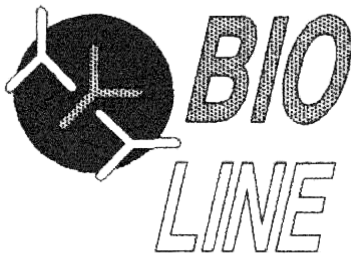


Read entire protocol before use.

FSH-Elisa

Bio-Line S.A. - Rue André Fauchille.17 - B-1150 Bruxelles - Belgium

I. INTENDED USE

Immunoenzymetric assay for the *in vitro* quantitative measurement of human Follicle Stimulating Hormone (FSH) in serum and plasma.

II. GENERAL INFORMATION

A. Name: Bio-Line **FSH-Elisa** Kit
B. Catalogue number: **BL-22-E:** 96 tests
C. Manufactured by: Bio-Line S.A.
Rue André Fauchille.17 - B-1150 Bruxelles - Belgium
For technical assistance or ordering information contact:
Tel: +32-2-736.62.18. Fax: +32-2-742.13.15.

III. CLINICAL BACKGROUND

A. Biological Activity

Human Follicle Stimulating Hormone (FSH) is a glycoprotein (M.W. approximately 30000d) comprising two associated subunits alpha and beta, both containing peptide and carbohydrate molecules. The alpha-subunit is identical for all gonadotropins and for TSH whereas the β-subunit determines the immunological and biological properties of each hormone.
FSH is secreted by the basophilic cells of the anterior pituitary under the control of gonadotropin releasing hormone (GnRH) produced in the hypothalamus.
In the female during menstrual cycle, FSH is responsible for the proliferation of the follicular cell, for the development of the graafian follicle and for ovum maturation. Estradiol, produced by the follicle, has a negative feedback on FSH secretion. In postmenopausal women, FSH and LH are elevated due to the absence of negative feedback.
In the male, FSH is required along with LH and Testosterone for maintaining spermatogenesis in the seminiferous tubules. The Leydig cells of the testes secrete testosterone and Estradiol. Circulating levels of FSH are maintained by Testosterone, which appears to control the response of the pituitary to the GnRH.
Further, it has been reported that seminiferous tubules secrete a specific peptide named inhibin, which specifically regulates FSH secretion from the pituitary.

