A Brief Review of Follicle-Stimulating Hormone (FSH, Follitropin) and Shibayagi’s Rat FSH ELISA KIT

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What is FSH?

FSH is a glycoprotein hormone produced and stored in basophilic gonadotropin-producing cells (gonadotrophs) in the anterior pituitary together with LH, and is secreted by stimulation by a hypothalamic GnRH/LHRH. FSH is found in all the vertebrates, from fishes to mammals. FSH content in male rat pituitary (~5 μg/gland) is far more than in female (~1 μg/gland) (expressed in terms of NIDDK rat FSH RP-2 standard preparation). The content in female pituitary changes according to the stage of sexual cycle. In fishes, FSH-like gonadotropins called GTH1, and is produce in different cells which produce LH-like hormone, GTHII. It has been also reported that FSH is expressed in rat/mouse ovary and testis (1-3).

FSH is a hetero-dimer glycoprotein of 35kDa which is composed of α-subunit which is common to LH and TSH and β-subunit peculiar to FSH. The three-dimensional structure shows “cystine knot structure which is formed with 3 big hear-pin loops and 3 disulfide bonds in the central area. This structure is common to LH, TSH and CG (4, 5). The cystine knot structure is present also in PDGF, VEGF, TGF-β, and NGF, and such a group is called cystine knot growth factor (CKGF) super-family

Amino acid sequence of rat FSH

Each subunit of FSH has two oligosaccharide chains, and all of them bind to asparagine.

The precursor of subunits are shown below. The signal peptides are shown in red color.

α-subunit

mdcyrryaav ilvm1smvlh ilh1pddgl iiqgcpck1 kenkyfsklg apiyqcmgcc

β-subunit

mmksiqlcil lwclrvaccch sceltinis vekeercfci sin1ttwcgy cytrdlvkyk

Amino acid sequences(105~109,111~112) are thought to be receptor-binding area.
FSH shows microheterogeneity owing to its sugar chains, and some isomers/components having different isoelectric points are found (6).

**Physiological actions of FSH**

The targets of FSH are granulosa cells of ovarian follicles in females and seminiferous tubules of testis in males. It causes follicular growth and maturation, enhances production and secretion of estrogens, and enhances growth of seminiferous tubules and spermatogenesis. FSH receptor penetrates cell membrane 7 times and G-protein coupling-PKA type. Deficiency of FSH causes insufficient spermatogenesis and oocyte maturation, hypogonadism, obesity, decrease of estrogen secretion, and hair growth defect, while excessive FSH causes hyperplasia of secondary sexual organs, hyper ovarian follicle maturation, and hyper secretion of estrogens.

Diseases showing low FSH blood levels: hypogonadotropic eunuchoidism, primary central amenorrhea, Sheehan’s syndrome, Chiari-Frommel syndrome, anorexia nervosa, folliculoma, adrenogenital syndrome, craniopharyngioma, etc.

Diseases showing high FSH blood levels: azospermia, Kleinfelter’s syndrome, Turner’s syndrome, pubertus precox, ovarian hypoplasia, castration, menopause.

**FSH assays**

**Bioassay of FSH**

Mouse uterine weight method (7)
This method is based on weight increase of uterus due to estrogen-secreting action of FSH. FSH samples are injected to infant 21 days old mice twice a day for 4 days, and in the morning of day 5, and measure the weight of uterus in the evening.

Mouse or rat ovarian weight method (HCG augmentation method) (8)
Ovarian weight action of FSH is made by cooperation with LH. So, the effect is influenced by contaminant LH in the sample. In order to minimize the effect of such LH, action of FSH is made maximum by injection of FSH samples together with HCG which mimics LH action. To infant female rat or mouse (21~22days old), FSH samples mixed with HCG (total 20IU) are injected three times a day for 3 days, and measure the weight of ovaries at 72 hours after the first injection.

**Radio-receptor assay, RRA**

RRA shares common competitive assay principle with radioimmunoassay (RIA) using hormone receptor as a binding reagent instead of antibody.

