dilution plate

Fig. 3.3: Assay plate

Figures 3.2 and 3.3: Schematic presentation of dilution pattern of NIBSC Standard/IRS/ samples.

3.1.6.6 Procedure to read the plate

1. After five hours of incubation decant all media from the plate.
2. Prepare 1X lysis buffer from 5X stock solution and add 25 µl lysis buffers to all the wells except the empty wells.
3. Incubate plate for 15 minutes at room temperature and read the plate using a Luminometer (Turner Biosystem) after adding 30µl of substrate using the injector with 2 sec delay time.

3.1.6.7 Reporting of results (calculation, data interpretation / analysis)

1. Calculate EC50 (Parameter C of a 4-PL equation from 4-PL graph) using SoftMax® Pro v5.
2. Analyze the data by PLA 2.0 software.
3. Calculate the parameters and check for assay acceptance criteria as well as system suitability criteria.
4. The specific activity obtained from the PLA 2.0 analysis complying or non-complying to the defined specifications is shown in the sample report.

3.2 Determination of Potency of rHu-FSH by *In-vivo* bioassay

To determine the potency of recombinant human Follicle Stimulating Hormone (rHu-FSH) by *in-vivo* bioassay using rodent model as per the method described in FSH monograph number 2285, *Pharm Europa*, July 2009.

3.2.1 Materials and Instrumentations

3.2.1.1 Materials

1. Human Chorionic Gonadotrophins (hCG) (Technopool Bopharma Co. Ltd., P.R. China)
2. Di Sodium hydrogen phosphate (Nice chemicals Pvt. Ltd.,Cochin)
3. Sodium Chloride (Spectrum Reagents and Chemicals Pvt. Ltd., Edayan, Cochin)
4. Orthophosphoric acid (88 %) (Spectrum Reagents and Chemicals Pvt. Ltd., Edayan, Cochin)
5. Phenol (SD Fine Chem Ltd., Mumbai)
6. Bovine serum albumin (BSA) (Rolex Chemical Industries, Mumbai)

7. Water for injection (WFI) (Hitech Biochemicals, Tumkur)
8. Microcentrifuge tubes, 2.0 ml, sterile (Tarsons Products Pvt. Ltd., Kolkata)
9. Serological pipettes, 2, 5, 10 and 25 ml, sterile (Tarsons Products Pvt. Ltd., Kolkata)
10. Glass bottles and Measuring cylinder (Borosil)
11. Filter System, pore size 0.22 µm, sterile, low protein binding (Merck-Millipore)
12. 15 and 50 ml Centrifuge tubes (Tarsons Products Pvt. Ltd., Kolkata)
13. 1.0 ml 26G ½ syringe with needle (BD, REF 301321)

Reference standard materials

The International Reference Standard for rHu-FSH (NIBSC Reference Material, Standard Code: 92/642) is not available in enough quantity as NIBSC only provides 6 vials per laboratory per year hence commercially available Innovators product is considered as Reference Standard.

- Commercial reference standard (CRS):

Name: Gonal *f*™

Source: India

Brand: Merck Serono S.P.A.

Storage: 2–8 °C for 2 years (Shelf-life)

Amount required per assay: 2.0 ml of 75 IU/ml (5.5 µg/ml)

- Internal reference standard (IRS):

To be used as a reference standard to determine potency of samples whenever available. Internal Reference Standard (IRS) is currently used standard for all routine analysis is the commercially available Gonal-*f* –pen 300 IU/ml (22µg) (Reference Medicinal Product). The rHu FSH drug substance (0.6-0.8 mg/ml) is similar to gonal-*f* in terms of identity and purity profile.

The material manufactured for pre-clinical study is qualified as IRS. The material is not only tested for the physicochemical parameter but also for efficacy in humans. Therefore, this material is a true representative and reference for the quality assessment of batches manufactured in future and stability studies. This material is serve as reference for trending

the impurity profile, identifying any new impurity (due to un-noticed deviations), and assessing impact of manufacturing changes.

IRS has advantage over the reference medicinal product.

- Batch to batch variation will not be a concern as the IRS will be of a single batch.
- It will be useful in qualifying a new IRS in case of this lot being exhausted.
- The impurities observed will be of the process for rHu FSH rather than the reference medicinal product which would most likely be from a different process or over host.

3.2.1.2 Instrument / Equipment

Note: Suggested vendor catalog/ model numbers are listed below for most equipments and instruments. Alternate vendor or models are acceptable if deemed equivalent.

1. Calibrated single channel pipettes, which can pipette volumes between 20 and 1000 µl. Two pipette types are required – 100 µl and 1000 µl pipettes. Appropriate sterile tips recommended by the pipette manufacturer to be used
2. Biological Safety Cabinet (BSC) having HEPA filtered unidirectional airflow (Enviro systems Pvt. Ltd., Hyderabad)
3. Pipette-Aid (Vitilab)
4. Refrigerator with a temperature adjusted to 2 to 8°C (Godrej)
5. Weighing balance (Oharus-AR2140, Sartorius-GPA3202)
6. pH meter (Equinox)
7. PLA 2.0 Software, Stegmann systems,Germany.(Note: Use of different software requires qualification prior to use)

3.2.2 Procedure / Method details

3.2.2.1 General instructions

1. Perform all the work in accordance with standard operating procedure.
2. All work should be recorded in appropriate record formats.

3.2.2.2 Assay

The assay as described in *Pharm Europa* monograph is based on measurement of stimulation of ovary weight gain in immature female rats. The follicle stimulating activity of rHu-FSH is estimated by comparing under the given conditions (as described below) its effect in enlarging the ovaries of immature rats treated with chorionic gonadotrophin with that of the weigh gain observed with the Reference Standard preparation of human recombinant follicle stimulating hormone.

3.2.2.3 Assay Parameters:

The parameters (as per the method described in monograph number 2285, *Pharm Europa*, July 2009) to be used for the assay has been given below:

- ❖ Animal Species : Rat
- ❖ Strain : Wistar albino
- ❖ Sex : Female
- ❖ No. of dose groups : 8 (1 for no dose, 1 for vehicle, 3 for reference standard and 3 for sample)
- ❖ No. of rats per dose group : 10
- ❖ Animal age : 19 - 28 Days old (differing in age by not more than 3 days)
- ❖ Body weight range of animals : Difference between the heaviest and the lightest rat should not more than 10 g.
- ❖ Randomization of animal : As per SOP of Bioneeds
- ❖ Diluent used for making dilutions : Phosphate-albumin buffered saline pH 7.2
- ❖ Route of administration : Subcutaneous
- ❖ Site of injection : Dorsal region

- ❖ Injection volume : 0.5 ml
- ❖ **Follicle Stimulating Hormone (FSH)**
  - Dosage per animal : 0.5, 1.0 and 2.0 IU per animal per Day for 3 days
  - Total Dosage per animal : 1.5, 3.0 and 6.0 IU per animal.
  - No. of dose/sample to be injected : 3 (1.5, 3.0 and 6.0 IU / animal).
  - No. of Dose preparations/dilutions : 3 (1.0, 2.0 and 4.0 IU / ml).
- ❖ **Chorionic gonadotrophin (To ensure complete luteinisation)**
  - Dosage per animal : 14 IU per animal per day for 3 days.
  - Total Dosage per animal : 42 IU per animal.
  - No. of dose/sample to be injected : 1 (42 IU / animal).
  - No. of Dose preparations/dilutions : 1 (28 IU / ml).
- ❖ Method of testing : Ovarian weight gain assay
- ❖ Method of statistical analysis : Parallel line assay (PLA 2.0)
- ❖ Reference standard : Gonal *f*™
- ❖ Assay acceptance criteria : Should meet all acceptance criteria under section 8.0.

3.2.2.4 Shipment of samples from IBPL (Intas Biopharmaceuticals Ltd) to Bioneeds (Contract Animal Testing Laboratory):

IBPL people will perform this activity.

Inform contract animal facility people (Bioneeds, Bangalore) 1 week prior to the date of shipment.

Reference standard (2.0 ml of 75 IU/ml) and sample (2.0 ml of 5.5 ± 0.55 µg/ml) shall be kept in 2.0 ml micro centrifuge. Labeled rHu FSH samples shall be kept in 50 ml falcon tube and sealed with parafilm.

Shipping of Reference standard and samples from IBPL to Bioneeds shall be done at 2-8 ℃ by placing the samples between the cool packs in to the cold box (cool packs should be in the frozen state).

3.2.2.5 General note for all dose preparations to be done for standard or samples:

1. Use Phosphate-albumin buffered saline pH 7.2 as diluent for all dose preparations.
2. Dose preparation must be done inside the BSC.
3. Arrange and label appropriately dosing tubes.
4. Use fresh tip at each step of the dose preparation.
5. Reference standard and sample should be stored at 2-8 ℃ until dose preparations.
6. All dose preparations should be stored at 2-8 ℃ until dosing.

3.2.2.6 Reconstitution of human chorionic Gonadotrophin (hCG):

This activity will be carried out at Bioneeds.

Add 1 ml of phosphate-albumin buffered saline pH 7.2 (diluents) in hCG vial (HUCOG-2000 IU/Vial) and dissolve contents of vial completely. After reconstitution vial contains hCG of 2000 IU/ml. Reconstitute two vials of hCG for one assay. After reconstitution mix the solution of both vials and use this properly mixed solution (2.0 ml) of hCG (2000 IU/ml) for dose preparation.

3.2.2.7 Dose preparation of vehicle:

Bioneeds people will perform this activity.

1. Perform dose preparations as per Table 3.3.

2. Add 252 µl of hCG (2000 IU/ml) and then add 17.8 ml of phosphate-albumin buffered saline pH 7.2 (diluents) in dose preparation tube. Total 18.0 ml vehicle contains hCG of 14 IU/ 0.5 ml or 28 IU/ml. Schematic presentation of this dose preparation is shown in Figure 3.4.

3. Store at 2-8°C until dosing.

3.2.2.8 Dose preparation of CRS/IRS/in-house sample:

Bioneeds people will perform this activity.

1. Perform dose preparations as per Table 3.3.
2. Add 252 µl of hCG (2000 IU/ml) in all 3 dose (Dose 1, Dose 2 and Dose 3) preparation tubes.
3. For Dose 1 (0.5 IU/Animal/Day), add 240 µl of CRS or IRS or Bulk sample of FSH (75 IU/ml) and then add 17.5 ml of phosphate-albumin buffered saline pH 7.2 (diluent) in dose preparation tube. Total 18.0 ml of Dose 1 contains hCG of 14 IU/ 0.5 ml or 28 IU/ml and FSH of 0.5 IU/ 0.5 ml or 1 IU/ml. Schematic presentation of this dose preparation is shown below in Figure 3.4.
4. For Dose 2 (1 IU/Animal/Day), add 480 µl of CRS or IRS or Bulk sample of FSH (75 IU/ml) and then add 17.3ml of phosphate-albumin buffered saline pH 7.2 (diluent) in dose preparation tube. Total 18.0 ml of Dose 2 contains hCG of 14 IU/ 0.5 ml or 28 IU/ml and FSH of 1 IU/ 0.5 ml or 2 IU/ml. Schematic presentation of this dose preparation is shown below in Figure 3.4.
5. For Dose 3 (2 IU/Animal/Day), add 960 µl of CRS or IRS or Bulk sample of FSH (75 IU/ml) and then add 16.8 ml of phosphate-albumin buffered saline pH 7.2 (diluent) in dose preparation tube. Total 18.0 ml of Dose 2 contains hCG of 14 IU/ 0.5 ml or 28 IU/ml and FSH of 2 IU/ 0.5 ml or 4 IU/ml. Schematic presentation of this dose preparation is shown in Figure 3.4.
6. Store at 2-8°C until dosing.

Table 3.3: Dose preparation for Vehicle, CRS or IRS or sample.

Dose No.	Daily Dose (IU/Animal/ Day)		Total Dose (IU/Animal)		Volume of hCG (2000 IU/ml) solution (µl)	Volume of FSH (75 IU/ml) solution (µl)	Volume of Diluent (ml)	Total volume (ml)	Concentration of dose solution (IU/ml)	
	hCG	FSH	hCG	FSH					hCG	FSH
Vehicl	14	0.0	42	0.0	252	0	17.748	18	28	0
1	14	0.5	42	1.5	252	240	17.508	18	28	1
2	14	1.0	42	3.0	252	480	17.268	18	28	2
3	14	2.0	42	6.0	252	960	16.788	18	28	4

Note: This scheme is for 100 % potency level; similarly other potencies will be prepared.

All the dilution should prepared in polypropylene tube and stored at 2-8°C until dosing.

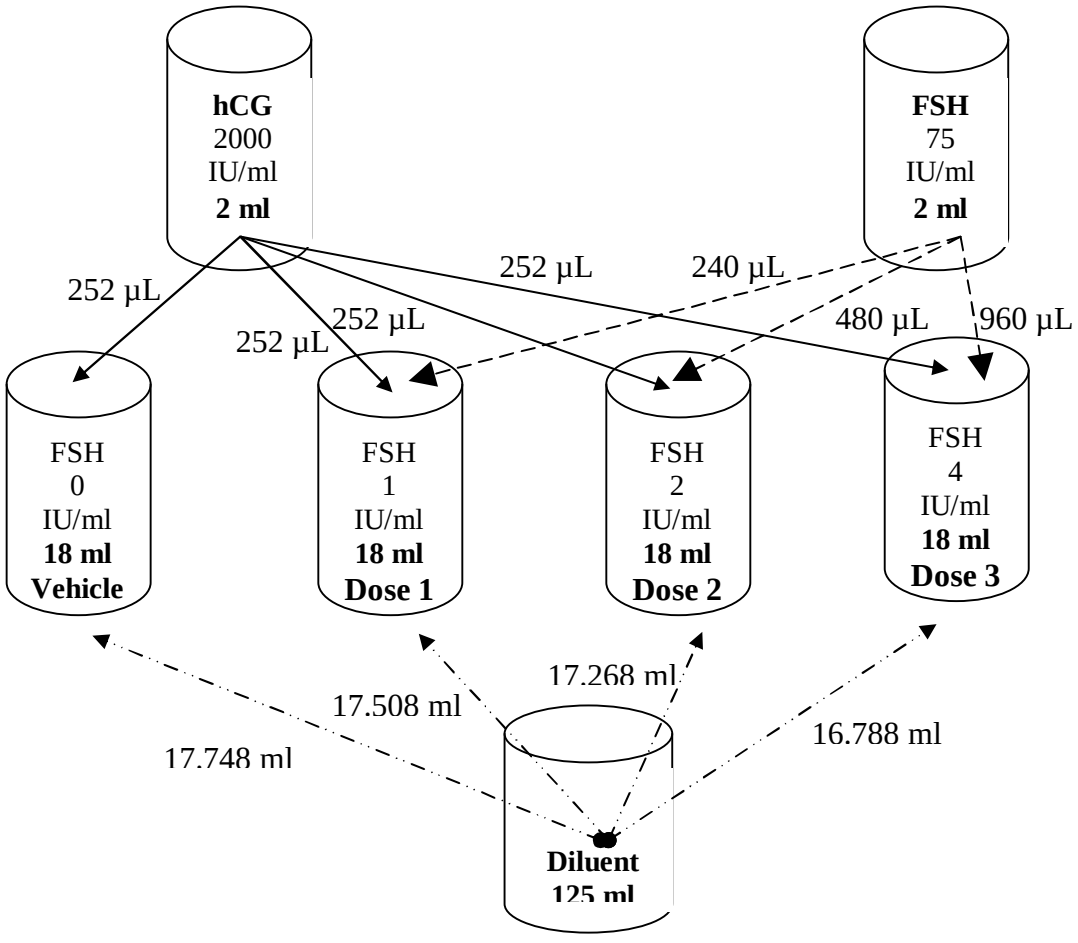


Figure 3.4: Schematic presentation of dose preparation of CRS/IRS/Sample.

3.2.2.9Animal selection, dosing and ovary collection:

Bioneeds people will perform this activity.

The animals for bioassay will be selected on the basis of age and body weight and will be randomized. Dosing and ovary collection will be carried out at Bioneeds.

Individual body weight will be recorded at the beginning of the study on day 1 and final body weight on day 4 at the terminal sacrifice. The animals will be weighed and recorded.

The formulated doses of reference standard and test items (samples) will be administered through subcutaneous route on the dorsal region of the animal body daily for 3 consecutive days. Injection of each dose will be repeated at 24 hrs and 48 hrs after the first injection. The daily dose will be administered in a volume of 0.5 ml per animal and will contain 14 IU of chorionic gonadotrophin to ensure complete luteinisation. On day 4, 24 hrs after the last injection, the animals will be humanely sacrificed and both ovaries will be dissected and collected. Any extraneous fluid and tissues will be removed from ovaries. The ovaries (paired) will be weighed immediately and recorded as raw data.

Raw data will be sent to IBPL for further analysis and potency estimation using PLA 2.0 and other statistical software.

3.2.2.10 Reporting of Results (calculation, data interpretation / analysis)

1. Analyze the data by PLA 2.0 software and compare the results obtained against the acceptance criteria and report the potency.
2. The potency obtained from the PLA 2.0 analysis is compared to the defined acceptance criteria, based on which it will be inferred whether the sample is complying or non-complying.

3.3 Iso-electric Focusing followed by Immunoblotting as an Identity test for rHuFSH

The objective of this method is to establish the identity of rHu FSH on the basis of its isoelectric point in comparison with the reference standard.

3.3.1 Rational

IEF is an analytical method which separates proteins based on their isoelectric point (pI). pI of the protein is the pH, when the net-charge on protein is zero. So, depending on the charge on protein, focusing of protein takes place on the IEF gel.

The test will be used as an identity test in which Gonal-*f* (market standard for rHuFSH) will be used as reference standard. rHu-FSH is produced in Chinese Hamster Ovary (CHO) cell line and undergoes post translational modification which gives different isoform pattern based on the charge. FSH has four N-linked glycosylation sites (two on alpha and two on beta subunits). All of these sites together can contribute 16 sialic acids on a single FSH molecule (If all of them are tetra sialylated). However, FSH molecules have different levels of sialic acids, which offer different charge distribution. This distribution gives rise to isoforms of FSH, which differ from one another with respect to the number of sialic acid residues, and thus the charge (sialic acid is a negatively charged sugar). These charge differences can be resolved on IEF.