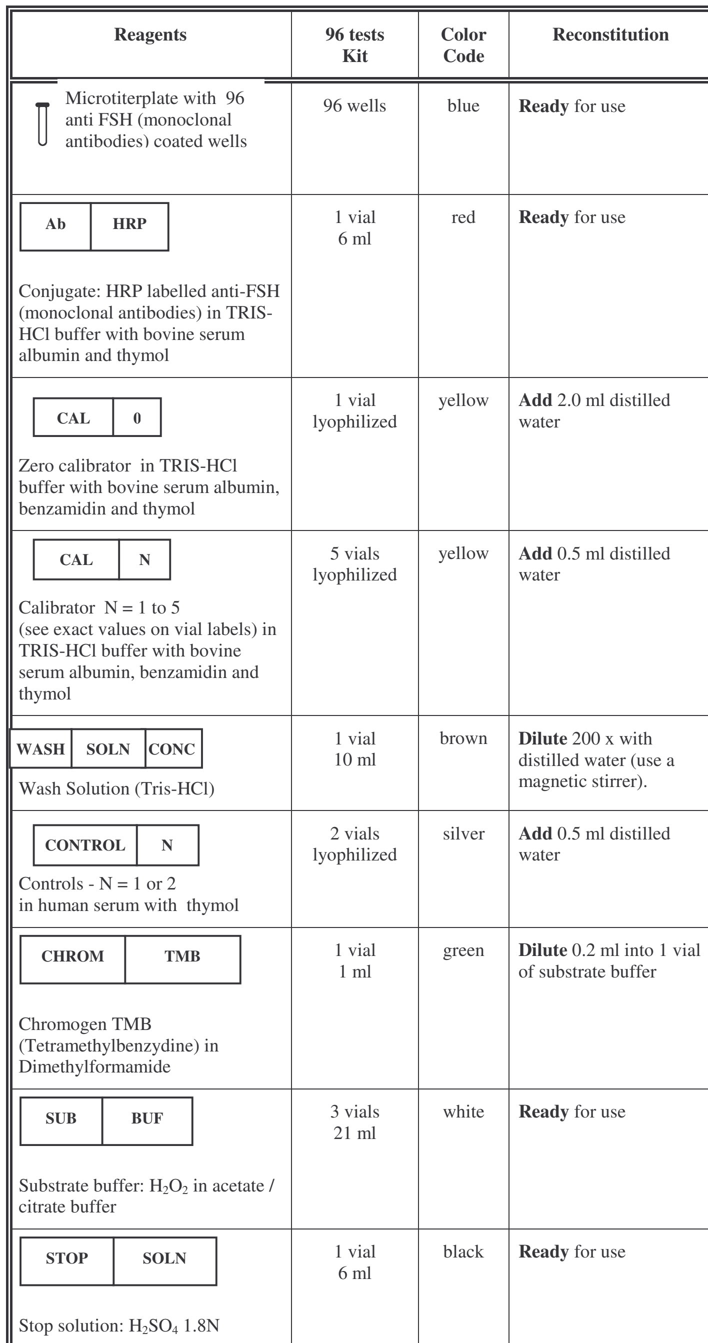
B. Clinical Application

Generally, both FSH and LH are measured in patients under a variety of circumstances such as suspected hypogonadism, ovulation timing, fertility investigation, monitoring of ovulation induction and clinical administration of gonadotropin.
The major clinical applications of LH and FSH measurement in females are: the detection of primary of secondary amenorrhoea and anovulatory cycles. Elevated LH and FSH levels, combined with low Estradiol levels, reflect a primary ovarian failure commonly associated with primary amenorrhoea. Pituitary tumors, granulomas and Sheehan's Syndrome can induce secondary amenorrhoea, in which circulating levels of estrogens as well as FSH and LH are low.
Clinical applications of FSH and LH measurement in males are in the detection of primary testicular failure where there is damage to germ cell production such as after infection or viral orchititis. Primary testicular failure leads to testosterone deficiency with high LH and FSH concentrations. In pure Sertolli Cells syndrome, spermatogenesis is defective with elevated levels FSH but normal testosterone and LH.

IV. PRINCIPLES OF THE METHOD

The Bio-Line FSH-ELISA is a solid phase Enzyme Amplified Sensitivity Immunoassay performed on microtiterplate. The assay uses monoclonal antibodies (MAbs) directed against distinct epitopes of FSH. Calibrators and samples react with the capture monoclonal antibody (MAb 1) coated on microtiter well and with a monoclonal antibody (MAb 2) labelled with horseradish peroxidase (HRP). After an incubation period allowing the formation of a sandwich: coated MAb 1 – human FSH – MAb 2 – HRP, the microtiterplate is washed to remove unbound enzyme labelled antibody. Bound enzyme-labelled antibody is measured through a chromogenic reaction. Chromogenic solution (TMB – H_2O_2) is added and incubated. The reaction is stopped with the addition of Stop Solution and the microtiterplate is then read at the appropriate wavelength. The amount of substrate turnover is determined colourimetrically by measuring the absorbance, which is proportional to the FSH concentration.
A calibration curve is plotted and FSH concentration in samples is determined by interpolation from the calibration curve. The use of the ELISA reader (linearity up to 3 OD units) and a sophisticated data reduction method (polychromatic data reduction) result in a high sensitivity in the low range and in an extended calibration range.

V. REAGENTS PROVIDED



Reagents	96 tests Kit	Color Code	Reconstitution
Microtiterplate with 96 anti FSH (monoclonal antibodies) coated wells	96 wells	blue	**Ready** for use
Ab HRP Conjugate: HRP labelled anti-FSH (monoclonal antibodies) in TRIS-HCl buffer with bovine serum albumin and thymol	1 vial 6 ml	red	**Ready** for use
CAL 0 Zero calibrator in TRIS-HCl buffer with bovine serum albumin, benzamidin and thymol	1 vial lyophilized	yellow	**Add** 2.0 ml distilled water
CAL N Calibrator N = 1 to 5 (see exact values on vial labels) in TRIS-HCl buffer with bovine serum albumin, benzamidin and thymol	5 vials lyophilized	yellow	**Add** 0.5 ml distilled water
WASH SOLN CONC Wash Solution (Tris-HCl)	1 vial 10 ml	brown	**Dilute** 200 x with distilled water (use a magnetic stirrer).
CONTROL N Controls - N = 1 or 2 in human serum with thymol	2 vials lyophilized	silver	**Add** 0.5 ml distilled water
CHROM TMB Chromogen TMB (Tetramethylbenzydine) in Dimethylformamide	1 vial 1 ml	green	**Dilute** 0.2 ml into 1 vial of substrate buffer
SUB BUF Substrate buffer: H_2O_2 in acetate / citrate buffer	3 vials 21 ml	white	**Ready** for use
STOP SOLN Stop solution: H_2SO_4 1.8N	1 vial 6 ml	black	**Ready** for use

Note: 1. Use the zero calibrator for sample dilutions.
2. 1 mIU of the calibrator is equivalent to 1 mIU of the 1st IS 92/510.