As a large amount of FSH receptor is present in the testis and the ovary, these tissues are used as the practical source for the receptor. The author’s group established a radio-receptor
assay system by using ovarian homogenates of infant female rats that had been treated with PMS (pregnant mare serum gonadotropin with FSH action) to have many maturated follicles (9). We found that addition of merthiolate to the assay system improved FSH-receptor binding, and this made FSH in the serum samples possible (10). We also found that the ratio of RRA/RIA assay values changed according to physiological state in rat and human (11,12).

**Immonoassays (RIA, ELISA)**

National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) supplies FSH RIA kits for various animals. In using those kits, researchers have to make radioiodination of the purified hormone by themselves. Commercial immunoassay kits are available for human FSH. Shibayagi Co. Ltd. has started to supply ELISA kit for rat FSH that is introduced in the last part of this review.

**International standard preparations of FSH and its unit**

**Human FSH international standard preparations**

- WHO National Institute for Biological Standards and Control (NIBSC) supplies international standards for human FSH.

- The 1st IRP-HMG (HMG-24) was prepared from human menopausal urine and issued as a biological standard for FSH and LH, however, the purity is not enough. In 1964, 2nd IRP-HMG (Pergonal 23) was supplied. It contained 9mg HMG (human menopausal gonadotropin, and the biological potencies were defined as 20IU FSH and 40IU LH per vial. This preparation was essentially intended for biological assay. But when RIA for human gonadotropins were established in commercial base, 2nd IRP-HMG was used as standard preparations for FSH and LH. Because this preparation derived from human menopausal urine, its biological activity/immunological activity ratio was different from pituitary gonadotropin preparations. So, when assay values obtained by using 2nd IRP-HMG were compared with those obtained with LER 907 (human pituitary origin distributed by NIH), the former gave FSH IU value twice as much as those of the latter. In the case of LH, the values were 5 times.

  - Now, following standard preparations for human FSH are distributed.
  - Human FSH, pituitary origin: 1st International Standard 1986 (83/575)
  - α-Subunit, pituitary origin: 1st International Standard 1984 (78/554)
  - Human FSH for immunoassay, recombinant: 1st International Standard (92/510)

**Standard preparations for animal FSH**

NIH-FSH S1 (“S” means sheep) was first distributed as an standard preparation for biological
studies, and 1mg of the preparation was defined as 1 NIH Unit. But the problem was in its purity, 0.67% of the highly purified preparation used for radioiodination in RIA. So, it is not recommended to express the assay value by the weight of NIH FSH S1, or NIH Unit. It would give terribly high values. It would be better to express the results in terms of immunoassay standard preparations stated below.

**Standard immunoassay preparations for animal FSH**

NIDDK reference preparations of FSH are supplied which are included in various NIDDK FSH assay kits. For rat FSH, NIDDK rat FSH RP-1 was first supplied in early period of distribution, however the purity was very low, as much 2 NIH Unit/mg. Many reports published in 1970s to 1980s expressed the assay results in terms of the weight of RP-1, so we should be careful in interpretation of the assay values. Later, RP-2 and RP-3 were supplied, and they were prepared from highly purified preparation, I-series for radioiodination, by adding protective agent and buffer components, and we can prepare standard solution containing a fixed concentration of pure FSH by adding a fixed volume of water. The biological potency of RP-3 (or I-8) is 100xNIH-1FSH S1 or 4714IU -2ndIRP/mg by HCG augmentation method (Ref. Instruction Sheet of NIDDK rat LH RIA kit, 19-Jan-95, by A. F. Parlow).

**Regulation of FSH production and secretion**

GnRH/LHRH from the hypothalamus is the well-known direct regulating factor of FSH. GnRH causes the release of gonadotropin granules as well as increases transcription of FSH $\beta$-subunit as the results of serial reactions after receptor binding (13). Another regulation factor is activin. Activin is also thought to enhance transcription of FSH $\beta$-subunit (14-16) FSH secretion are increased in proestrous and follicular growth period, at low blood sexual steroid levels, and throughout climacterium and post menopausal period. Inhibitory factors on FSH section are inhibin and follistatin. Inhibin lowers FSH production, while follistatin binds activin to inhibit its action (17, 18). PACAP (pituitary adenylatecyclase activating peptide), an indirect inhibitory factor, also inhibits FSH production by increasing follistatin production (19, 20).