3.3.2 Equipment and equipment parameters

1) Dry IEF Phast Gels**:** Dry IEF Phast Gel™, Amersham Biosciences, Catalog #17-0677-01, PHAST System, Amersham Biosciences

2) Pharmalyte 2.2-5 for IEF amersham Biosicences Cat.# 17-0451-01

3) ZOOM Carrier Ampholytes 4-7, Invitrogen, Cat. No.:ZM0022

4) Submerge electro-transfer unit (e.g. Biorad).

5) Mounting block

6) Gel backing remover

7) Transfer electrode cassette

8) PVDF membrane

9) Power pack

3.2.3 Procedure

Initializing the system: - Switch on the system and set on standby temperature (15ºC).

Preparation of gel compartment:-

- Switch the system on and set to standby temperature.

- Adjust the electrode assembly to the higher position by pressing upon both red eccentric levers until they click into place.
- Raise the electrode assembly to the vertical position.
- Wipe off the separation bed with tissue paper.

Positioning of the gels:-

Take the rehydrated PHAST IEF gels from the petri plate.

- Put a drop of water on the separation bed and place the gel within the red margin shown, so that a film of liquid, free from air bubbles forms between the gel and separation bed. Remove any air bubbles by sliding the gel around.
- Position the gel so that its edges are in prefect alignment with the red lines.
- Remove any excess liquid with absorbent paper.
- Lower the electrode assembly so the inner and outer electrode rest evenly on the gel.
- Lower the sample applicator arm.

Sample Application

- Choose the 6 well lanes.
- Hold the sample applicator in one hand at the eye level.
- Fill the sample applicator wells with 4 µl of sample volume and reference standard (Gonal-*f*) to load 0.5 µg of protein. Make sure there is no air bubbles trapped in these samples.
- Lower the applicator to the surface of the samples. Avoid spillage of sample on the sides of the applicator.
- Slide the loaded sample applicator into the slot nearest the cathode on the sample applicator arm.
- Close the separation compartment lid.

Separation Run

- Press “SEP start/stop” and then enter the number of gels for the run.
- Press “do” to confirm.
- Enter the “Sep Method”, to be run.

- Press “do” to confirm.
- To monitor the separation run-press “SEP real condition”.

Table 3.4 below gives detail of separation method

Table 3.4: Gel running parameters.

Sample Application	Down At	9.1	0000Vh			
Sample Application	Up at	9.1	0000Vh			
Extra Alarm to Sound at	70Vh					
Separation	9.1	2000V	2.5mA	3.5W	15ºC	0075Vh
Separation	9.2	200V	2.5mA	3.5W	15ºC	0015Vh
Separation	9.3	2000V	2.5mA	3.5W	15ºC	0410Vh
Separation	9.4	0000V	0.0mA	0.0W	15ºC	0000Vh

- Press “SEP start/stop” to stop the method. And then press “do” to confirm.
- The entire run would take approximately 35min.
- Remove the gel from the separation unit and put it in transfer buffer. Proceed with transfer after 10 minutes.

Transfer and blotting the gel

- Place the gel on gel holder slab and fix the edge of the gel in the holder.
- Once the gel is properly fit in the gel holder, place the fine wire of the cutting frame on the gel at the margin from where the separating gel starts. Properly fit the cutting frame on the gel holder.
- Now gently slide the cutting frame horizontally. The gel will come out of the hard plastic base.
- Place a PVDF membrane, cut to the size of the gel (activated with methanol and equilibrated in the transfer buffer for 5 min), on the gel. Avoid trapping of any air bubbles in between the gel and membrane.
- Hold the entire sandwich by a forceps and invert it.

- Roll a clean glass rod dipped in transfer buffer on to gel of the gel to get rid of any air bubbles trapped between the gel and the membrane.
- Close the cassette. Make sure that the gel is facing black plate while membrane is facing the white plate.
- Place the cassette in the running unit in such a way that the black plate of cassette faces the cathode (Black side) while white plate faces the anode (Red side).
- Connect the electrodes with the power pack and set following parameters for transfer:

Current: 400mA

Voltage: 100V

Time 45 Minutes

- Transfer the membrane to blocking solution (do not touch with bare hand; wear gloves). Incubate the membrane in blocking buffer (5% skim milk powder (non-fat) in (1x) TBS) for 1hr with gentle shaking at RT. If membrane is not to be developed immediately or same day, it can be stored in blocking solution at 2ºC-8ºC overnight.

Developing the membrane

- Decant the blocking solution after one hour, and add 1:250 diluted mouse anti FSH Intact and beta2 antibody (Primary antibody) on the membrane.
- Incubate for one hour at room temperature with gentle agitation.
- Decant the primary antibody and wash the membrane thrice (5 min each wash) with 1X TBS. Add 1:250 diluted goat anti mouse ALP conjugate (secondary antibody) and incubate for one hour at room temperature.
- Decant the secondary antibody and wash the membrane thrice (5 min each) with 1X TBS.
- Now add 2ml-3 ml of BCIP/NBT substrate solution (ready to use solution).
- Incubate the membrane with the substrate till 7-8 isoforms of Gonal-*f* is available.
- Decant the solution and wash the membrane with water to stop further reaction.
- Scan the membrane, dry and preserve it.

3.4 Size exclusion chromatography (SEC-HPLC) for determining content of rHu-FSH

In order to investigate the content of rHu-FSH (µg/ml) the size exclusion chromatography has been used. SEC-HPLC was carried out with the Agilent 1100/1200 series on Phenomenox Bio-Sep-SEC-S 2000(Diameter 7.8 mm, Length 300 mm, particle size of 5 micron pore size of 125 Å). The mobile phase was sodium phosphate buffer (pH 6.7; 0.1 M), with a flow rate of 1.0 ml/min.

3.5 HPAEC-PAD (High Performance Anion Exchange Chromatograph with Pulsed Amperometric Detection):

HPAEC is an improved chromatographic technique developed to separates carbohydrates coupled with pulsed amperometric detection (PAD) which permits direct quantification of non-derivatized carbohydrates at low pico-mole level. The released sialic acid was separated on CarboPac PA10 column (Dionex). Sialic acid was detected and quantified by pulsed amperometric detection.

3.6 Desialylation of rHu-FSH:

The first aim of this experiment was to generate a range of increasingly desialylated rHuFSH samples. These samples are then used for comparison of results between *in-vivo* and *in-vitro* assays. The rHu-FSH was dissolved in buffer A (providedin Kit Prozyme GK80110) to give final concentration 2.18 mg/ml. This solution was then vortexed and equal volume of different aliquots (90 µl i.e. 196 µg/90µl) were incubated for different time point (such as 0 hrs, 12 hrs, 24 hrs, 36 hrs, and 48 hrs) with Sialidase A (Provided in Kit Prozyme GK80110) in water bath at 37^{o}C and removed from the water bath at different time points as shown above. After removal from the water bath the samples were immediately transferred to -20^{o}C to stop/arrest the reaction of Sialidase A.

4. Results and Discussion

4. Results and Discussion

4.1 The development and qualification of an *in- vitro* cell based bioassay to determine the biological activity of recombinant human follicle stimulating hormone (rhu-FSH).

The *in-vitro* method was developed in different stages using different plate formats as follows:

1) 24 well plates: The initial method was developed in 24 well plate. The method worked well, but since the number of wells is only 24, it is laborious and time consuming. Therefore, it was decided to adapt it to a 96 well plate.
2) 96 well Plate: To make method more convenient and high throughput "96 well plates" were used. However, the well sizes (32 mm^2 well diameter) of a "96 well plate" consumes a lot of reagents and therefore it was further adapted to a "96 well half area plate".

"96 well half area plate": The "96 well half-area plate" has wells which are smaller in diameter (15mm^2). This helps save on the volume of the reagents (and hence the assay cost) without affecting the outcome of the assay. The following assay parameters were optimized in "96 well half area plate (15mm^2):

- Dose range optimization.
- Luminometer integration time optimization.

4.1.1 Method development in 24 (191 mm^2) well plate format

The FSH reporter gene assay was initially developed using 24 well plates. Briefly, the method was developed using the following conditions:

- Cell number: $5x10^5$ cells/well,
- Dose range: 60mIU/ml to 0.07 mIU/well, 2 fold dilution,
- Incubation time with the drug: 5 hrs
- Lysis buffer volume: 50µl

- Substrate volume: 100µl

In a reporter gene bioassay the crucial parameters are incubation time with the drug, cell numbers, incubation time for cell lysis, volume of lysis buffer, substrate incubation time and substrate volume. Considering the criticality of above mention parameter all incubation times were kept constant during method development when transferred to a "96 well plate" format, where as the volume of lysis buffer, volume of substrate and the cell number used per well have changed depending on the minimum holding volume of the 96 well plate. The surface area of 96 well plate is (32 mm^2) is approximately 6 times lesser compared to the surface area of 24 well plate (191 mm^2).

Experimental Design

Assays were performed in both the formats; 24 well and 96 well plates. Besides cell number the number of doses was changed to 8 ranging from 7.5 mIU/ml to 0.11 mIU/ml. Assay performance was compared using the dose response profiles obtained from both the formats.

Results

Figure 4.1 show a representative profile of dose response obtained from these experiments.

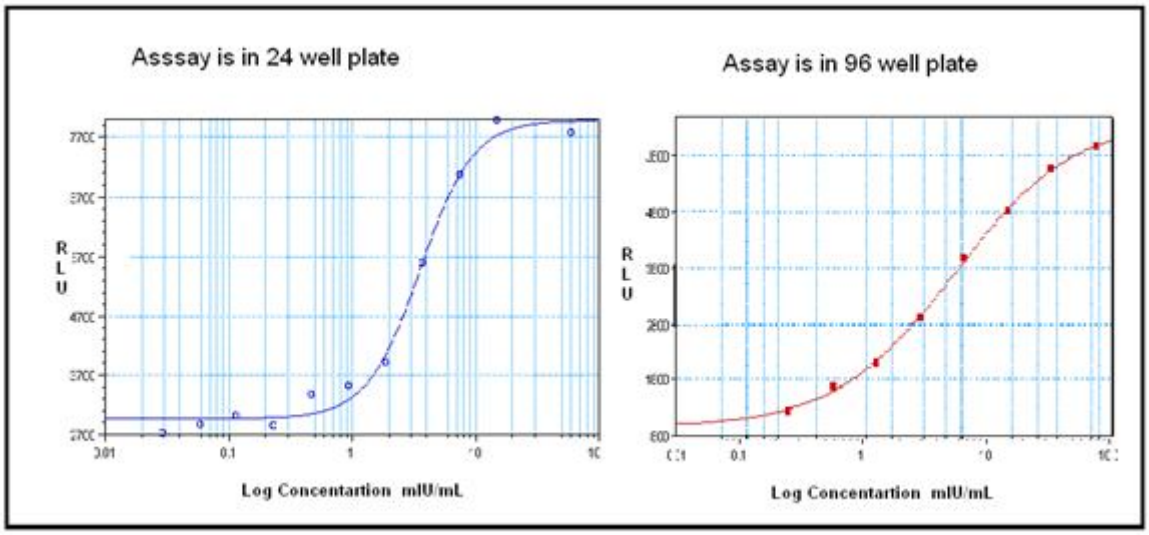


Figure 4.1: Dose response profiles from 24 well plate and 96 well plate formats

Discussion

In 24 well plate format total 12 doses were used 60 mIU,15 mIU,7.5 mIU,3.75 mIU,1.88 mIU,0.94 mIU,0.47 mIU,0.24 mIU,0.12 mIU,0.029 mIU,0.014 mIU and 0.0036 mIU and in

96 well plates only 8 doses were used starting from 7.5mIU followed by a 2 fold dilutions up to 0.12mIU/ml. A sigmoid dose response curve with clear upper and lower asymptotes was observed in both the plate formats. However, the curve obtained from 24 wells plate showed 4 linear doses whereas in the case of 96 well plate, 6 linear doses were obtained with a higher slope and S/N.

Conclusion

Dose response obtained from 96 well plate format is suitable for further assay development and is suitable for the potency estimation by an acceptable statistical model (Parallel Line Model)

The following assay conditions were taken into consideration:

Cell density: $10x10^4$cells/well

Cells incubation time in serum containing media: 30 mins

Incubation time of cells with the drug: 5hrs

Dose range:

- Dose 1 as 25.05 mIU/ml/well
- Dose 2 as 12.53 mIU/ml/well
- Dose 3 as 6.26 mIU/ml/well
- Dose 4 as 3.13 mIU/ml/well
- Dose 5 as 1.57 mIU/ml/well
- Dose 6 as 0.78 mIU/ml/well
- Dose 7 as 0.39 mIU/ml/well
- Dose 8 as 0.20 mIU/ml/well

4.1.2 Method optimization in 96 well plate format

With the expected dose response (dose dependent luciferase activation) observed in the 96 well plate format, the method was further optimized using the parameters mentioned below, but not necessarily in the order listed

4.1.2.1 Cell line used for the assay Source of CHO B5 cells

- Growth curve of CHOSI FSHR CRE luc B5 cells (B5-cells)

- Optimization of Sub culturing conditions

4.1.2.2 Optimization of Cell density

4.1.2.3 Optimization of Stimulation time with the drug

4.1.2.4 Optimization of Substrate volume

4.1.2.5 Optimization of Incubation condition

4.1.2.6 Luminometer systems (Bioteck and Turner systems)

4.1.2.1 Cell Line used for the assay

- **Source of CHOSI FSHR CREluc B5 cells**

CHOSI FSHR CREluc B5 cells (to be referred as B5-cells here after) (Commercially received from - Germany)

It is a genetically modified CHO cells adapted in the suspension culture in animal source free media.

Vials of frozen B5-cells were obtained during technology transfer.

Experimental Design to study the growth curve:

The aim of the study was to examine the growth pattern of B5-Cells. Growth curve was studied by seeding different cell densities of 6.25 $x10^4$, 12.5 $x10^4$, 25.0$x10^4$ and 50.0 $x10^4$ cells/ml. Cell count and percentage viability (% viability) were recorded at different time points (hrs). Cells were centrifuged at 350 x g for 3 min, and finally suspended in 15 ml of growth medium. Based on the cell count the suspension was further diluted to 3 different densities (6.25, 12.5, 25.0 and 50.0 $x10^4$ cells/ml) and 20 ml of each cell density suspension was seeded into T-75 cm^2 flask. An aliquot (0.1 ml) was collected from each flask at intervals of 24 hrs till 168 hrs and cell count was performed using the automated Vi-cell counter (Beckman).

Results

Figure 4.2 shows that seeding density of 0.5 x 10^6 cells/ml gave highest cell densities but also showed early decline in the viability (<90% after 96 hrs).

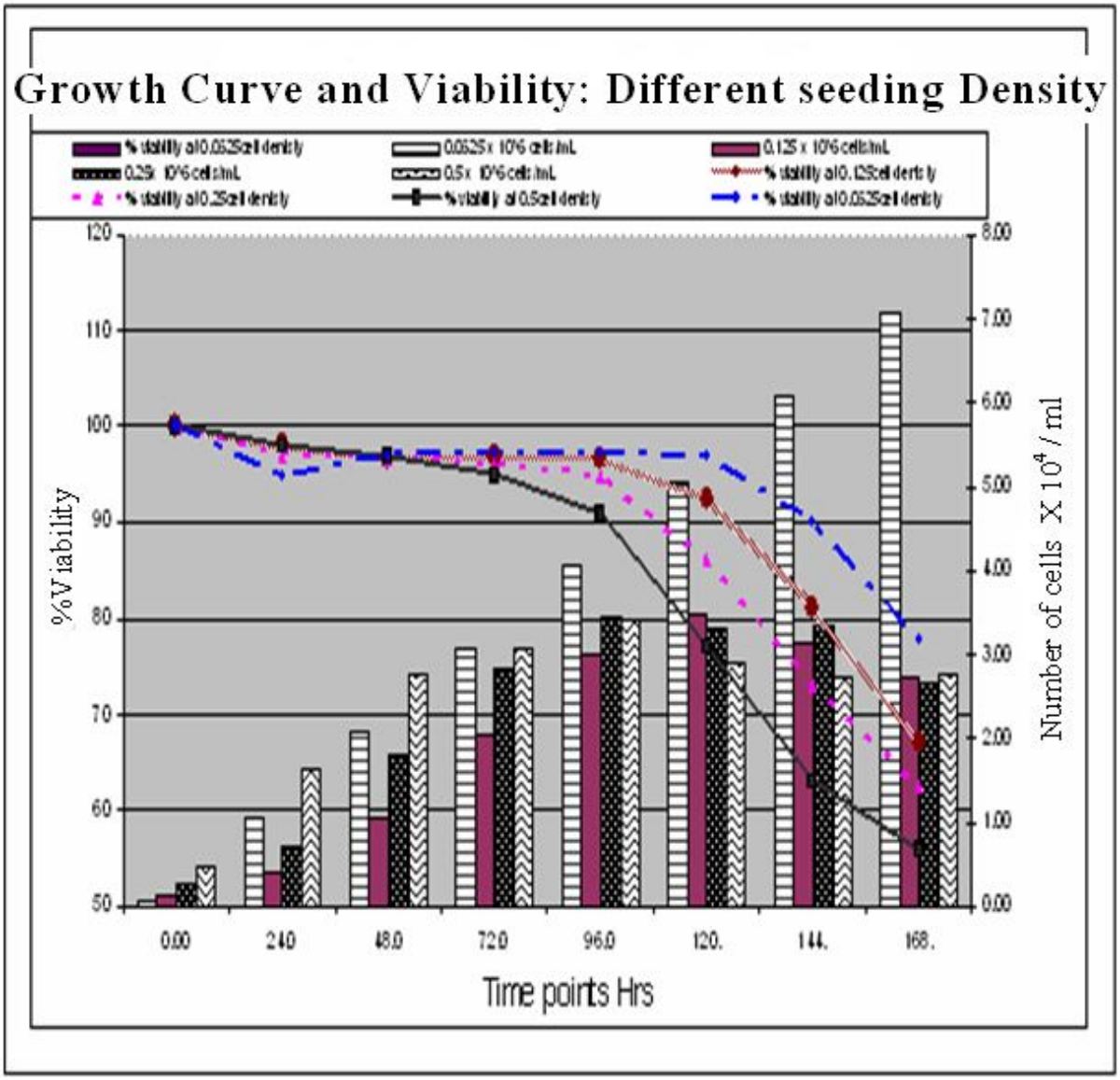


Figure 4.2: Growth pattern and viability profile of different seeding cell densities at different time points.

Table 4.1 : Total cells, viable cells and % viability of different cell seeding densities at different time points and doubling time obtained at each density.