Sexual steroid hormones also indirectly minimize FSH release by acting hypothalamus to inhibit GnRH release, and possibly act on FSH gene directly. Physiologically, FSH blood levels are low in infant, pregnancy, and confinement.

**Blood levels of FSH**

*Human (Express as WHO 2nd IRP-hPG)*

- Normal adult male: $\sim$5mIU/ml
- Normal adult female
Ovulatory phase: ~9mIU/ml, Follicular phase: ~9mIU/ml, Luteal phase: ~4mIU/ml
Menopausal period: ~55mIU/ml

Following data were obtained by RIA in the author’s laboratory.

Relationship between serum LH levels and age in normal human males (21)

<table>
<thead>
<tr>
<th>Age</th>
<th>Number</th>
<th>Serum LH ng x LER907/ml</th>
<th>Serum FSH ng x LER907/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SE</td>
<td>Mean</td>
</tr>
<tr>
<td>0–5</td>
<td>6.3</td>
<td>1.2</td>
<td>14.6</td>
</tr>
<tr>
<td>6–10</td>
<td>9.0</td>
<td>1.4</td>
<td>28.9</td>
</tr>
<tr>
<td>11–15</td>
<td>12.9</td>
<td>1.8</td>
<td>84.5</td>
</tr>
<tr>
<td>16–20</td>
<td>16.0</td>
<td>1.8</td>
<td>71.2</td>
</tr>
<tr>
<td>21–30</td>
<td>23.9</td>
<td>1.8</td>
<td>80.6</td>
</tr>
<tr>
<td>31–40</td>
<td>21.4</td>
<td>2.3</td>
<td>102.2</td>
</tr>
<tr>
<td>41–50</td>
<td>32.5</td>
<td>4.7</td>
<td>156.3</td>
</tr>
<tr>
<td>51–60</td>
<td>57.4</td>
<td>5.5</td>
<td>242.3</td>
</tr>
<tr>
<td>61–70</td>
<td>69.87</td>
<td>11.1</td>
<td>368.5</td>
</tr>
<tr>
<td>71–</td>
<td>60.9</td>
<td>6.0</td>
<td>297.9</td>
</tr>
<tr>
<td>16–40</td>
<td>63</td>
<td>22.3</td>
<td>89.5</td>
</tr>
<tr>
<td>51–</td>
<td>58</td>
<td>63.4</td>
<td>336.1</td>
</tr>
<tr>
<td>Aged women</td>
<td>30</td>
<td>163.0</td>
<td>1,138.5</td>
</tr>
</tbody>
</table>

Results are expressed in terms of LER 907 (human pituitary origin) which was included in the RIA for human gonadotropin distributed by NIDDK. Its hormone contents are FSH 20IU/mg and LH 48IU/mg (IU: 2nd IRP HMG).

Relationship between human FSH and spermatogenetic dysfunction

Serum FSH levels in Kleinfelter’s syndrome and Oligo-Azo-spermia patients (22)

<table>
<thead>
<tr>
<th></th>
<th>No.</th>
<th>Mean ng x LER 907/ml</th>
<th>SE ng x LER 907/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal (17-39 yr)</td>
<td>11</td>
<td>104.1</td>
<td>11.6</td>
</tr>
<tr>
<td>Normal (47-75 yr)</td>
<td>6</td>
<td>379.2</td>
<td>121.2</td>
</tr>
<tr>
<td>Oligospermia</td>
<td>7</td>
<td>145.3</td>
<td>22.5</td>
</tr>
<tr>
<td>Azospermia</td>
<td>7</td>
<td>354.1</td>
<td>82.6</td>
</tr>
<tr>
<td>Chromatin positive</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kleinfelter’s syndrome</td>
<td>18</td>
<td>881.6</td>
<td>91.8</td>
</tr>
<tr>
<td>Hypogonadotropic eunuchoidism</td>
<td>21</td>
<td>43.6</td>
<td>8.0</td>
</tr>
</tbody>
</table>