Time points	6.25 x 10^4 cells/mL			12.5 x 10^4 cells/mL			25.0 x 10^4 cells/mL			50.0 x 10^4 cells/mL		
Hrs	Total cells	Viable cells (x10^6/mL)	% viability	Total cells (x10^6/mL)	Viable cells (x10^6/mL)	% viability	Total cells (x10^6/mL)	Viable cells (x10^6/mL)	% viability	Total cells (x10^6/mL)	Viable cells (x10^6/mL)	% viability
0	0.06	0.06	100	0.13	0.125	100	0.25	0.25	100	0.5	0.5	100
24	1.12	**1.06**	**95**	0.41	**0.4**	**98**	0.74	**0.72**	**97**	1.66	**1.63**	**98**
48	2.13	**2.06**	**97**	1.09	**1.05**	**97**	1.88	**1.82**	**97**	2.86	**2.77**	**97**
72	3.15	**3.06**	**97**	2.12	**2.05**	**97**	2.95	**2.84**	**96**	3.23	**3.07**	**95**
96	4.18	**4.06**	**97**	3.11	**3**	**97**	3.64	**3.45**	**95**	3.74	3.4	91
120	5.23	**5.06**	**97**	3.77	3.49	93	3.87	3.33	86	3.77	2.9	77
144	6.72	6.06	**90**	3.88	3.15	81	4.6	3.36	73	4.33	2.73	63
168	9.07	7.06	**78**	4.11	2.75	67	4.29	2.68	62	4.95	2.77	56
Average DT	19.6			18.8			16.9			22.7		

Note: The bold figures in the table above represent the log phase

Formula used for doubling time calculation:

$$\frac{0.693}{\text{Ln } (n2/n1)} \text{ X } t2\text{-}t1$$

Where,

t2 is the time point at n2
t1 is the time point at n1
n2 is number of cells at t2
n1 is number of cells at t1
0.693 is a factor (natural logarithm of 2)

Discussion

- Cells at different selected seeding densities followed the same growth pattern in terms of doubling time. Cells maintained maximum viability up to 120 hrs in case of the seeding

density of 6.25 $x10^4$ cells/ ml, while Cells maintain maximum viability up to 96 hrs in case of the 12.5 $x10^4$ cells/ ml, and up to 48 hrs in the case of the seeding densities of 25.0 and 50.0 $x10^4$cells/ml, respectively.

- Viability was consistent (over 90%) till 72 hrs with all the seeding densities.
- Except for the cell density of 6.25 $x10^4$ cells/ml all other cell densities showed a stationary growth phase from 72 hrs. In case of 6.25 $x10^4$ cells/ml cell densities a linear increase in cell density till 168 hours was observed, however the % viability declined below 90% at 120 hrs onwards.

Conclusion

All the selected seeding densities showed a similar viability profile till 72 hrs. The decline in viability is in direct proportion to the increase in seeding densities. The average doubling time determined with all the seeding densities was 19.6 to 22 hrs.

Optimization of Sub culturing conditions

Experimental Design

The aim of sub culturing optimization experiments was to fix the pre assay seeding density, time of sub culturing and harvest density before setting up a reporter gene assay for potency determination. These studies help to establish a near synchronous culture at the harvest with similar growth conditions which in turn reduces variability in the assay.

From the growth curve study, it was observed that B5- cells maintain log phase from 24 hrs to 72 hrs with mid log phase at 48 hrs. However, in the cases where higher seeding densities were used (50 $x10^4$ and $25x10^4$ cells/ml) there was an drop in the viability sooner. Considering these variations it was necessary to conduct bioassays with different seeding densities ($25.0x10^4$, $50.0x10^4$ and $75.0x10^4$ cells/ml) and two different sub-culturing periods (24 and 48 hrs) to identify the most sensitive phase.

Method Details

Three different cell densities (25.0 $x10^4$, 50.0 $x10^4$ and 75.0 $x10^4$ cells/ml) were seeded in duplicate in T-75 cm^2 flask. After 24 and 48 hrs of post-sub culturing each flasks were harvested and bioassays were performed using stability sample and NIBSC standard material as reference standard.

Results

Results of the experiments are shown below

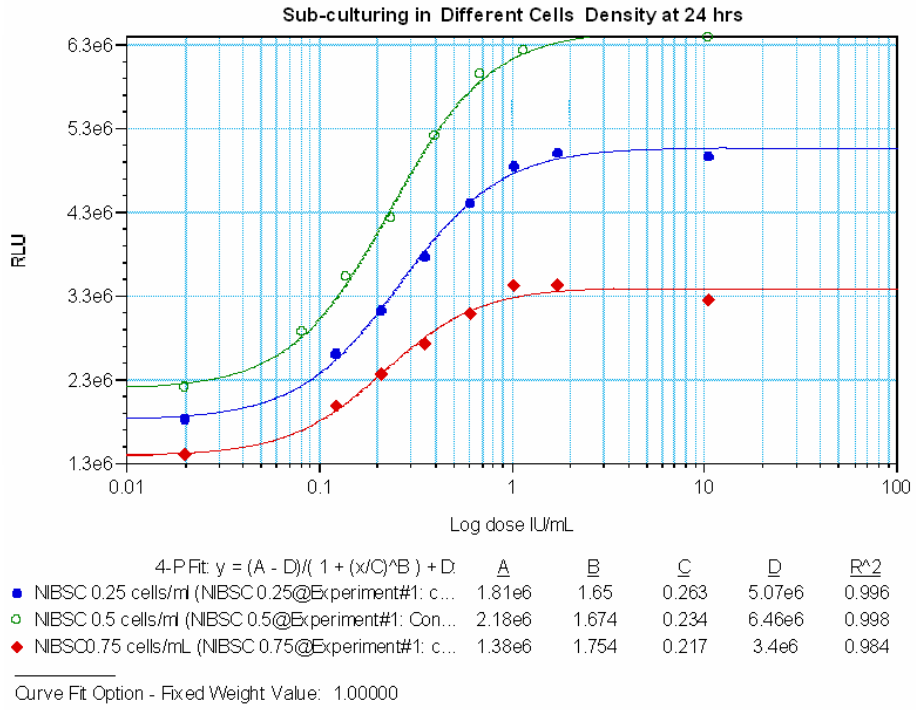


Figure 4.3: Full dose response curve (4-PL curve fit) obtained with 24 hrs sub cultured cells after growing them in T-75 flask using seeding densities of 25 x 10^4, 50x10^4 and 75x10^4cells/ml.

Table 4.2: Comparison of assay parameters on the response (assay sensitivity) after 24 hrs of sub culturing in T-75 flask with seeding densities of 25, 50 and 75 x10^4cells/ml.

Experiment	Cells density at seeding (1x 10^4)	Cell Harvest density (1X 10^4 cells/ml)	Slope	R^2	EC_{50} (mIU/ml)	Dose 1(Max)/ Dose 8(Min) Ratio
NIBSC	25.0	55	1.65	0.996	0.263	3.26
NIBSC	50.0	117	1.674	0.998	0.234	4.28
NIBSC	75.0	153	1.754	0.984	0.217	2.02

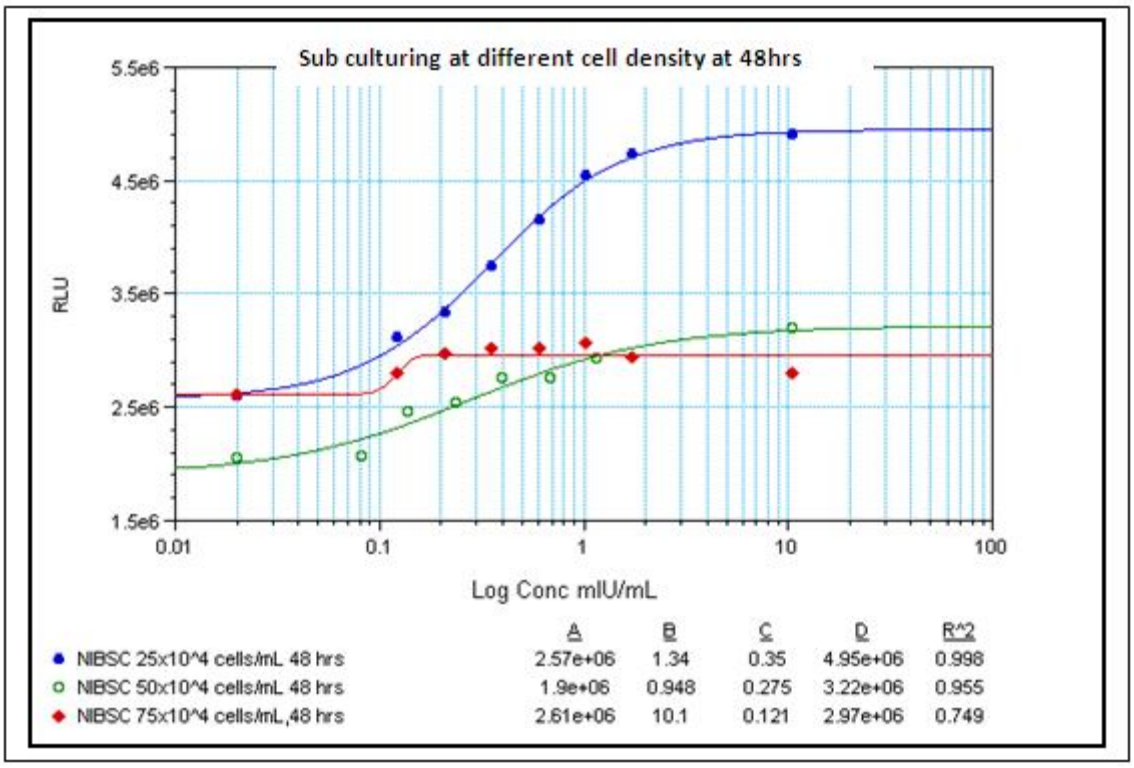


Figure 4.4: Full dose response curve (4-PL curve fit) obtained with 48 h sub cultured cells after growing them in T-75 flask using seeding densities of 25 x 10^4,50x10^4 and 75x10^4cells/ml.

Table 4.3: Comparison of growth response parameters on the response (assay sensitivity) after 48 hrs of sub culturing in T-75 flask with 25, 50 and 75 x10^4cells/ml seeding densities.

Experiment	Cells density at seeding (1x 10^4)	Cells Harvest density (1X 10^4 cells/ml)	Slope	R^2	EC_{50} (mIU/ml)	Dose 1(Max)/ Dose 8(Min) Ratio
NIBSC	25.0	181	1.34	0.998	0.35	2.38
NIBSC	50.0	197	0.948	0.995	0.275	1.32
NIBSC	75.0	238	10.1	0.749	0.121	0.36

Discussion:

Higher growth rate was observed with lower seeding density, i.e. 25x 10^4 cells/ml as compared to other (50 and 75 x 10^4 cells/ml) densities. Sigmoid dose response profiles with well-defined upper and lower asymptotes were obtained in all the subculture densities after 24 hrs sub culturing. The 24 hrs sub cultured cells showed EC_{50} values; slope and regression values similar with all densities.

Sigmoid dose response profiles with well-defined upper and lower asymptotes were obtained in 25x 10^4 cells/ml after 48 hrs post seeding. Sigmoid dose response profile was found with 50 x10^4 cells/ml. As the cells density increased the EC_{50} and the ratio of Max response /Min response (upper asymptote value / lower asymptote value) decreased. There was no dose response seen with 75x10^4 cells/ml after 48 hrs of sub culturing.

Observable difference was found in the growth rate at higher seeding density. This difference may be attributed to the fact that there is no FBS in the growth media (Seen Appendix A) and that the CHO cells have higher metabolic activity which leads to depletion of the media components in the flask, which in turn may be causing variations in the expression of the FSH receptors on the cell surface.

Conclusion

Based on these experiments it is concluded that lower cell densities i.e. 25x10^4 cells/ml or lesser than 25x10^4 cells/ml and 24 and 48 hrs of sub culturing time gave better dose response.

Looking to the practical feasibility it is recommended to use a cell density of $50x10^4$ cells/ml and 24 hrs of sub culturing time for all subsequent assays to be performed.

4.1.2.2 Optimization of Cell Density to be used per well in the assay plate

Experimental Design

To determine the optimal cell number to be used for the assay, experiments were done using two different cell numbers per well as 10,000 and 15,000 cells/100µl in a 96 well bioassay plate.

Method Details

Assays were performed with 10,000 and 15,000 cells/well plates with eight doses of FSH ranging from 7.81 to 0.12 mIU/well with 2-fold serial dilutions were incubated for 30 min in CO_2 incubator. After 5 hrs of exposure to FSH, 50µl lysis buffer was added in the all the wells. Luminescence was measured and dose response quantified in comparison to that seen with NIBSC Reference Standard using 4-PL software. RLU for the blank was used as background (noise). RLU for the first dose was used as signal. These values were then used to calculate signal to noise ratio.

Results

Figure 4.5 shows dose response curves with different cell densities. A summary of dose response parameters is presented in Table 4.4.

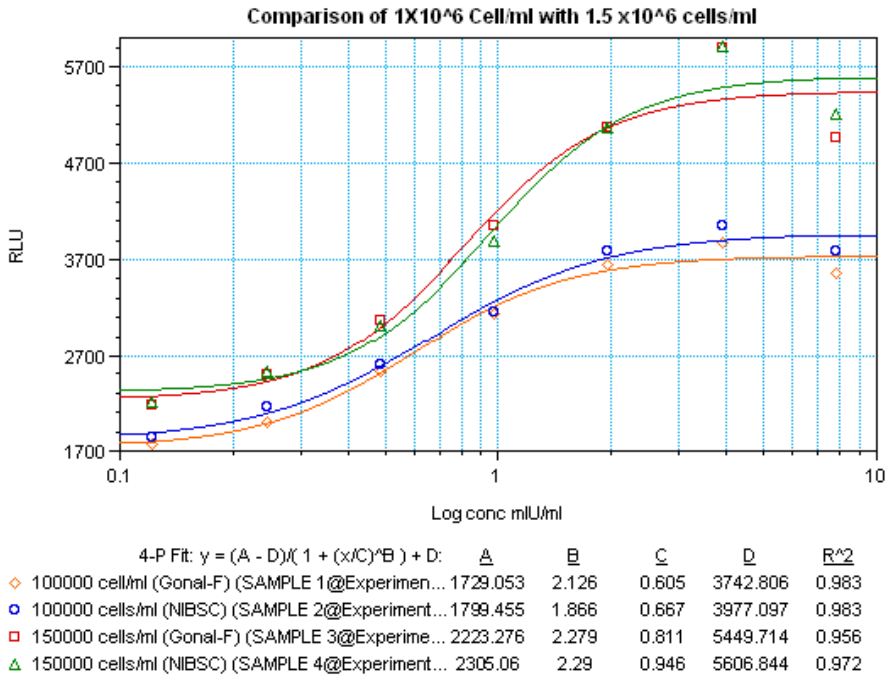


Figure 4.5: Dose response profiles (4-PL curve fit) with 10 x10^4 cells/well and 15 x10^4cells/well.

Table 4.4: Compiled representative cell response parameters with different cell densities.

Cell Density		Regression	Slope	S/N	EC50
10,000 cells/well/100µl	NIBSC	0.98	2.12	2.01	0.60
	Gonal-*f*	0.98	1.86	2.07	0.66
15,000 cells/well/100µl	NIBSC	0.95	2.27	2.29	0.81
	Gonal-*f*	0.97	2.29	2.35	0.94

Discussion

Different response was observed with two different seeding densities. Slope values were found to belittle higher in the case of 15,000 cells/well/100µl but not significantly different from that of 10,000 cells/well/100µl. Therefore, 10,000 cells/well/100µl was chosen for all further optimization experiments.

Conclusion

Similar dose response was observed with both 10,000 cells/well/100µl and 15,000 cells/well/100µl densities. Hence, it was decided to use cell density of 1 x10^6 cells/ml and seeding number of 10,000 cells/well.

4.1.2.3 Optimization of Stimulation Time with the Drug (FSH)

Experimental design/ Approach

To optimize the stimulation time for maximizing luciferase activity.

Method details

Assays were performed in 96 wells plate with eight doses from 7.81mIU/ml to 0.12 mIU/well using 2-fold serial dilutions, with a seeding density of $10x10^4$ cells/well, before inducing the cells with the drug for 30 mins. After addition of different concentrations of the drug, the assay plates were incubated at 37^0C in CO2 incubator for different time points (4 hrs, 5 hrs and 6 hrs), the dose response was quantified.

Results

Figure 4.6 shows representative dose response curves and Table 4.5 shows influence of stimulation time on various assay parameters.

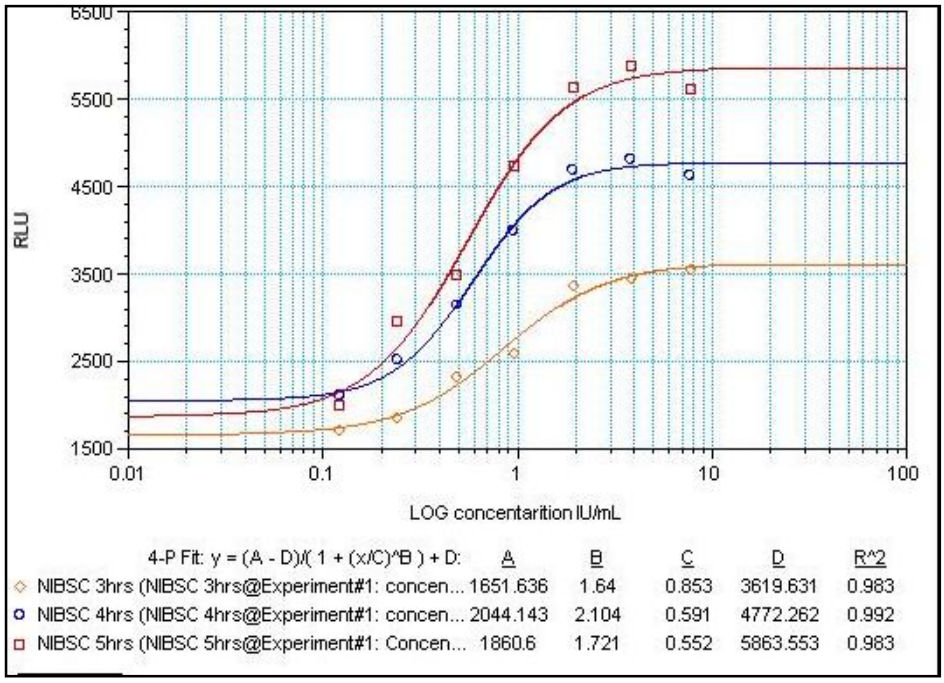


Figure 4.6: Dose response profiles (4-PL curve fit) with different stimulation time with the drug (FSH).

Table 4.5: Summary of results obtained with different stimulation time with drug.

Incubation Time		Regression	Slope	S/N
3 hours	NIBSC	0.95	1274	2.0
4 hours	NIBSC	0.97	1947	2.5
5 hours	NIBSC	0.91	2475	2.4

Discussion

Different stimulation time showed different dose-response curve. With increasing stimulations time the slope values were found to increase, while regression and S/N ratio were not affected by different stimulation periods tested.

Conclusion

Maximal luciferase stimulation by rHu-FSH was obtained at 5 hrs. Good slope, S/N ratio and response (in terms of RLU) were observed after 5 hrs of stimulation time. Hence, stimulation time was kept at 5 hrs in all subsequent optimization experiments.

4.1.2.4 Optimization of Substrate Volume

Experimental design/ approach

To determine the minimum volume of substrate to be used that gives maximum response.

Method details

The aim was to study the effect of substrate volume on assay parameters and to minimize the use of the substrate, there by lowering the cost of the assay. Assays were performed with two different volumes of substrate (50 µl and 100 µl). Dose response was then quantified by Promega Luciferase kit.

Results

Results from these experiments are presented in the following Figure 4.7 and Table 4.6. Figure 4.7 show the dose response profiles with different volumes of substrate (i.e. 50 µl and 100 µl) .Table 4.6 shows a comparison in dose-response parameters such as slope, EC_{50}, S/N ratio between two different volumes of substrate.

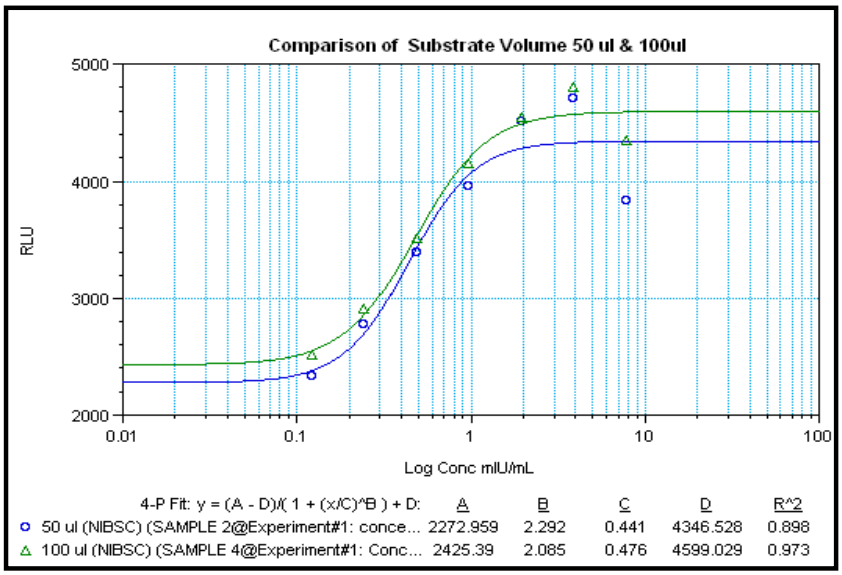


Figure 4.7: Dose response profiles (4-PL curve fit) with different volumes of substrate.

Table 4.6: Summary of results with different volumes of substrate.

Substrate volume		Regression	Slope	EC_{50}	S/N
50 µl	NIBSC	0.94	2.258	0.441	2.0
100 µl	NIBSC	0.98	1.853	0.476	2.0

Discussion

Although 100 µl showed higher signal (in terms of RLU) the S/N, slope and regression was not significantly different.

Conclusion

Comparable slope and regression values were observed with both 50 µl and 100 µl of substrate. Therefore, considering the cost factor, 50µl substrate was considered for all subsequent optimization experiments.

4.1.2.5 Plate-incubation conditions

Experimental Design/ Approach

Method details

The aim was to study the effect of incubation conditions on the assay parameters. According to the assay procedure, cells should be in an adherent state before stimulation with the drug. This is achieved by adding 4% FBS. The transition from suspension to the adherent cells is highly influenced by temperature. During incubation with the drug uneven heat transfer to

the plates kept at the bottom and the top can occur. This could result in higher assay variation. To avoid these occurrence two dummy plates were kept at the bottom and top of the assay plates. Dose response was then quantified as described previously.

Results

Results from these experiments are presented in table 4.7.

Table 4.7: Compiled parameters with different volume of substrate.

Incubation Conditions		Regression	Slope	S/N	%CV in Potency
Without dummy plates on top and at the bottom	NIBSC	15.6 (n=12)	47.1 (n=12)	14.6 (n=12)	23.7 (n=18)
	Gonal-*f*	25.9 (n=18)	41.8 (n=18)	29.3 (n=18)	
With dummy plates on top and at the bottom	NIBSC	14.9 (n=09)	26.9 (n=09)	11.0 (n=09)	11.69 (n=18)
	Gonal-*f*	8.7 (n=18)	21.5 (n=18)	12.9 (n=18)	

Discussion

Assays with dummy plates showed lesser variation in regression, slope, S/N ratio and potency values compare to the assays without the dummy plates.

Conclusion

Based on the experiments it is concluded that dummy plates should be used in further assay to reduce direct contact with the incubator shelf or atmosphere.

4.1.2.6 Assay performance on different Instruments system.

Experimental design/ Approach

To compare the RLU values measured on two different luminometers and calculate the difference.

Method details

The aim was to compare the RLU values measured on two different luminometers and calculate the difference. The experiment was carried out on the same day and the dose response quantified by Promega Luciferase kit. RLU values were measured in two different makes of Luminometers - Bioteck Multimode Reader and Turner Biostem.

Results

Table 4.8 and 4.9 shows comparative RLU and %CV in Potency between two different instruments - Bioteck Multimode reader and Turner biosystem.

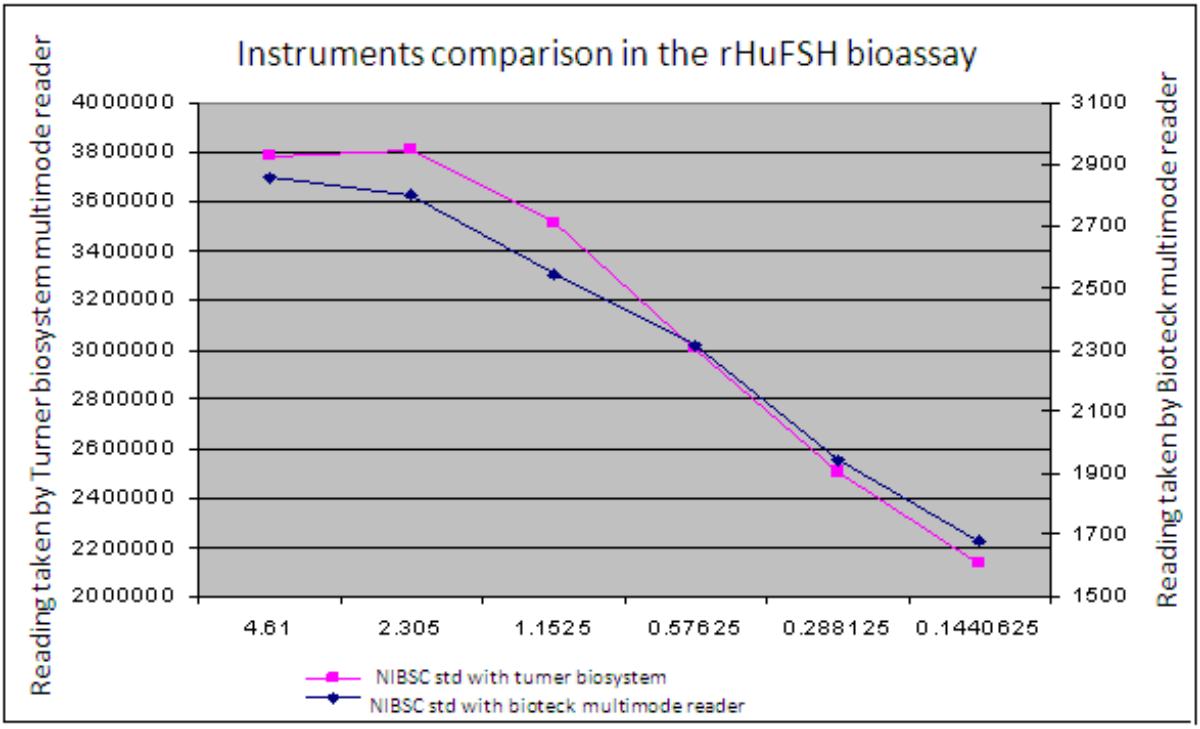


Figure 4.8: Dose response (4-PL curve fit) with different Luminometers.

Table 4.8: Compiled parameters with aspiration and without aspiration.

Fold difference between Bioteck Multimode Reader and Turner Biosystem in RLU*		
NIBSC		*Gonal-f*
1324		1234
1361		1358
1381		1316
1297		1355
1289		1377
1271		1243
1116		1118
967		1119
1027		1056
1089		1118
1042		1060
1110		1153
AVERAGE	1190	1209
SD	145	120
%RSD	12	10

*****The values represent the RLU from Bioteck Instrument divided by the values from Turner Biosystem.

Table 4.9: % Activity with aspiration and without aspiration.

% Activity		
Bioteck		Turner
100		75
99		76
86		80
60		105
68		77
		84
Average SD	82.6 18.08	82.8 11.3
CV	22	13.7

Discussion

A thousand fold differences in RLU values were observed between the two luminometer systems. However, there is no significant difference was observed in terms of percent activity as measured by both systems. The activity obtained from the two systems cannot be absolutely compared as these are two different experiments done on two different days by two different analysts. However, it is apparent that the average variation between the two instruments is not very significant when the same sample was assayed many times independently.

Conclusion

Turner Biosystem systems is user friendly as well as less time demanding compared to the Bioteck system as the former has an out-injector which can add light sensitive substrate to individual wells. In all subsequent assays Turner Biosystem was used.

4.1.3 Method transfer to 96 well (34mM) plate to 96 half area (15mM) well plate

rHu-FSH in-vitro bioassay method was optimized in 96 well (34 mm) plates. In order to reduce the consumption of reagents (cells, volume of drug, lysis buffer and substrate) and thereby the cost of the assay, half area plates were planned to be used.

Experimental design/ approach

The objective behind the use of half area plates for method optimization was to save all reagents used. Viz.; cell numbers used 5 $x10^3$ cells/well/50µl instead of 10 $x10^3$ cells/well/100 µl, substrate volume (30 µl instead of 50µl), Lysis buffer (25 µl instead of 30µl) Although this is not exactly half it is required to cover the full surface area of the well. Based on the dose range determined with the 96 well (34mm) plates it was decided to use the same dose range (10.5, 1.75, 1.03, 0.61, 0.36, 0.21, 0.12 and 0.02 mIU/ml/well) for half-well plates. The linear range is 1.75 to 0.12 mIU/ml with 1.7 fold dilutions between 1.75 to 0.12 mIU/ml with the highest and lowest doses of 10.5 and 0.02 mIU/ml to get upper and lower asymptotes with 6 points in the linear range.

Method details

Assays parameters were the same for 96 well half-area, After 5 hrs stimulation with the drug, the plates were read in Turner biosystem and compared with the results.

Results

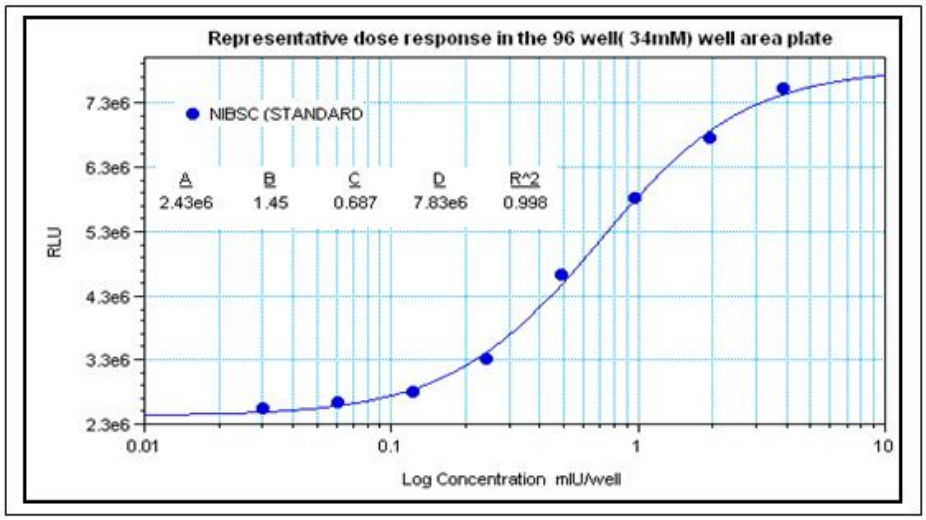


Figure 4.9: Dose response profiles (4-PL curve fit) with a dose range of 25.8 mIU/ml/well to 0.2mIU/ml/well.

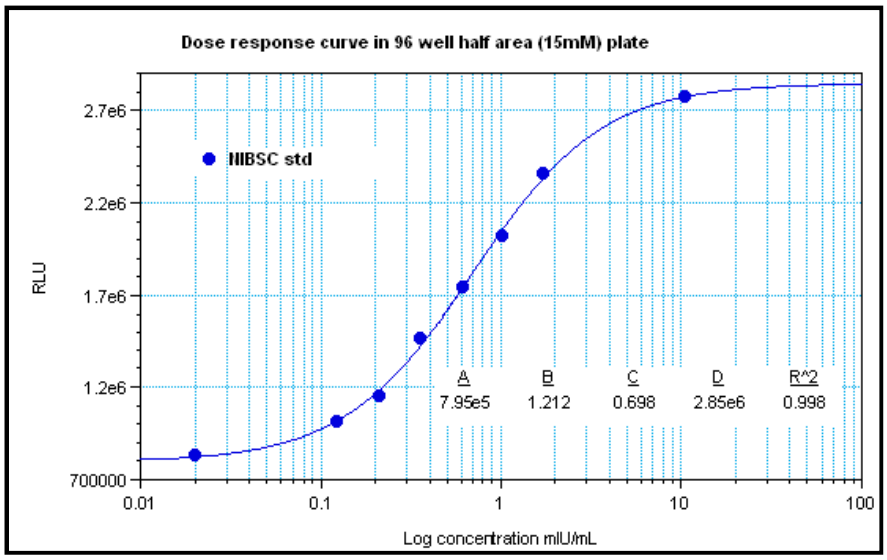


Figure 4.10: Dose response profiles (4-PL curve fit) with dose range of 10.5mIU/ml/well to 0.02 mIU/ml/well.

Discussion

As shown in the results both the figures showed typical sigmoid curve with clear upper and lower asymptotes for 96 well plates as well as 96 well half area plates. In the case of the two fold dilution from 25.8 mIU /ml to 0.2 mIU/ml the data points were scattered and did not exactly fit the sigmoidal curve. While in the case of the 1.7 fold dilution from 10.5mIU/ml/well to 0.02mIU/ml, the data fitted well with the sigmoidal curve with a clear linear dose range and well defined upper and lower asymptotes.

Conclusion

Based on the above results the dose range of the assay was optimized to be consisting of 8 doses from 10.5 mIU/ml/well to 0.02 mIU/ml/well with 1.7 fold dilution steps from dose 2 to dose 7 as shown below using 96 well half area plates.

Dose 1 as 10.5 mIU/ml/well

Dose 2 as 1.75 mIU/ml/well

Dose 3 as 1.03 mIU/ml/well

Dose 4 as 0.61 mIU/ml/well

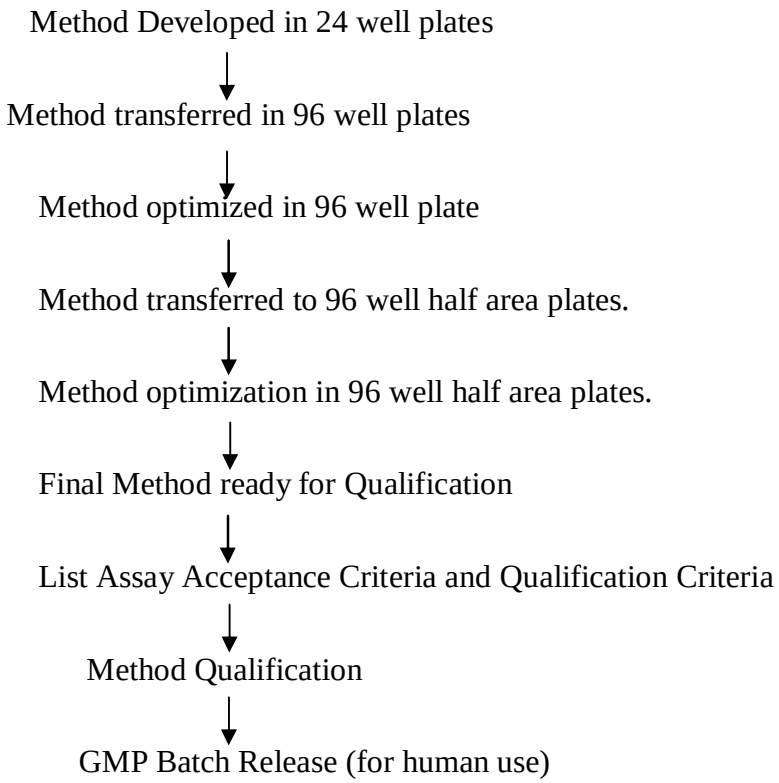
Dose 5 as 0.36 mIU/ml/well

Dose 6 as 0.21 mIU/ml/well

Dose 7 as 0.12 mIU/ml/well

Dose 8 as 0.02 mIU/ml/well

4.2 Final conclusion of method development flow for arriving at the final method



4.3 Reference standard and test sample details

- In-house Drug Substance,
    - Type: Test Solution
    - Manufactured Date: December 2008
    - Stored at: -20ºC
    - Concentration: 0.571 mg/ml
- NIBSC standard, 92/642
    - Type: WHO International Reference material
    - Stored at: -20ºC
    - Concentration: 10 µg/ampoule
    - Assigned Potency: 138 IU/ampoule

4.4 Biological assay Qualification

Bioassays are one of several physicochemical and biologic tests with procedures and acceptance criteria that control critical quality attributes (measured) of a biological drug product. Assay qualification is the process of demonstrating and documenting that the performance characteristics of the procedure and its underlying method meet the requirements for the intended application and that the assay is thereby suitable for its intended use. Assessment of bioassay performance is a continuous process, but bioassay qualification should be performed when development has been completed and the bioassay standard operating procedure (SOP) has been approved. Bioassay qualification is guided by a qualification protocol describing the goals and design of the qualification study. Qualification parameters discussed include relative accuracy, specificity, intermediate precision, repeatability, linearity, range and robustness. Laboratories may use dilution linearity to verify the relative accuracy and range of the method (Kathy et.al., 2006, Anscombe 1973, ICH, Q2(R1), Center for Drug Evaluation and Research, 2001, Center for Biologics Evaluation and Research, 2000, Muire-Sluis, 2004, USP 30 <1225>, Pharmacopoeial Forum 32 <1226>, Lucy *et.al.*, 2003, Nguyen *et.al.*, 2003, American Society for Testing and Materials, 2002, Capen, 2004, Krause, 2007).