Serum FSH and injured spermatogenesis caused by accidental $^{192}$Ir irradiation (23)

In 1971, an accidental irradiation happened in a shipyard in Chiba city, Japan.
192Ir has been used for examination of welding. The 192Ir case dropped off from the unlocked lead container, and a workman, by chance, has picked up it, slipped it into his hip pocket, and went home. After searching, the case was found and recovered, however, the workman received gamma ray irradiation of 175rad to his testes. He was accommodated in the hospital attached to National Institute of Radiological Sciences where the author had been working. He was examined in detail by a specially organized team, and the author measured his serum gonadotropins. About 100 days after irradiation, we found the only FSH started to increase, and that it went up to more than 2SD over the average of normal male levels. Around this period, sperm count went down to 1/10 and complete disappearance. The irradiation influenced spermatogenesis but not androgen production, and resulted in enhancement of FSH secretion without changing LH level. The FSH level stayed high more than 400 days (23).

Physically estimated gonadal dose was 175rad.

**Serum levels and pituitary contents in proestrus and estrus in female rats (6)**

In female rats, a LH surge is observed in the evening of proestrous day. FSH also starts to increase in the same time. LH level comes back low within several hours, however, FSH level stays high until midnight then shows a second upraising. As the graph shows the mean of 20 rats, the second peak is not clear. FSH content of the pituitary gland decreases coinciding with the first peak, then it is switched to increase, and returns almost to the initial level until the morning of diestrus I. The second peak of the blood level may be due to activin.

Animals: Female rats of 8 weeks old Wistar-Imamichi strain

Mean of 20 rats and SE
Changes in serum FSH levels in male rats after orchidectomy (unpublished data)

Animals: male rats of 8 weeks old Wistar strain, assayed by RIA
Mean of each 5 rats and standard error
After castration, serum FSH increases to nearly twice in the next day, then it gradually decreases to the bottom until 1 week, and goes up again with a very small slope, but never reaches the highest level (that of the next day) even 6 weeks later.

FSH content of the pituitary decreases until 5 days to about half of the initial level due to enhancement of secretion, then turns to increase to the level 1.5 times more than initial content. Compared with LH content, increase in FSH content is less.

**Influence of stress and anesthesia on blood FSH levels**

**Immobilization stress**

○ Eight hours’ immobilization for 10 days lowered blood FSH only slightly but not significantly, but pituitary content was increased. In such a situation, response of FSH secretion to GnRH+TRH injection was enhanced probably due to the increased content (24).

**Starvation**

○ Fasting lowered blood FSH (25).

**Ether anesthesia**

○ Ether inhalation in ovariectomized rat increased plasma FSH level within 2 minutes. In this case, plasma levels of prolactin and LH were also increased. The order of increase was prolactin>LH>FSH (26).

○ Rats were taken out from an ether container when they became unconscious, and decapitated when partially returned conscious. Blood FSH increased significantly (27).
Shibayagi’s [Rat FSH ELISA Kit]
(Code No.: AKRFS-010)

Intended use

Rat FAH ELISA kit is a sandwich ELISA system for quantitative measurement of rat FSH (Follicle Stimulating Hormone), and is intended for research use only.

Features

(1) Highly sensitive assay with the standard range of 0.4~20 ng/ml.
(2) This kit is for FSH in rat serum or plasma (use EDTA-2Na for anticoagulant at the final concentration of 1 mg/ml).
(3) Assay sample volume is 20µl in the standard procedure.
(4) Assay format is 96 wells.
(5) Standard preparation is rat origin.
(6) Components of the kit are provided ready to use or in concentrated form.

Storage and expiration

When the complete kit is stored at 2-8°C, the kit is stable until the expiration date shown on the label on the box.

Opened reagents should be used as soon as possible to avoid less than optimal assay performance caused by storage environment.

Assay principle

In Shibayagi’s Rat FSH ELISA Kit, biotin-conjugated anti-FSH and standard or sample are incubated in monoclonal anti-FSH antibody-coated wells. After 15~18 hours incubation and washing, HRP (horse radish peroxidase)-conjugated avidin is added, and incubated for 30 minutes. After washing, HRP-complex remaining in wells are reacted with a chromogenic substrate (TMB) for 30 minutes, and reaction is stopped by addition of acidic solution, and absorbance of yellow product is measured spectrophotometrically at 450 nm (sub-wavelength is 620nm). The absorbance is nearly proportional to FSH concentration. The standard curve is prepared by plotting absorbance against standard FSH concentrations. FSH concentrations in unknown samples are determined using this standard curve.

Reagents supplied

<table>
<thead>
<tr>
<th>Components</th>
<th>State</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>(A) Anti-FSH-coated plate</td>
<td>Use after washing</td>
<td>96 wells/1 plate</td>
</tr>
<tr>
<td>(B) Standard rat FSH (200 ng/ml)</td>
<td>Concentrated. Use after dilution</td>
<td>200 µl/1 vial</td>
</tr>
<tr>
<td>(C) Buffer solution</td>
<td>Ready for use.</td>
<td>60 ml/1 bottle</td>
</tr>
<tr>
<td>(D) Biotin-conjugated anti-FSH</td>
<td>Concentrated. Use after dilution</td>
<td>100 µl/1 vial</td>
</tr>
<tr>
<td>(E) HRP-conjugated avidin</td>
<td>Concentrated. Use after dilution</td>
<td>200 µl/1 vial</td>
</tr>
<tr>
<td>(F) Chromogenic substrate reagent (TMB)</td>
<td>Ready for use.</td>
<td>12 ml/1 bottle</td>
</tr>
<tr>
<td>(H) Reaction stopper (1M H₂SO₄)</td>
<td>Ready for use.</td>
<td>12 ml/1 bottle</td>
</tr>
<tr>
<td>(I) Concentrated washing buffer (10x)</td>
<td>Concentrated. Use after dilution</td>
<td>100 ml/1 bottle</td>
</tr>
</tbody>
</table>

Plate cover: Use after dilution.

Biotin-conjugated anti-FSH (D), and HRP-conjugated avidin (E): Vials contain more than volumes shown in the list so that you can take out 50 µl from vials.

Preparation of reagents

❖ Bring all reagents of the kit to room temperature before use.
❖ Prepare reagent solutions in appropriate volume for your assay. Do not store the diluted reagents.
❖ Do not use the reagent after expiration date.
**Reagents ready for use after return to room temperature**

[Anti-FSH-coated plate]

Storage and stability
If seal is not removed, put the strip back in a plastic bag with zip-seal originally used for well-plate container and store at 2-8 °C. The strip will be stable until expiration date.

[Buffer solution] and [Chromogenic substrate reagent]

Storage and stability
If not opened, store at 2-8 °C. It maintains stability until expiration date. Once opened, we recommend using them as soon as possible to avoid influence by environmental condition.

[Reaction stopper (1 M H$_2$SO$_4$)]

Storage and stability
Close the stopper tightly and store at 2-8 °C. It maintains stability until expiration date.

**Concentrated reagents**

[Concentrated washing buffer (10x)]

Dilute 1 volume of the concentrated washing buffer (10x) to 10 volume with purified (distilled) water to prepare working solution. Example: 100 ml of concentrated washing buffer (10x) and 900 ml of purified (distilled) water.

Storage and stability
The rest of undiluted buffer: if stored tightly closed at 2-8 °C, it is stable until expiration date.
Dispose any unused diluted buffer.

**Master Standard rat FSH (200 ng/ml)**

Make a serial dilution of master standard solution to prepare each standard solution.

An example of preparation.