Method Qualification Parameters

As per ICH guideline ICH-Q2 R14, following parameters were considered for qualifying the method:

4.4.1 Specificity

4.4.2 Linearity

4.4.3 Accuracy

4.4.4 Precision

4.4.4.1 Repeatability

4.4.4.2 Intermediate precision

4.4.5 Range

4.4.6 Robustness

4.4.6.1 Cell Density

4.4.6.2 Cell Incubation Time

4.4.6.3 FSH Incubation time

4.4.6.4 Different System

4.5 System suitability/Assay Acceptance criteria

Table 4.10 system suitability/assay acceptance criteria

Sr. No.	Parameter	Limit Value
1	Test of Regression, 95 % (F Test)	$F_{Regression} > F_{Critical}$ ($p<0.05$)
2	Test of Linearity, 95 % (F Test)	$F_{Critical} > F_{Regression}$ ($p>0.05$)
3	Test of Parallelism, 95 % (F Test)	$F_{Critical} > F_{Regression}$ ($p>0.05$)
4	RLU Ratio of Dose 1 (10.5 mIU/ml/well) to Dose 8 (0.02 mIU/ml/well) of standard	≥ 2
5	RLU Ratio of Dose 8 (0.02 mIU/ml/well) to Cell Control of standard	>1.0

4.4.1 Specificity

Specificity is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present. Typically these might include impurities, degradants, matrix, etc.

Experimental Design/ approach

Specificity is the ability of an analytical method to assess unequivocally the analyte of interest in the presence of other constituents in the sample and also in presence of any unrelated protein.

As per ICH guidelines, in order to establish that the test method has the ability to measure and differentiate the analyte in the presence of components that may be expected to be present (e.g., matrix components, product related impurities, etc.).

Qualification criteria

Except for rHu-FSH, the formulation buffer (placebo) and other unrelated proteins should not show any dose response when added to the assay diluents. Formulation buffer spiked with test sample or reference standard should give the same response as that seen in assay diluents, with intra plate assay variation of not more than 15%.

Results

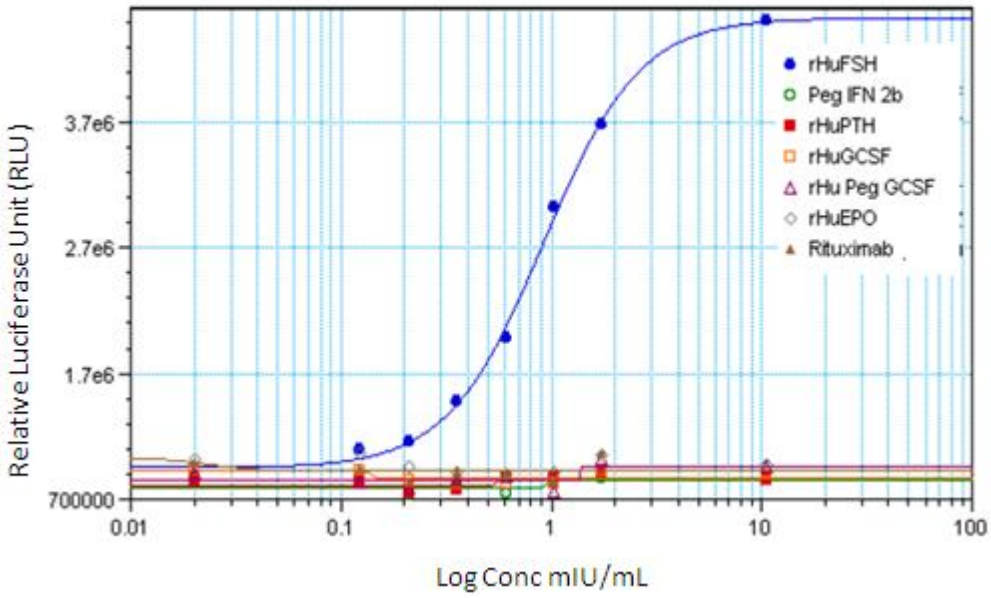


Figure 4.11: Dose response of rHu-FSH, Peg IFN 2b, rHuPTH, rHu-GCSF, rHu PEG GCSF, rHuEPO, Rituximab.

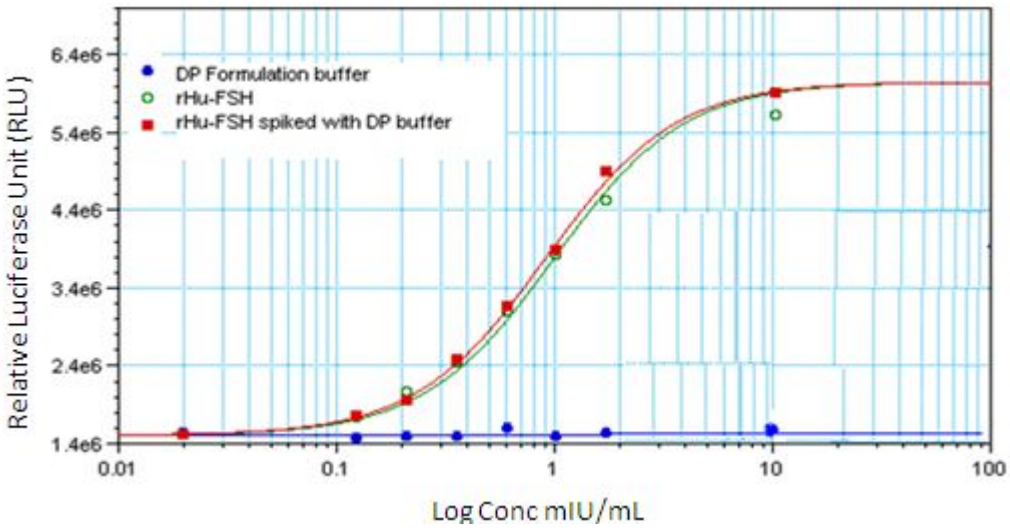


Figure 4.12: Dose response of Drug Product formulation buffer and rHu- FSH spiked with DP buffer.

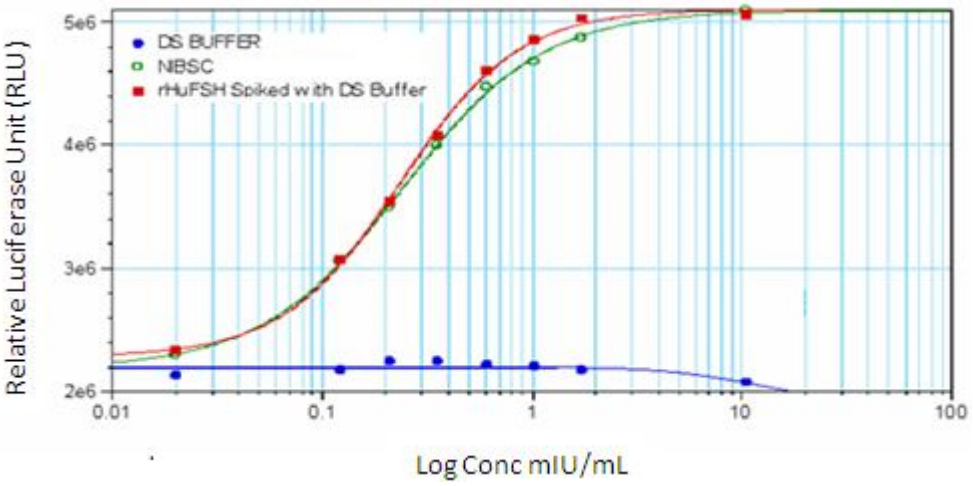


Figure 4.13: Dose response of Drug Substance formulation buffer and rHu-FSH spiked with DS buffer.

Table 4.11: Results of method specificity.

Sr.No.	Sample	Dose-response
1	rHu FSH(NIBSC standard)	Observed
2	rHu IFN alpha 2b	Not observed
3	rHu PTH	Not observed
4	rHu Peg GCSF	Not observed
5	rHu EPO	Not observed
6	rHu Rituximab	Not observed
7	DS and DP Formulation buffer (FB)	Not observed

Discussion

Results from these experiments are represented in Figures 4.11, 4.12, 4.13 and Table 4.11.

Figure 4.11 shows the representative profiles of rHu-FSH, Peg IFN 2b, rHuPTH, rHu-GCSF, rHu PEG GCSF, rHuEPO, Rituximab.

Figure 4.12 shows the representative profiles of DP Formulation buffer and DP buffer spiked with rHu FSH Figure 4.13 shows the representative profiles of DS Formulation buffer and DS buffer with rHu-FSH spiked.

Table 4.11 shows the compiled dose response observed.

Conclusion

Assay Acceptance criteria were met and the method was found to be specific for rHu-FSH

4.4.2 Linearity

The linearity of an analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentration (amount) of analyte in the sample.

Experimental Design/ approach

Five different 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 levels.

Test dilutions were prepared in assay media to get above mentioned potencies, namely 160 % (8.80µg/ml), 140 % (7.15 µg/ml), 100 % (5.5 µg/ml), 80 % (4.4 µg/ml) and 60 % (3.3µg/ml). Volumes required for each potency were prepared and stored as 25 µl aliquots at -20 °C.

Since the reference standard was diluted to 5.5 µg/ml, required volumes for each potency were prepared and stored at -20 °C in 25µl aliquots. The above 5 samples were analyzed in bioassay as if they were at the concentration of 5.5 µg/ml. Nine independent assays were performed by three different analysts.

Qualification Criteria

1. Linearity of the method should be with R^2 value greater than or equal to 0.95.
2. A random pattern of residuals, about zero should be observed in the residual plot.

Data analysis

Data from linearity experiments were analyzed as described below:

1. Observed relative potency converted into observed % relative potency.
2. Percent relative potency values transformed into natural-log values.
3. Percent expected relative potency and values transformed into natural-log values.

Note: (Step 1, 2, 3 are common for analysis of linearity, intermediate precision, accuracy and range) Calculated the mean of natural-log transformed data at each potency level.

4. Plot graph of natural log transformed observed average % relative potency versus natural log transformed expected average % relative potency performed linear regression analysis and reported slope, y-intercept and regression (R^2) values.
5. Regression output (R^2 value, slope value and intercept value) was obtained from the linear regression analysis. For each potency level (i.e. observed y value), from the linear equation a predicted Y-value was calculated respectively for each potency level. Residual value was obtained based on the difference between observed y value and predicted y value. Based on the values obtained a residual plot was plotted.

Results

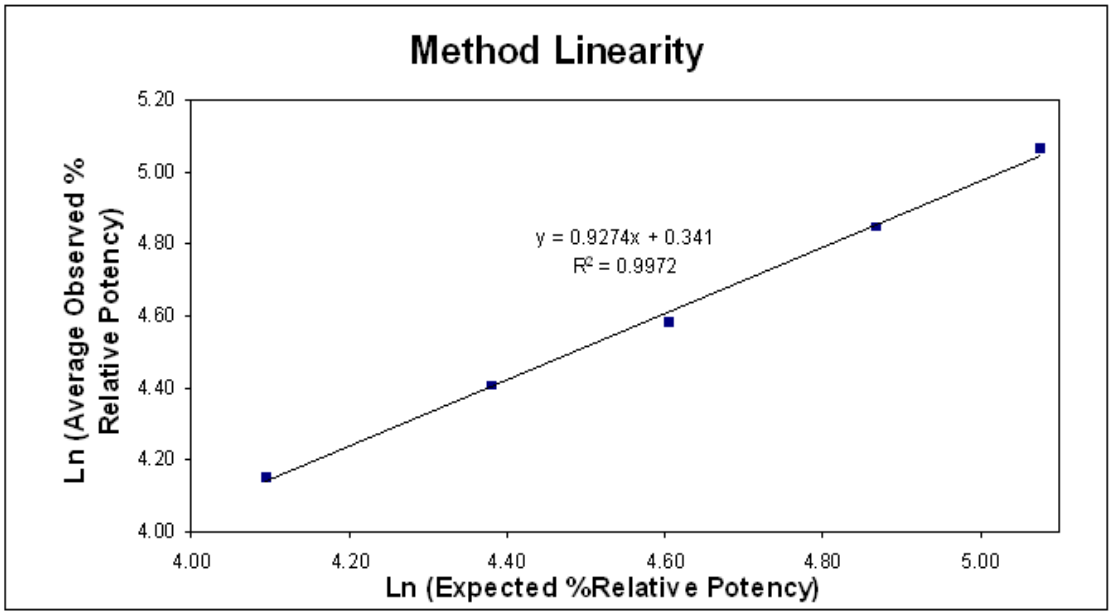


Figure 4.14: Plot for Linearity of the method.

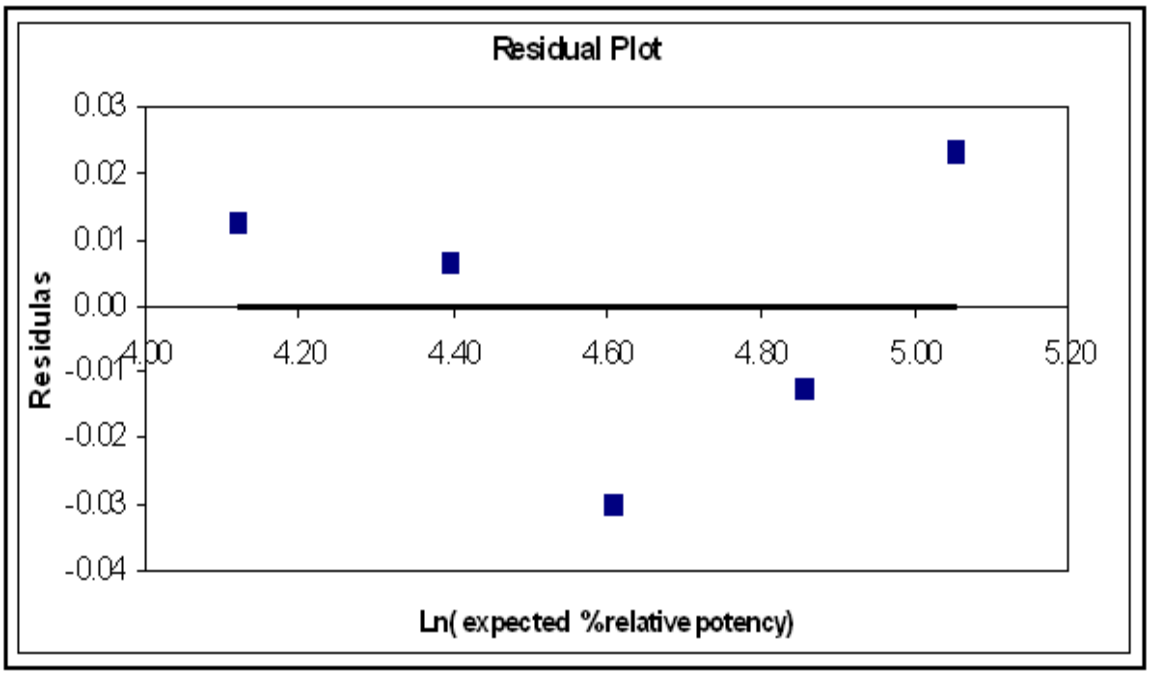


Figure 4.15: Residual Plot.

Table 4.12: Regression (R^2), Intercept and Slope output for method linearity.

R^2	0.997
Intercept	0.341
Slope	0.927

Table 4.13: Table showing Residual values.

RESIDUAL OUTPUT		
Observation	Predicted Y value	Residuals
1	5.05	0.0234
2	4.86	-0.0123
3	4.61	-0.0301
4	4.39	0.0065
5	4.12	0.0124

Discussion

The slope, Y-intercept, and R^2 are 0.927, 0.341 and 0.997 respectively (Table 4.12). The plot of the residuals versus the expected potency level from the regression analysis shows a random pattern of residuals about zero (Figure 4.15).

Conclusion

A linear relationship between the expected natural log (Ln) relative potencies and observed natural log (Ln) relative potencies was demonstrated (Figure 4.14).

A random pattern of residuals about zero indicates unbiased estimation of relative potencies. Acceptance criteria were achieved.

4.4.3 Accuracy

Accuracy is the measure of exactness of an analytical method, or the closeness of agreement between the values that is accepted either as a conventional true value or an accepted reference value and the actual value estimated. As per ICH, accuracy of the method should be assessed over the specified range of the method.

Experimental design / approach

Data obtained from testing of the five different simulated potency samples from the method linearity assessment were utilized to assess the accuracy.

Qualification criteria

The average % recovery must be 100 % ± 15 % per potency level.

Data analysis

1. Calculated % recovery per potency level by taking percentage of ratio of observed average potency to expected potency at each potency level.
2. Calculated average accuracy of the method by averaging % recovery at each potency level.
3. Calculated Lower and Upper 95 % confidence limit for the % recovery.

Results

Results are summarized below in Table 4.14.

Table 4.14: Percent recovery per potency level and average percent recovery.

% Potency level						Average
	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

Discussion

The % recoveries for 160, 130, 100, 80 and 60 % samples were 105.4 %, 105.8 %, 104.8 %, 108.2 %, 112.2 %; respectively. Average % recovery was 102.9 %.

Conclusion

Acceptance criterion for all samples tested was achieved. The method meets the acceptance criteria in terms of accuracy in the range of 60 % to 160 %.

4.4.4 Precision

The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same

homogeneous sample under the prescribed conditions. Here precision includes repeatability and intermediate precision.

4.4.4.1 Repeatability

Repeatability of an analytical method describes the closeness of individual measures of an analyte when the procedure is applied repeatedly under the same operating conditions.