<table>
<thead>
<tr>
<th>Volume of standard solution</th>
<th>Buffer</th>
<th>Concentration (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Master solution 50μl</strong></td>
<td>450 μl</td>
<td>20</td>
</tr>
<tr>
<td>20 ng/ml solution 200 μl</td>
<td>200 μl</td>
<td>10</td>
</tr>
<tr>
<td>10 ng/ml solution 200 μl</td>
<td>200 μl</td>
<td>5.0</td>
</tr>
<tr>
<td>5.0 ng/ml solution 200 μl</td>
<td>200 μl</td>
<td>2.5</td>
</tr>
<tr>
<td>2.5 ng/ml solution 200 μl</td>
<td>300 μl</td>
<td>1.0</td>
</tr>
<tr>
<td>1.0 ng/ml solution 200 μl</td>
<td>300 μl</td>
<td>0.4</td>
</tr>
<tr>
<td>0 (Blank)</td>
<td>200 μl</td>
<td>0</td>
</tr>
</tbody>
</table>

**Preparation of assay samples**

This kit is principally intended to measure FSH in rat serum or plasma.

○ For preparation of plasma, we recommend to use EDTA-2Na at final concentration of 1 mg/ml.

○ Deep ether anesthesia at blood sampling may lower blood FSH level.

○ High hemolysis (more than 120 mg/dl hemoglobin) may influence assay results.

○ If sample is turbid or contains insoluble materials, centrifuge and use clear supernatant fluid.

○ Organic solvents may influence assay results.

○ Assay samples soon after preparation.

○ Sample dilution should be carried out with the buffer solution of the kit using small test tubes before assay.

  Mix well, and pipette 50 μl of diluted sample into a well. In the standard assay procedure, the dilution rate is 2.5x. Undiluted (neat) serum and plasma are not suitable because their impurities may influence assay results.

○ Frozen stored samples should be thawed just before assay and mixed well to make them homogenous.

○ If the presence of any interfering substances is suspected, confirm dilution linearity using more 2 different dilutions or more.

**Stability and storage of samples**

In immediate assay, samples can be kept in a refrigerator, and brought to room temperature just before assay. If they have to kept for a long period, tightly close the container and store lower than -35 °C.

Avoid repeated freezing and thawing.
### Summary of assay procedure [Standard procedure]

<table>
<thead>
<tr>
<th>Day 1</th>
<th>Anti-FSH-coated plate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>↓ Washing 4 times</td>
</tr>
<tr>
<td></td>
<td><strong>Biotin-conjugated anti-FSH</strong> 50μl</td>
</tr>
<tr>
<td></td>
<td>↓ Shaking</td>
</tr>
<tr>
<td></td>
<td><strong>Standard or diluted sample solution</strong> 50μl</td>
</tr>
<tr>
<td></td>
<td>↓ Shaking, Reaction at 2<del>8°C, 15</del>18hours (Standing)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Day 2</th>
<th>Dilution of HRP-conjugated avidin with buffer of room temperature to 200x</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>↓ Washing 4 times</td>
</tr>
<tr>
<td></td>
<td><strong>HRP-conjugated avidin</strong> 100μl</td>
</tr>
<tr>
<td></td>
<td>↓ Shaking, reaction at room temp. 30min (standing)</td>
</tr>
<tr>
<td></td>
<td>↓ Washing 4 times</td>
</tr>
<tr>
<td></td>
<td><strong>Chromogenic substrate reagent (TMB)</strong> 100μl</td>
</tr>
<tr>
<td></td>
<td>↓ Shaking, reaction at room temp. 20min (standing)</td>
</tr>
<tr>
<td></td>
<td>**Reaction stopper (1 M H₂SO₄) **Careful 100μl</td>
</tr>
<tr>
<td></td>
<td>↓ Shaking</td>
</tr>
<tr>
<td></td>
<td><strong>Measurement of absorbance (450nm, sub 620nm)</strong></td>
</tr>
</tbody>
</table>

*Room temp : 20~25°C*

**FSH assay standard curve (an example above)**
Absorbance may change due to assay environment.
**Assay performance characteristics**

**Specificity**

Monoclonal antibodies specific to FSH are used in this kit. Reactivity for closely related substances to FSH is shown below.