As per ICH Q2 R1, repeatability should be assessed using a minimum of nine determinations that cover the specified range (e.g. three concentrations using three replicates each) or by using a minimum of six determinations at 100 % of the test sample. It is acceptable to perform repeatability using the maximum number of samples allowed per method, which may be less than six. It is recommended that minimum six assays shall be performed to capture the overall repeatability.

Experimental Design/ Approach

To assess the repeatability of the method, three analysts performed the assay on two different days and thus total 3 independent assays were performed.

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 (Geometric Relative Standard Deviation).

Qualification Criteria

1. Repeatability per assay: % GRSD $\leq$ 20 %
2. Average repeatability: % GRSD $\leq$ 15 %

Data analysis

1. Calculated intra plate variation in terms of % GRSD between two determinations of each plate.
2. Calculated repeatability per assay in terms of % GRSD for 6 determinations of each assay.
3. Calculated average repeatability in terms of % GRSD for total 3 assays.

Results

Table 4.15: Repeatability of *in-vitro* Cell based 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%
Intra-assay	5.1%	7.2%	6.9%
Overall Repeatability	6.5%		

Discussion

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.

Conclusion

Acceptance criteria were achieved for all the assays. The method is therefore consistent, showing reproducible results.

4.4.4.2 Intermediate Precision

Intermediate precision is the degree of reproducibility of results obtained under different operating conditions like different days and different analysts.

Experimental Design/ Approach

Three different analysts performed experiments over multiple days in order to assess the intermediate precision. Data obtained from the analysis of five different simulated potency samples from the method linearity assessment were utilized to calculate intermediate precision.

Qualification criteria

1. Per potency level, % GRSD $\leq$ 20.
2. Per analyst per potency, % GRSD $\leq$ 20.

Data analysis

1. Calculated intermediate precision per potency level in terms of % GRSD of values of each analyst and at each potency level.

2. Calculated intermediate precision per analyst and potency level in terms of % GRSD at each potency level.

3. Calculated average intermediate precision of the method in terms of % GRSD for all potency levels.

Results

Table 4.16: Intermediate Precision of *in-vitro* cell based 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

Observation and Discussion

The % GRSD per analyst per potency ranged from 5.2 % to 11.8 % for analyst 1, 3.4 % to 17.6 % for analyst 2, 6.8 % to 10.9 % for analyst 3 the % GRSD per potency level ranged from 6.8 % 10.9 %. The variation with average intermediate precision is 9.4 %.

Conclusion

Acceptance criteria were achieved for all assays. The method is consistent, showing reproducible results between 60 to 160% potency levels.

4.4.5 Range

The range of the method is the interval between the upper and lower potencies, where it has been demonstrated that the method has a suitable level of precision, accuracy and linearity. When method linearity, precision and accuracy have been successfully demonstrated, the range of the method is established. When feasible, the range of method should encompass the width of product specification limits.

Experimental Design/ Approach

Data obtained from the testing of five different simulated potency samples from the method linearity assessment were utilized to assess the range.

Qualification criteria

Range of method will be demonstrated if linearity, precision, and accuracy of the method meet the respective acceptance criteria.

Results

The linearity, accuracy and precision of the method met the qualification target of analytical method within the range of 60 % to 160 % potency levels.

Conclusion

Acceptance criteria were achieved. The range for the method is 60 % to 160 % potency levels.

Acceptance criteria

The linear range of the method must at a minimum cover the "width" of the product specification. Hence range of method should be at least 80 % to 125 % of relative potency.

4.4.6 Robustness

Robustness is defined as the method's capacity to remain unaffected by small, but deliberate variations in the optimized parameters and provides an indication of its reliability during normal usage. Four critical parameters identified during assay development (Number of cells per well, Cell incubation time, Time of incubation with drug, Reading at two different systems) were tested to assess robustness of the method.

4.4.6.1 Number of Cells Per Well

Two assays were performed using 40000 and 60000 cells per well along with the control of 50000 cells per well.

Results

Overall results are summarized below in Table 4.17.

Table 4.17: Results for robustness parameter, number of cells per well

Condition	Specific Activity (E+05 IU/mg)	Max/Min	Slope	EC_{50} (IU/ml)
40000	0.15	2.3	1.65	0.15
50000	0.12	2.4	1.36	0.20
60000	0.13	2.3	1.33	0.16
Mean	0.13	2.33	1.44	0.17
% variation	12.30	0.70	12.38	13.47

Discussion

Specific activity remained unchanged for all three cell densities.

Slope values for 60000 cells/well were slightly low compared to 50000 and 40000 cells/well.

Ratio of Max/Min remained unchanged for all three cell densities.

Percent variation for all parameters was well below 15%; hence it could be said that no significant difference was observed.

Conclusion

Altering cell number by 20% did not affect the final result i.e. specific activity. However, it is recommended to keep the cell density within ± 10% of the control cell density of 50000 cells/well.

4.4.6.2 Cell incubation Time

The parameter “Cell incubation time” was tested in order to find out a time range within which assay can be done without affecting the final results. The incubation time is 30 min. Hence for robustness the time range chosen was 30 (control) ± 6 Min (i.e.± 20%).

Experimental Design / Approach

Assays were performed with 24 and 36 Min incubation along with 30 Min of incubation as control.

Results

Table 4.18: Results for Robustness Parameter, cell incubation time

Time in Minutes	Specific Activity (E+08 IU/mg)	Max/Min	Slope	EC_{50} (IU/mg)
24 min	0.14	2.43	1.46	0.26
30 min	0.13	2.50	1.43	0.30
36 min	0.16	2.59	1.30	0.29
Mean	0.14	2.51	1.39	0.28
% variation	9.88	3.11	5.97	7.73

Discussion

EC_{50} and Specific Activity have not much difference and the overall percentage variation is also 10%.

Ratio of Max/Min and slope value remained unchanged for all three incubation time.

Conclusion

Cell incubation 24 or 36 Min did not bring about any additional variation in the specific activity compared to the control incubation time of 30 Min. Overall percentage variation of slope values, Max/Min, Slope and EC_{50} were unaffected. Therefore, it is recommended to keep the target incubation time as 30 Min ± 6 Min.

4.4.6.3 Time of Incubation with Drug

The parameter "time of incubation with drug" was tested in order to find out a time range within which assay can be done without affecting the final results. The time of incubation with drug has been set for 5 hrs. Hence for robustness the time range chosen was 5 hrs (control) ± 1 hrs (i.e.± 20%).

Experimental Design / Approach

Assays were performed with 4 and 6 hrs incubation along with 5 hrs of incubation as control.

Results

Table 4.19: Results for robustness parameter, time of incubation with drug

Condition	Specific Activity (E+08 IU/mg)	Max/Min	Slope	EC_{50} (IU/mg)
4 hrs	0.15	2.20	2.07	0.30
5 hrs	0.14	2.84	1.58	0.38
6 hrs	0.14	2.97	1.30	0.37
Mean	0.14	2.65	1.62	0.34
% variation	**1.81**	**15.48**	**23.83**	**13.19**

Discussion

No difference in specific activity was observed.

Max/Min and EC_{50} values is less compared with 5 and 6 hrs, while Slope value is high compared to 5 and 6 hrs incubation time with drug.

Variation of 23% indicated, slope values increased significantly with decrease in incubation time.

Conclusion

Incubation with the drug for 4 or 6 hrs did not bring about any additional variation in the specific activity compared to the control incubation time of 5 hrs. However, slope values were affected. Therefore, it is recommended to keep the target incubation time as 5 hrs ± 1 hrs.

4.4.6.4 Difference between Turner Biosystem and Biotech Luminometer

The parameter “Different System” was tested in order to find out a difference in RLU values and the effect on overall assay parameter of two different instrument types for reading. Hence for robustness the Different System chosen was Turner Biosystem and biotech luminometer.

Experimental Design / Approach

Assays were performed with two Different System that is Turner Biosystem and biotech luminometer.

Results

Table 4.20: Results for robustness parameter, Different system for quantification.

Condition	Specific Activity (E+08 IU/mg)	Max/Min	Slope	EC_{50} (IU/mg)
Synergy HT microplate Reader	0.139	2.71	1.03	0.75
TURNER BIOSYSTEM	0.137	2.48	1.27	1.27
Mean	0.14	2.59	1.14	0.98
STDEV	0.00	0.16	0.17	0.36
% variation	0.93	6.30	14.88	37.19

Discussion

No difference in specific activity was observed.

Max/Min and Slope value are unchanged with two different instrument types.

Variation of 37% indicated, EC_{50} values increased significantly with Turner Biosystem.

Conclusion

Assays were performed with two Different System that is Turner Biosystem and biotech luminometer did not bring about any additional variation in the specific activity. However, EC_{50} values increased were affected. Therefore, it is recommended to take reading in Turner Biosystem.

4.6 Scientific evidence to suggest that *in-vivo* bioassay can be replaced by an *in-vitro* bioassay:

FSH preparations are heterogeneous and contain multiple isoforms, which differ in their carbohydrate structure (Ulloa-Aguirre *et. al.,* 2003). Carbohydrates (Sialic Acid) are important to prevent degradation and to delay clearance of FSH from the circulation (Masaomi *et. al.,* 2007*)*. Carbohydrate plays an essential role for the *in-vivo* biological activity of FSH (Renato *et.al.,* 1996).

Activity of FSH (*in-vivo*) is completely abolished when it is fully desialylated but in case of *in-vitro* assay, higher activity was observed. This is because when FSH molecule is desialylated, due to decrease in stearic hindrance, the interaction between FSH receptor and FSH is facilitated resulting in elevated *in-vitro* activity (Samar *et. al.,* 2013).

It indicated that there is direct correlation between degree of Sialylation (Figure 4.16) and *in-vivo* activity because liver expressed asialoglycoprotein (ASGPR) plays an important role in clearing the incompletely sialylated glycoprotein from the circulation (Signe *et.al.,* 2003, Ann *et.al.,*1992). In contrast, an inverse relationship has been observed in case of *in-vitro* bioassay (Zambrano *et.al.,* 1996).

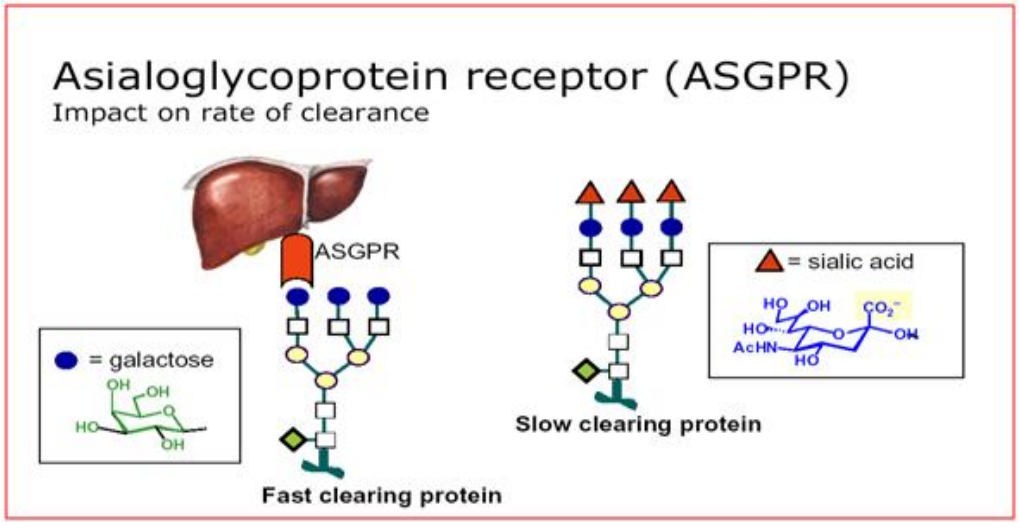


Figure 4.16: Role Asialoglycoprotein Receptors (ASGPR) present in liver.

Ref. www.crucell.com

There are several complementary methods during either manufacturing of rHu-FSH drug substance or in final drug product e.g. CZE, IEF, Sialic acid estimation etc. that can ensure proper control of molecular heterogeneity of FSH (C.M.Timossi *et.al*., 2000, Weirong *et.al.*, 2001). If results of these analyses are in accordance with predefined acceptance criteria then it can be technically concluded that *in-vitro* activity obtained is a function of FSH molecule (varying level of binding to the receptor).

Physico-chemical and functional analysis (using *in-vivo* and *in-vitro* bioassays) of more than 40 batches (data shown in annexure 01) of rHu-FSH demonstrated that the specific activity by *in-vivo* bioassay, isoforms patterns, dissociation of subunits by isoelectric focusing as well as degree of sialylation were highly consistent (Mary *et.al.*, 1994). This fact is a strong evidence of a controlled manufacturing process that complies to predefined acceptance criteria consistently. Thus the validated *in-vitro* alternative of the *in-vivo* bioassay should be acceptable by the regulatory agencies.

As a historical case in a similar fashion (that is of by demonstration of controlled manufacturing history) the Pharma European monograph based *in-vivo* bioassay of rhFSH molecule has been replaced by the innovator with a physicochemical test.

During development of *in-vitro* method, special rHu-FSH samples were generated by forced degradation (thermal and enzymatic) in order to find the capability of the *in-vitro* method to detect such changes (Vitt *et.al*., 1998). This includes:

4.6.1 Assay response on Thermally Dissociated Samples with respect to kinetics

4.6.2 Assay response on partial Enzymatic desialylation using Prozyme kit

It was clearly demonstrated that there are consistent and reproducible relationship between assay response and different types of sample that is equivalent or not inferior in comparison with *in-vivo* bioassay.

4.6.1 Assay response on Thermally Dissociated Samples with respect to kinetics

The method is intended for analyzing stability samples; hence it is important to demonstrate the stability indicating property of the assay. To assess this, a rHu-FSH sample was exposed to different stress condition to generate impurities and tested in the bioassay, along with appropriate physico-chemical analysis (Leo *et.al.*, 1975).

Dissociation of rHuFSH

Purpose of this experiment was to achieve 0%, 25%, 50%, 75%, and 100% dissociation. All the test samples were analyzed by non-reducing SDS-PAGE followed by silver staining. Test sample were stored at 5^{o}C.

Experimental Design/ Approach

Samples of FSH drug substance (0.25mg/ml) were kept at 55^{o}C for 1, 2, 3, 4 and 6 hrs. After 1, 2, 3, 4, and 6hrs samples were removed from the water bath and analyzed for specific activity using *in-vitro* bioassay.

Result

Specific activity IU/mg against % dissociation is shown in Table 4.21 and Figure 4.17.

Figure 4.18 shows SDS-PAGE profiles of samples treated at 55°C in incubator. Lane 1 has 20µg intact FSH and Lane 2 has 20µg of boiled FSH (with dissociated alpha and beta subunits).

Table 4.21: Specific Activity (Potency Ratio) of 55^{0} C treated samples.

Specific activity (IU/mg)	%dissociation observed	%dissociation expected
15804	0(assumed)	0
14061	11.03	10
11617	26.49	25
7154	54.73	50
4868	69.20	75
793	94.98	100

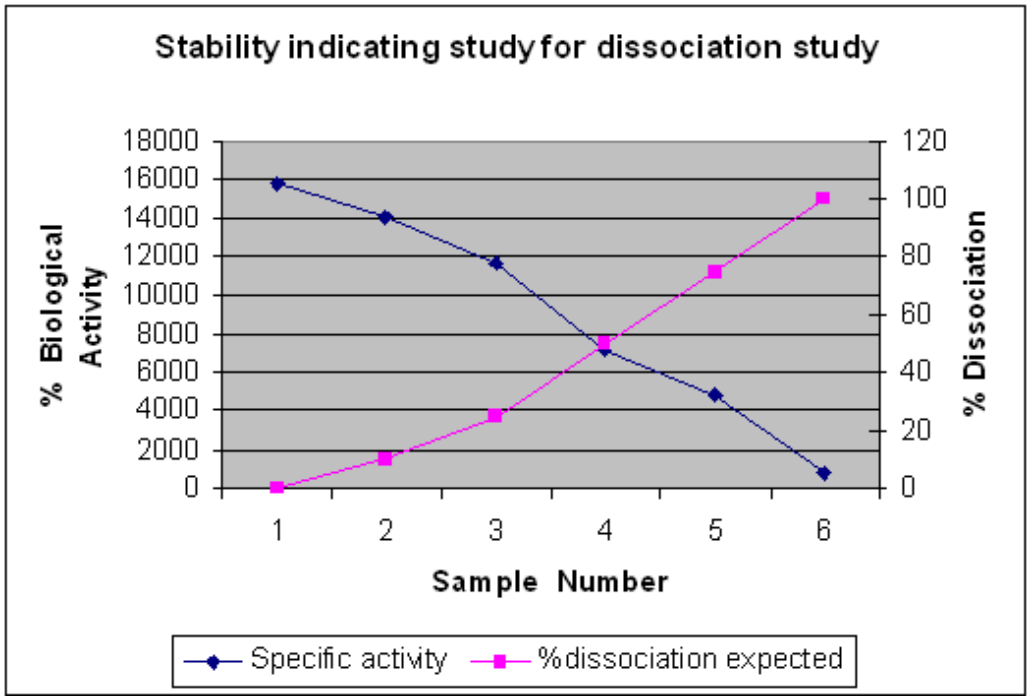


Figure 4.17: % Biological activity at different % dissociation.

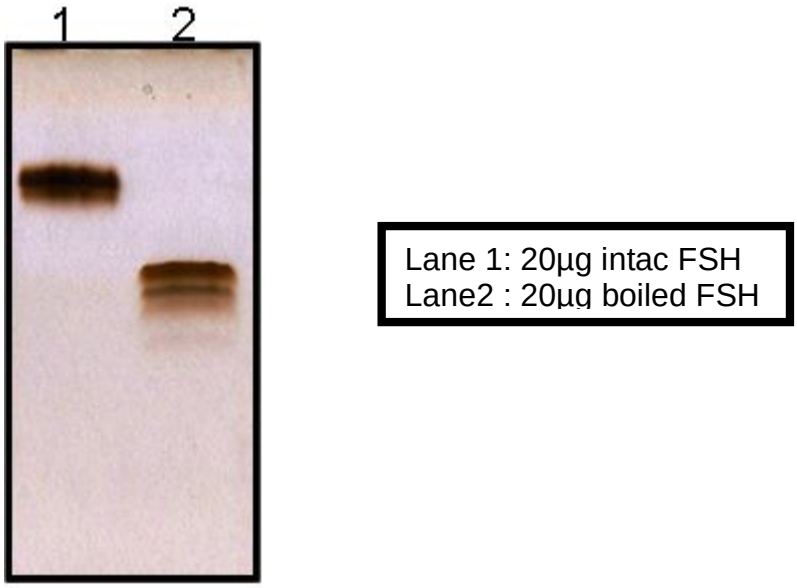


Figure 4.18: SDS-PAGE profile of samples treated at 55°C in water bath.

Discussion

It has been shown that as the percentage dissociation increases, the specific activity decreases and at 100% dissociation the protein almost fully lost its biological activity (from nearly 16000 IU/mg the activity dropped to 739 IU/mg), which indicated that the sample failed potency specification.

Conclusion

The result of dissociation indicates that the *in-vitro* method can capture the dissociation.

4.6.2 Assay response on partial enzymatic desialylation using Prozyme kit

4.6.2.1 Isoelectric Focusing of desialylated rHu-FSH samples

The aim of this experiment was to generate a range of increasingly desialylated rHu-FSH samples. These are then used for comparisons of results between *in-vivo* and *in-vitro* assays. Figure 4.16 shows the gradual increase in the basic isoforms of rHu-FSH which correlates with the length of incubation with sialidase. As rHu-FSH loses negatively charged sialic acids (i.e. becomes more positive) it migrates towards the negative electrode (cathode). Without enzymatic treatment rHu-FSH sample (at '0' hrs) had all the isoforms of normally sialylated FSH sample. The longer the samples were incubated with sialidase, the lesser acidic isoforms were generated through removal of sialic acid. Incubation for 48 hrs with sialidase resulted in a sample having the highest basic isoforms.

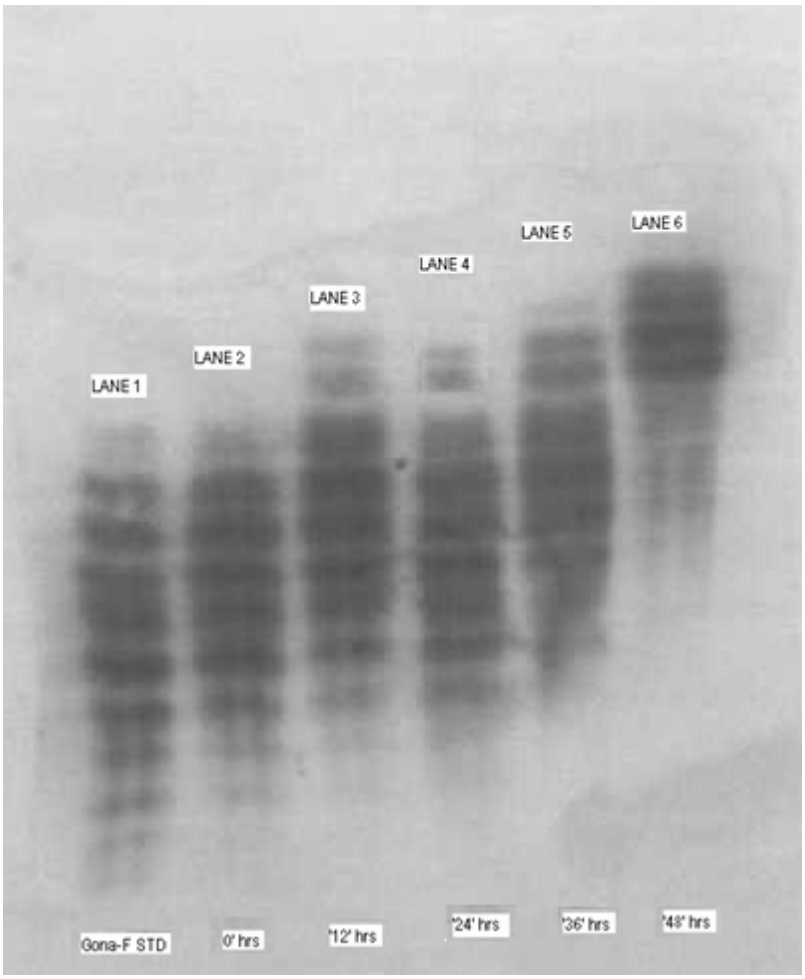


Figure 4.16: Isoelectric focusing gel with anti-FSH antibody of rHu-FSH treated with sialidase for increasing lengths of time. IEF analysis over the pH range 3 to 6 was performed on a thin-layer 6% polyacrylamide gel in the presence of urea and the protein was visualized by immunobloting. Lane 1 represents standard Gonal-*f* and Lane 2 to 6 represent the rHuFSH treated with sialidase for increasing lengths of time (t=0, t=12 hrs, t=24 hrs, t=36 hrs, t=48 hrs).

4.6.2.2 SEC-HPLC

Size Exclusion Chromatography (SEC) is a method which separates molecules based on their hydrodynamic volume. Molecules pass through the column and, depending on their sizes, elute through a stationary phase at different rate. SEC was used for quantifying amount of FSH.

The follicle stimulating hormone is a heterodimer molecule containing one alpha and one beta subunit. Herein, the objective of the study is to quantify total rHu-FSH present in the protein. The SEC method dose not resolves the alpha and beta subunits; rather they elute intact as along with aggregates and free subunits in single peaks. Table 4.22 represent the content determination of rHuFSH treated with sialidase for increasing lengths of time.

Table 4.22: Content determination of rHuFSH treated with sialidase by SEC-HPLC.

Time Sialidase treatment time (Hrs)	rHuFSH Conc.(mg/ml)
0	2.72
12	2.80
24	2.60
36	2.52
48	2.33

4.6.2.3 CHO-hFSHR-Luc *in-vitro* and Steelman–Pohley *in-vivo* bioassay

The *in-vitro* and *in-vivo* activities of the rHu-FSH treated with sialidase for increasing lengths of time in the CHO-hFSHR-Luc *in-vitro* bioassay and Steelman–Pohley *in-vivo* assay are shown in Figure 4.20. The gradual increase in the basic isoforms ('48' hrs sample) of rHu-FSH which correlating with the length of incubation in sialidase A. Sialic acid containing carbohydrate can affect receptor binding activity of rHu-FSH. As rHu-FSH losses negatively charged sialic acids (i.e. Becomes more positive) it shows higher Specific Activity in the CHO-hFSHR-Luc *in-vitro* bioassay (t=12 and t=48 hrs samples). Whereas the less acidic rHu-FSH were less potent in the Steelman-Pohley *in-vivo* assay (i.e. t=48 hrs compared to t=12 hrs) (Table 4.23 and Figure 4.20). These results indicate that sialic acid containing carbohydrates was required for maintenance of *in-vivo* activity and the *in-vivo* activity loss were proportional to the amount of sialic acid loss from the rHuFSH with increasing lengths

of time (table 4.23). Figure 4.21 shows the rat ovary weight in mg in the Steelman-Pohley *in-vivo* assay which was found to be significantly less while moving from '12' hrs to '48' hrs disialidase enzyme treated samples. This result confirms the direct relationship between sialic acid moles/mole of FSH, circulating half –life, and *in-vivo* biological activity, but an inverse relationship with receptor affinity *in-vitro*.

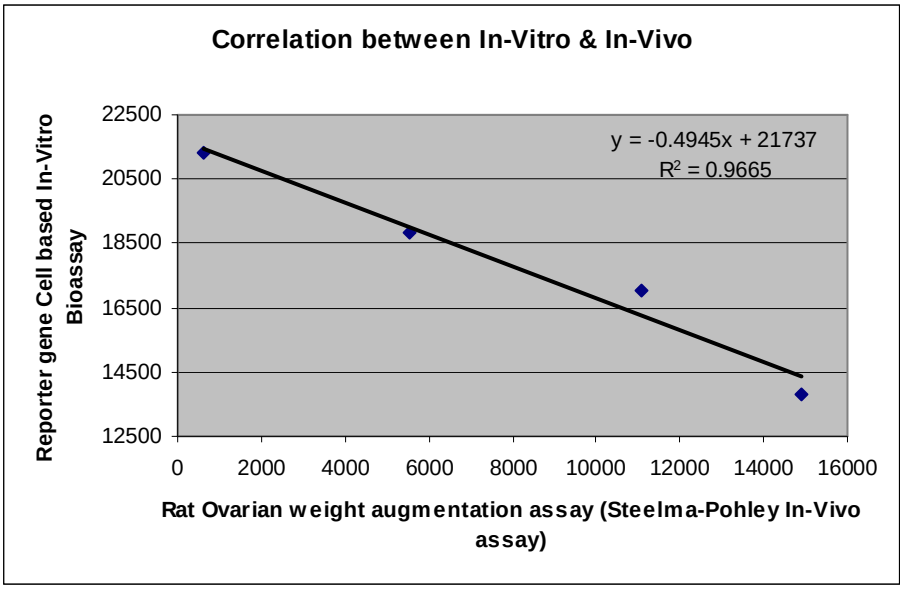


Figure 4.20: Specific Activity of sialidase enzyme treated samples with control in the CHO-hFSHR-Luc *in-vitro* bioassay and Steelman-Pohley *in-vivo* Bioassay. There is inverse relationship found between sialic acid content and receptor-binding activity. *in-vivo* activity decrease with the sialic acid content of the glycohormone, indicating the amount of sialic acid containing carbohydrate chain regulates clearance while the *in-vitro* bioactivity increase due to the highest affinity for the FSH Receptor (FSHR).

Table 4.23: Estimates of the moles of sialic acid /mole of FSH of the sialidase-treated rHuFSH samples and its correlation with Steelman–Pohley *in-vivo* assay and *in-vitro* bioassay.

Time in (Hours)	moles of sialic acid permole of FSH	*in-vivo* Potency (IU/mg)	*in-vitro* Specific Activity (IU/mg)
12	0.21	14889	13827
24	0.22	11103	17061
36	0.44	5561	18839
48	0.60	601	21320

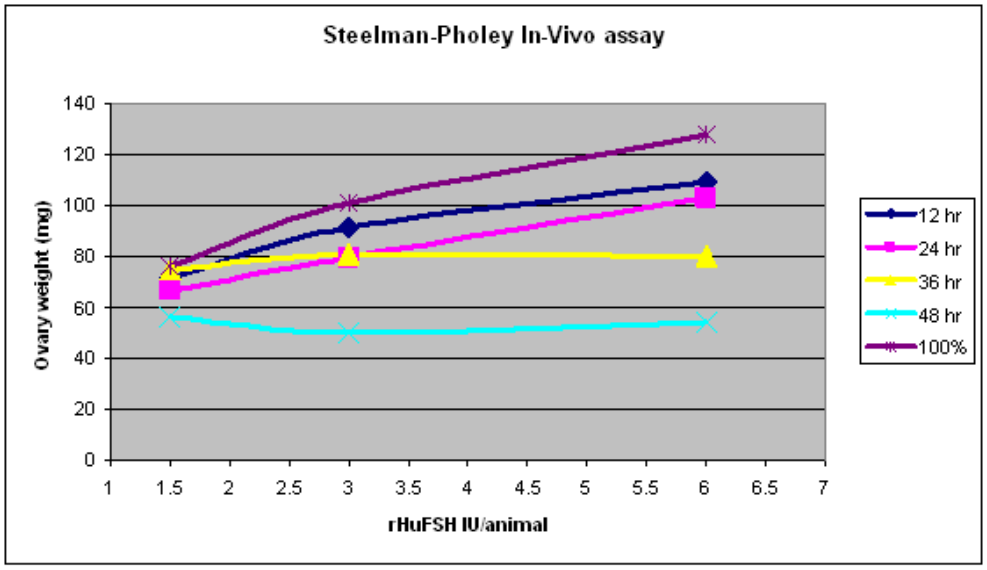


Figure 4.21: The Steelman-Pohley *in-vivo* (rat ovary weight gain) assay after administration of sialidase enzyme treated samples *in-vivo*. As samples lose it's negatively charged sialic acid its circulatory half life decreased leading to progressive decrease in the rat ovary weight.

4.6.2.4 Moles of sialic Acid/Moles of FSH by Dionex

Figure 4.22 represents the comparison of different chromatograms (Dionex) of rHu-FSH treated with sialidase for increasing lengths of time.

Figure 4.23 represents moles of sialic acid/mole of FSH in the sialidase treated samples based on the retention time of respective sialidase treated sample window (t=12, t=24, t=36 and t=48 hours). It shows that at the time of incubation with sialidase enzyme is increased the moles of sialic acid/moles of FSH are also increased. Overall we can say that there is a direct relationship between moles of sialic acid /mole of FSH and *in-vivo* activity. These results indicated that as increasing lengths of time with sialidase enzyme the sample released more sialic acid and became less acid thereby having less *in-vivo* activity and more receptor binding activity *in-vitro*.

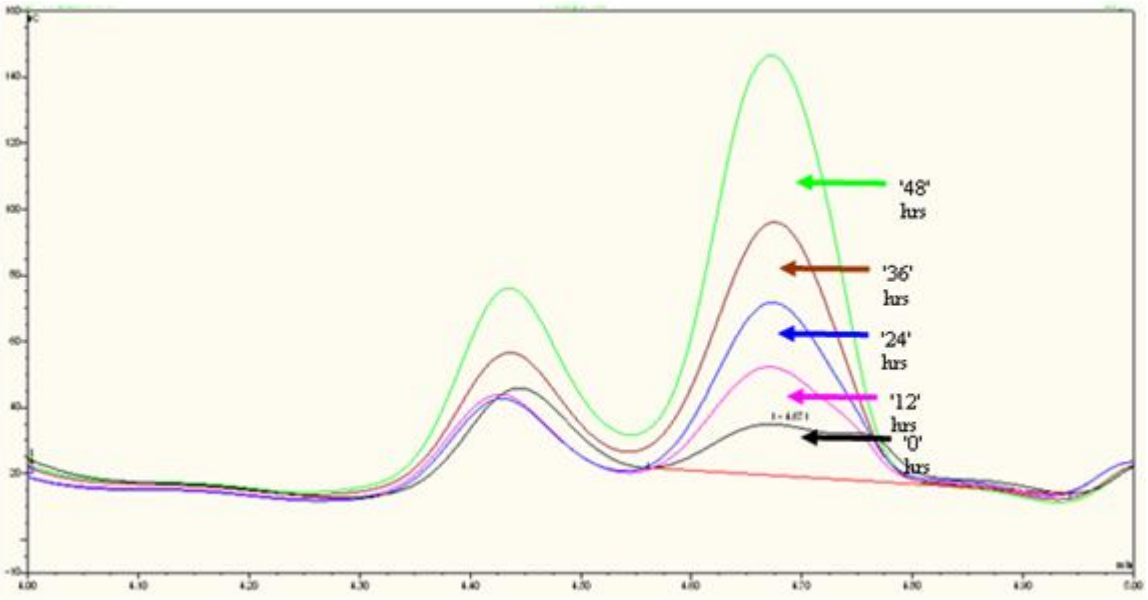


Figure 4.22: Shows a comparison of different chromatogram (Dionex) of rHu-FSH treated with sialidase for increasing lengths of time. Sialic acid moles/mole of FSH profile analysis of recombinant human follicle stimulating hormone (rHu-FSH). Sialic acid released from 2.18mg/ml rHu-FSH was analyzed by high performance anion exchange chromatography (HPAEC-PAD). Chromatograms of samples treated for t=0, t=12, t=36, t=48 hrs with sialidase are shown.

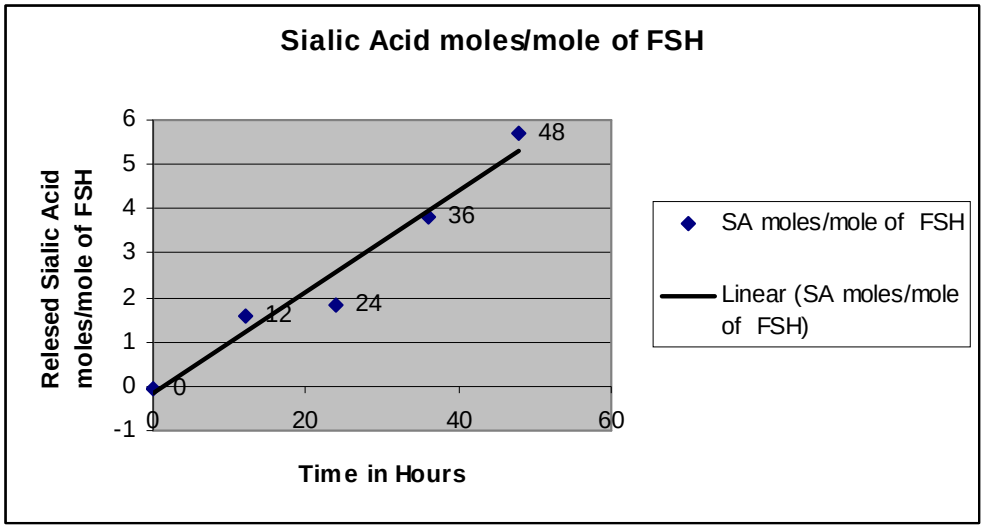


Figure 4.23: Correlation between moles of sialic acid/moles of FSH versus time of sialidase treatment. Longer the samples were incubated with sialidase, the higher the loss of sialic acid content.

Table 4.23 shows a negative correlation between *in-vitro* and *in-vivo* bioassays indicating that the loss of sialic acid from the glycan structure correlates with the loss of *in-vivo* biological activity. Steelman-Pohley assay showed decrease in the rat ovary weight gain after sialidase treatment in comparison to FSH which has intact glycan structure or more acidic isoforms. In contrast, the less acidic isoforms (i.e. t=48 hrs enzyme treated sample) retained full *in-vitro* receptor binding and showed higher specific activity as compared to the untreated samples. The apparent paradox, that samples with higher sialic acid /mole of FSH showed lower *in-vitro* receptor-binding activity but increase *in-vivo* activity, is explained by the counter acting effects of sialidase treatment on receptor binding and clearance from circulation. These results indicate that sialic acid containing molecules are required for maintenance of *in-vivo* activity and the *in-vivo* activity loss was proportional to the length of sialidase treatment. These results indicate that higher the amount of free sialic acid (beyond those found in t=0 hrs sample), could decrease the *in-vivo* activity and that there was a direct relationship between the number of sialic acid moles/mole of FSH and *in-vivo* activity. The number of sialic acid moles/mole FSH increased, receptor binding (*in-vitro*) bioactivity also increased in a linear-log relationship (Anatol *et.al.*,1971).

4.6.2.5 Method comparability exercise between existing *in-vivo* method and proposed *in-vitro* method

• Sample which is out of specification in potency (46 hrs) analyzed by *in-vivo* assay, due to improper isoform distribution pattern (desialylation of the molecule), showed comparable results in the IEF (Figure 20). When analyzed by *in-vitro* bioassay the batch showed enhanced activity beyond the upper specification limit (Figure 21) (due to inverse relationship between *in-vivo* and *in-vitro* bioassay).