<table>
<thead>
<tr>
<th>Substance</th>
<th>Cross reactivity</th>
<th>Substance</th>
<th>Cross reactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat FSH</td>
<td>100%</td>
<td>Rat TSH</td>
<td>No cross reaction</td>
</tr>
<tr>
<td>Rat LH</td>
<td>No cross reaction</td>
<td>Rat GH</td>
<td>No cross reaction</td>
</tr>
</tbody>
</table>

**Repeatability, Precision**

**Intra-assay variation**

<table>
<thead>
<tr>
<th>Well</th>
<th>Sample. A</th>
<th>Sample B</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>12.5</td>
<td>2.73</td>
</tr>
<tr>
<td>2</td>
<td>12.3</td>
<td>2.82</td>
</tr>
<tr>
<td>3</td>
<td>12.6</td>
<td>2.64</td>
</tr>
<tr>
<td>4</td>
<td>12.8</td>
<td>2.69</td>
</tr>
<tr>
<td>5</td>
<td>12.7</td>
<td>2.81</td>
</tr>
<tr>
<td>mean.</td>
<td>12.6</td>
<td>2.74</td>
</tr>
<tr>
<td>SD</td>
<td>0.19</td>
<td>0.08</td>
</tr>
<tr>
<td>CV(%)</td>
<td>1.5</td>
<td>2.8</td>
</tr>
</tbody>
</table>

**Intermediate precision**

(Inter-assay variation)

<table>
<thead>
<tr>
<th>Sample.</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
<th>Day 4</th>
<th>mean.</th>
<th>SD</th>
<th>CV(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>5.02</td>
<td>5.04</td>
<td>5.07</td>
<td>5.03</td>
<td>5.04</td>
<td>0.02</td>
<td>0.41</td>
</tr>
<tr>
<td>D</td>
<td>1.18</td>
<td>1.19</td>
<td>1.17</td>
<td>1.18</td>
<td>1.18</td>
<td>0.01</td>
<td>0.99</td>
</tr>
<tr>
<td>E</td>
<td>0.615</td>
<td>0.592</td>
<td>0.588</td>
<td>0.618</td>
<td>0.603</td>
<td>0.02</td>
<td>2.56</td>
</tr>
</tbody>
</table>

**Spike recovery test**

<table>
<thead>
<tr>
<th>Sample H</th>
<th>Added</th>
<th>Found</th>
<th>Recovered</th>
<th>Recovery(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.000</td>
<td>2.58</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>1.60</td>
<td>4.09</td>
<td>1.51</td>
<td>94.4</td>
</tr>
<tr>
<td></td>
<td>2.94</td>
<td>5.57</td>
<td>2.99</td>
<td>102</td>
</tr>
<tr>
<td></td>
<td>5.01</td>
<td>7.45</td>
<td>4.87</td>
<td>97.2</td>
</tr>
</tbody>
</table>

Sample I

<table>
<thead>
<tr>
<th>Added</th>
<th>Found</th>
<th>Recovered</th>
<th>Recovery(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.00</td>
<td>4.48</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>6.02</td>
<td>10.3</td>
<td>5.82</td>
<td>96.7</td>
</tr>
<tr>
<td>7.22</td>
<td>11.7</td>
<td>7.22</td>
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</tr>
<tr>
<td>8.32</td>
<td>12.4</td>
<td>7.92</td>
<td>95.2</td>
</tr>
</tbody>
</table>
Dilution linearity test

![Graph showing linearity test results](image)

$R^2 = 0.9994$

$R^2 = 0.9986$

N=3

Rat reference assay data

Serum FSH level in male rats
Thirty rats: Crl:CD(SD), males, 8 weeks of age, fed ad libitum, bled: 14:00-18:00
Plasma samples obtained using EDTA
Assay results: mean 3.10 ng/ml, SD: 1.7 ng/ml (triple assays)

Serum levels of FSH through proestrus to diestrus

Wistar-Imamichi female rats of 8 weeks of age. Eight rats for each group.

![Graph showing rat FSH levels](image)
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