• For lower potency sample (sample with 80 % potency), results with *in-vitro* method are similar (with respect to specific activity) to the results obtained by the *in-vivo* method. This shows that the low potency samples can be accurately estimated by the proposed *in-vitro* method.

• For higher potency sample (sample with 125% potency) results obtained by *in-vitro* method (see Table 21) are similar (with respect to specific activity) to the results obtained by *in-vivo* method. This shows that, like the lower potency sample, the higher potency sample also can be accurately estimated by the proposed *in-vitro* method.

• All drug substance and drug product samples, analyzed by the *in-vitro* bioassay method, resulted in similar potency value as obtained by *in-vivo* method. (Table 4.24) (Figure 4.24).

From the above conclusions it can be summarized that the proposed *in-vitro* bioassay method is comparable for potency estimation with *in-vivo* bioassay which is being carried out at Contract laboratories (Bioneeds). In addition, the *in-vitro* assay is reliable and results obtained are reproducible confirming the non-inferiority of this assay over *in-vivo* bioassay. Therefore, proposed *in-vitro* bioassay can be used to analyze routine lot release samples of DS and DP as well as stability samples.

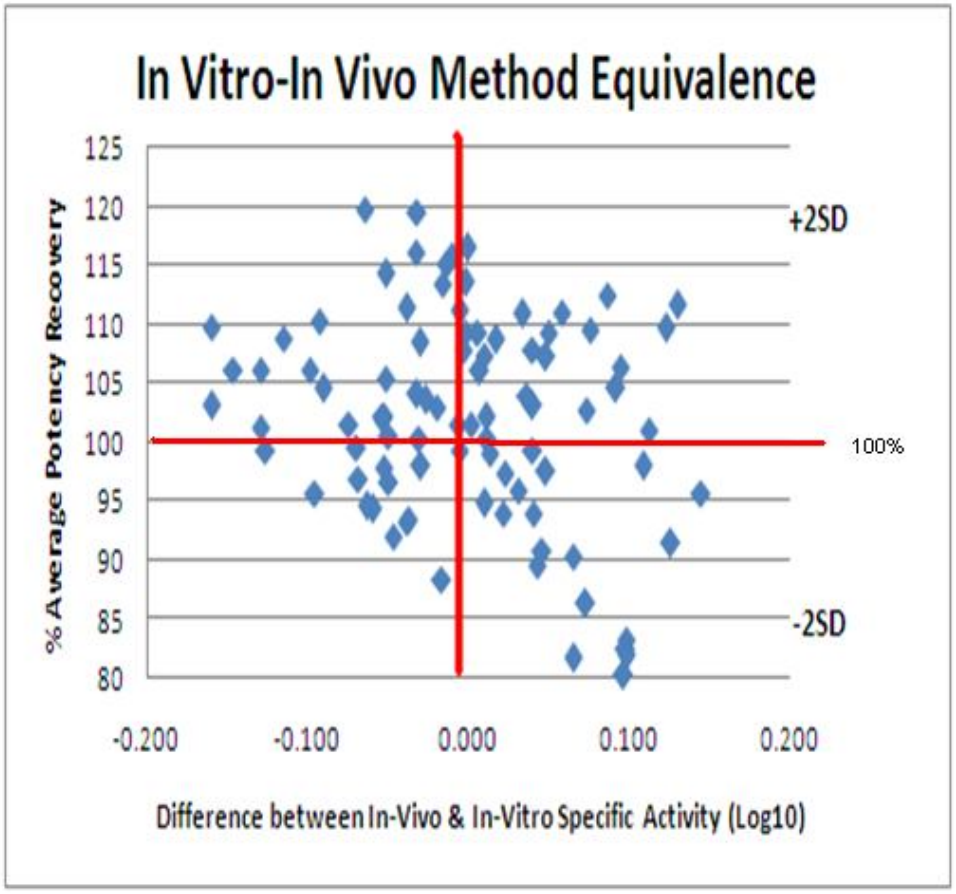


Figure 4.24: Residual plot of Difference between means of potency of the methods against average percentage potency recovery for each sample tested.

Table 4.24: Analysis of statistical validity for equivalence.

	Specific Activity (Log10) in-vivo Bioassay	*Specific Activity (Log10) in-vitro Bioassay*		
Mean	4.117	4.212	Mean D	-0.004
Sum of Squares of errors (SSE)	0.169	0.307	D+2SD	0.135
Number of assay (N)	89	89	D-2SD	-0.143
Degree of freedom (df)	176			
Lower acceptance limit	80.0			
Upper acceptance limit	125.0			
Lover 90% confidence Interval (CI) of DM	96.2			
Upper 90% confidence Interval (CI) of DM	102.1			
Mean	176			
Sum of Squares of Errors (SSE)	80.0			
EQUIVALENCE	EQUIVALENT			

5. Summary and Conclusions

5. Summary and Conclusions

The objective of this study was to show that the *in-vitro* bioassay of rHu-FSH is equivalent in terms of potency recovery to that of the *in-vivo* bioassay described in the *Pharm Europan* monograph. This was necessary to replace the *in-vivo* bioassay with the *in-vitro* alternative. Different samples of rHu-FSH DS (Drug Substance) and DP (Drug Product) were analyzed by both the methods and the estimated potencies were compared. The results were tested for statistical significance between the differences of mean potency values obtained from both the methods. This analysis established the validity of the *in-vitro* alternative method and showed how close the agreement was between the results obtained with the two methods. From the analysis it is concluded that both the methods are equivalent for potency recovery (with a precision of <11% of 90% Confidence Interval of Difference between Mean Potency Values) and the *in-vivo* assay can be replaced selectively with that of the *in-vitro* alternative.

Based on the excellent correlation between *in-vitro* and *in-vivo* bioactivity assay as shown in the present study, it is clear that *in-vitro* receptor-binding assay using CHO-hFSHR-Luc assay can substitute for animal based-ovarian weight gain assay. The former can therefore be used to determine the potency of the FSH for different manufacturing lots and therefore can be used for batch release of drug substance and drug product. The *in-vitro* assay is sensitive and high throughput, making it easier and reliable to study variations in the manufacturing process. The study confirms that both the methods are equivalent in terms of potency recovery and thus the *in-vitro* bioassay can be used wherever suitable. It is recommended that few commercial batches should be analyzed by both the methods as per the availability, for potency determination and statistical validity tests similar to the one described here be done. From the large sample size (n=39) it is apparent that the replacement of *in-vivo* by *in-vitro* is justified.

6. Appendices

6. Appendices

I. Reagent and media Preparation

6.1 Preparation of Media and Reagents for *in-vitro* Cell based assay

Note: Volumes are suggested to achieve the desired concentration in the following solutions. Other volumes may be prepared by proportionally increasing or decreasing the amount of each component.

Table 6.1 Growth Media for *in-vitro* cell based assay

Basal Media / Supplements	**For 1000ml**
MAM PF-2	975ml
L Glutamine (100X)	10ml
Plurronic F-68	10ml
Phenol Red Solution	5ml
Gentamicin	1ml

Mix all the above components aseptically in a sterile reagent bottle. Vaccum filter through 0.22µ sterile filters. Remove an aliquot in a sterile $25cm^2$ tissue culture flask and incubate in CO_2 incubator for sterility check. Store the growth media at 2-8 °C. Use within one month from the date of preparation.

Table 6.2 Assay Media *in-vitro* cell based assay

Basal Media / Supplements	**For 1000ml**
MAM PF-2	940ml
Dialyzed FBS	40ml
L Glutamine (100X)	10ml
Plurronic F-68	10ml
Gentamicin	1ml

Mix all the above components aseptically in a sterile reagent bottle. Vacuum filter through 0.22µ sterile filters. Remove an aliquot in a sterile 25cm^2 tissue culture flask and incubate in CO_2 incubator for sterility check. Store the assay media at 2-8 ℃. Use within one month from the date of preparation.

Table 6.3 Freezing Media for CHO cells

Basal Media / Supplements	**For 100ml**
GM	90ml
DMSO	10ml

PREPARE FRESHLY AND FILTER PRIOR TO USE.

Table 6.4 Preparation of Phosphate buffer solution

Chemical / Solvent	**For 1000ml**
NaCl	8.0 g
KH_2PO_4	0.2 g
KCl	0.2 g
Na_2HPO_4	1.15 g
Water for injection	800ml

Dissolve and adjust the pH to 7.2 ± 0.2 with 1M NaOH and then make up the volume up to 1000ml with water for Injection (WFI). Aliquot in working volume and autoclave. Store at room temperature and use within one month from the date of preparation.

Table 6.5 Preparation of Phenol red solution

Chemical / Solvent	**For 50ml**
Phenol Red	25.6mg
PBS	50ml

Dissolve and filter it through 0.22µ filter and store it at 2-8℃ Temperature.

6.1.1 Reagent Preparation from kit.

Thaw all reagents prior to use at RT before 1 hr.

LUCIFERASE ASSAY SUBSTRATE (LYOPHILIZED)

10ML LUCIFERASE ASSAY BUFFER

30ML REPORTER LYSIS 5X BUFFER

Mix substrate buffer to lyophilized powder immediately before use in dark.

6.2 Reagent Preparation for IEF

Table 6.6 Preparation of rehydration solution

Ingredient	Volume/Amount
Urea	0.4 µl
Pharmalyte 2.5-5for IEF	10 µl
Zoom Carrier Ampholytes 4-7	50 µl
WFI	933 µl
Dissolve urea in 933 µl WFI by vortexing and then add 10 µl of Pharmalyte 2.5-5 for IEF and 50 µl of ZOOM carrier Ampholytes 4-7. Store at room temperature (RT) until used. Use within 2 hours.	

Table 6.7 Preparation of Tris-buffered saline (TBS) Buffer (10X)

Ingredient	For 1 L stock solution
Tris-base	30g
NaCl	80g
KCL	2mg
Dissolve the ingredient in 800 ml of WFI. Adjust the pH to 7.4 with 1N HCL and make up the volume to 1000 ml. The solution can be stored at RT for 4 month.	

6.2.1 Tris-buffer saline (TBS) buffer (1X, 100 ML)

To 10 ml of 10X TBS, add 90ml WFI to make up the volume to 100ml. The solution can be stored at RT for 2 months.

Table 6.8 Preparation of Tris-glycine Buffer for electro-transfer

Ingredient	For 100 ml stock solution
Tris glycine buffer (10X)	10ml
Methanol	10ml
Make up the volume to 100 ml with WFI. Prepare reagent freshly every time. The solution is to be chilled at 0 º C to -20 º C for a least 1 hour before use.	

Table 6.9 Preparation of Tris-glycine Buffer (10X, 100ML)

Ingredient	For 100 ml stock solution
Glycine	14.4 g
Tris	3.03 g
Dissolve in 80 ml of warm WFI. Make up the volume to 100 ml. Store at RT. Use within 3 months.	

Table 6.10 Preparation of Blocking Buffer (100ML)

Ingredient	For 100 ml stock solution
Skimmed Milk Powder	5g
1X TBS	q.s. to 100ml
Make fresh each time.	

6.2.2 Primary antibody Solution (1:250)

Take 60µl of mouse anti human beta 2 (Serotec, Cat# MCA 338) and 60 µl of mouse anti human FSH Intact (Serotec, Cat # MCA 337) antibody from the tube provided by the manufacturer and mix with 15 ml of blocking buffer. Store at 4ºC. It can be reused 3 times. Do not freeze.

6.2.3 Secondary Antibody solution (1:250)

Take 60 µl of Goat anti-mouse ALP conjugated antibody and mix with 15ml of blocking buffer. After use, transfer to a clean container. Store 4ºC. It can be reused 3 times. Do not freeze.

6.2.4 Substrate Solution

BCIP/NBT is used as the substrate solution. It comes as a 'ready to use' solution, and can be used directly. Store it in a dark container at 2-8ºC. Avoid long exposure to light.

II. Definition

Assay-: Analysis (as of a drug) to determine the quantity of one or more components or the presence or absence of one or more components.

Assay data set-: The set of data used to determine a single potency or relative potency for all samples included in the bioassay.

Bioassay, biological assay (these terms are interchangeable)-:Aanalysis (as of a drug) to quantify the biological activity(ies) of one or more components by determining its capacity for producing an expected biological activity, expressed
in terms of units.

Potency-: The specific ability or capacity of the product, as indicated by appropriate laboratory tests or by adequately controlled clinical data obtained through the administration of the product in the manner intended, to effect a given result.

Relative potency-: A measure obtained from the comparison of a test to a reference drug substance on the basis of capacity to produce the expected biological activity.

Reportable value-: The potency or relative potency estimate of record that is intended to achieve such measurement accuracy and precision as are required for use.

System suitability-: The provision of assurance that the laboratory control procedure is capable of providing legitimate measurements as defined in the validation report.

Accuracy-: An expression of the closeness of agreement between the value that is accepted either as a conventional true value or an accepted reference value and the value found.

Intermediate precision-: Expresses within-laboratory precision associated with changes in operating conditions.

Precision-: The closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions.

Repeatability-: The expression of the precision under the same operating conditions over a short interval of time.

Reproducibility-: Expresses the precision between laboratories.

Specificity-: The ability to assess unequivocally the analyte in the presence of components that may be expected to be present.

Detection limit-: The lowest amount of analyte in a sample that can be detected but not necessarily quantified or quantified to any given level of precision and accuracy.

Linearity, bioassay-: The ability (within a given range) of a bioassay to obtain log relative potencies that are directly proportional to the log relative potency of the sample.

Quantitation limits-: The limits of true relative potencies between which the assay has suitable precision and accuracy.

Range-: The interval between the upper and lower relative potencies for which the bioassay is demonstrated to have a suitable level of precision, accuracy, and assay linearity.

Robustness-: A measure of an analytical procedure's capacity to remain unaffected by small but deliberate variations in method parameters.

Validation, assay-: A formal, archived demonstration of the analytical capacity of an assay that provides justification for use of the assay for an intended purpose and a range of acceptable potency values.

Blocking-: The grouping of related experimental units in experimental designs.

III. Annexure

Determination #	Sample Detail	Sp. Activity In Vivo IU/mg	Sp. Activity In Vitro IU/mg	Sp. Activity In Vivo Ln	Sp. Activity In Vitro Ln	D	Average Potency Recovery	% Average Potency Recovery
1	P7 CB D01-BULK	14164	14239	4.151	4.153	-0.002	14202	109
2	P7 CB D02-BULK	12486	14122	4.096	4.150	-0.053	13304	102
3	P7 CB D03-BULK	14883	15253	4.173	4.183	-0.011	15068	116
4	P7 CB D04-BULK	13077	13700	4.117	4.137	-0.020	13389	103
5	P7 CB D05-BULK	13964	15748	4.145	4.197	-0.052	14856	114
6	P7 CB D01 (NIF-II)	12224	11082	4.087	4.045	0.043	11653	90
7	P7 CB D02 (NIF-II)	12800	15862	4.107	4.200	-0.093	14331	110
8	P7 CB D03 (NIF-II)	13829	15134	4.141	4.180	-0.039	14481	111
9	P07F-C01 (DP)	11219	15131	4.050	4.180	-0.130	13175	101
10	P07F-C02 (DP)	11664	16867	4.067	4.227	-0.160	14265	110
11	P07F-C03 (DP)	14016	12793	4.147	4.107	0.040	13404	103
12	P07F-C04 (DP)	12319	13825	4.091	4.141	-0.050	13072	101
13	P7 CB D04 25°C 7D S	12776	11647	4.106	4.066	0.040	12212	94
14	P7 CB D04 25°C 15D S	12055	9615	4.081	3.983	0.098	10835	83
15	P7 CB D01 5°C 1M AS	13100	13307	4.117	4.124	-0.007	13204	102
16	P7 CB D02 5°C 1M AS	14085	12966	4.149	4.113	0.036	13526	104
17	P7 CB D03 5°C 1M AS	14527	15682	4.162	4.195	-0.033	15105	116
18	P7 CB D01 5°C 3M AS	12523	11922	4.098	4.076	0.021	12223	94
19	P7 CB D02 5°C 3M AS	12916	12031	4.111	4.080	0.031	12474	96
20	P7 CB D03 5°C 3M AS	11416	9835	4.058	3.993	0.065	10626	82
21	P7 CB D01 -80°C 3M RT	11759	15844	4.070	4.200	-0.129	13802	106
22	P7 CB D02 -80°C 3M RT	12425	14089	4.094	4.149	-0.055	13257	102
23	P7 CB D03 -80°C 3M RT	12307	13183	4.090	4.120	-0.030	12745	98
24	P07F C03 300IU 40°C 7D S	14347	14547	4.157	4.163	-0.006	14447	111
25	P07F C04 900IU 40°C 7D S	12055	14328	4.081	4.156	-0.075	13192	101
26	P7 CB D01 NIF II 25C 7D S	13028	14040	4.115	4.147	-0.032	13534	104
27	P07F-C05 (DP)	12489	14107	4.097	4.149	-0.053	13298	102
28	P07F-C06 (DP)	13216	12876	4.121	4.110	0.011	13046	100
29	P07F C01 300IU 25°C 6M AS	11597	13596	4.064	4.133	-0.069	12597	97
30	P07F C02 300IU 25°C 6M AS	12463	12200	4.096	4.086	0.009	12332	95
31	P07F C03 300IU 25°C 6M AS	11944	13503	4.077	4.130	-0.053	12724	98
32	P07F C04 300IU 25°C 6M AS	14708	13170	4.168	4.120	0.048	13939	107
33	P7-BM-CT-0001	14401	12331	4.158	4.091	0.067	13366	103
34	P7 CB D01 5°C 6M AS	13048	15742	4.116	4.197	-0.082	14395	111
35	P7 CB D02 5°C 6M AS	11047	14776	4.043	4.170	-0.126	12912	99
36	P7 CB D03 5°C 6M AS	14726	11469	4.168	4.060	0.109	13098	101
37	G07CT0001	11065	13215	4.044	4.121	-0.077	12140	93
38	P7-BM-CT-0001 (1MRT)	14657	13384	4.166	4.127	0.039	14021	108
39	G07CT0002	14113	13619	4.150	4.134	0.015	13866	107
40	G07CT0002	14113	13619	4.150	4.134	0.015	13866	107
Mean		**12911**	**13509**	**4.111**	**4.130**	**-0.020**	0.108	0
SSE		**49168694**	**101853495**	**0.055**	**0.113**	0.064	-0.148	0
N		**39**	**39**			0.128		
df		**76**						

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