VI. SUPPLIES NOT PROVIDED

The following material is required but not provided in the kit:

1. High quality distilled water
2. Pipettes for delivery of: 50 µl, 200 µl, 500 µl and 2 ml (the use of accurate pipettes with disposable plastic tips is recommended)
3. Vortex mixer
4. Magnetic stirrer
5. Horizontal microtiterplate shaker capable of 700 rpm ± 100 rpm
6. Washer for Microtiterplates
7. Microtiterplate reader capable of reading at 450 nm, 490 nm and 650 nm (in case of polychromatic reading) or capable of reading at 450 nm and 650 nm (monochromatic reading)
8. Optional equipment: The ELISA-AID™ necessary to read the plate according to polychromatic reading (see paragraph XI.A.) can be purchased from Robert Maciels Associates, Inc. Mass. 0.2174 USA.

VII. REAGENT PREPARATION

A. **Calibrators** : Reconstitute the zero calibrator with 2.0 ml distilled water and other calibrators with 0.5 ml distilled water.
B. **Controls** : Reconstitute the controls with 0.5 ml distilled water.
C. **Working Wash solution** : Prepare an adequate volume of Working Wash solution by adding 199 volumes of distilled water to 1 volume of Wash Solution (200x). Use a magnetic stirrer to homogenize. Discard unused Working Wash solution at the end of the day.
D. **Revelation Solution**: pipette 0.2 ml of the chromogen TMB into one of the vials of substrate buffer (H_2O_2 in acetate/citrate buffer). Extemporaneous preparation is recommended.

VIII. STORAGE AND EXPIRATION DATING OF REAGENTS

- Before opening or reconstitution, all kits components are stable until the expiry date, indicated on the vial label, if kept at 2 to 8°C.
- Unused strips must be stored, at 2-8°C, in a sealed bag containing a desiccant until expiration date.
- After reconstitution, calibrators and controls are stable for 1 week at 2 to 8°C. For longer storage periods, aliquots should be made and kept at -20°C. Avoid successive freeze thaw cycles.
- The concentrated Wash Solution is stable at room temperature until expiration date.
- Freshly prepared Working Wash solution should be used on the same day.
- After its first use, the conjugate is stable until expiry date, if kept in the original well-closed vial at 2 to 8°C.
- The freshly prepared revelation solution is stable, before use, for maximum 15 minutes at room temperature and must be discarded afterwards.
- Alterations in physical appearance of kit reagents may indicate instability or deterioration.

IX. SPECIMEN COLLECTION AND PREPARATION

- Serum and plasma must be kept at 2 - 8°C.
- If the test is not run within 24 hours, storage in aliquots at -20°C is recommended. Avoid subsequent freeze thaw cycles.
- Prior to use, all samples should be at room temperature. It is recommended to vortex the samples before use.
- Serum, heparinized plasma or EDTA plasma provides similar results.
  Y(serum) = 1.21 x (EDTA plasma) - 0.85 r=0.994 n=34
  Y(serum) = 1.04x (Heparin plasma) + 0.25 r=0.996 n=36
- Do not use haemolysed samples.

X. PROCEDURE

A. Handling notes

Do not use the kit or components beyond expiry date.
Do not mix materials from different kit lots.
Bring all the reagents to room temperature prior to use.
Thoroughly mix all reagents and samples by gentle agitation or swirling.
Perform calibrators, controls and samples in duplicate. Vertical alignment is recommended.
Use a clean plastic container to prepare the Wash Solution.
In order to avoid cross-contamination, use a clean disposable pipette tip for the addition of each reagent and sample.
For the dispensing of the Revelation Solution and the Stop Solution avoid pipettes with metal parts.
High precision pipettes or automated pipetting equipment will improve the precision.
Respect the incubation times.
To avoid drift, the time between pipetting of the first calibrator and the last sample must be no longer than 30 minutes.

Prepare a calibration curve for each run, do not use data from previous runs.
The chromogenic solution should be colourless. If a dark blue colour develops within a few minutes after preparation, this indicates that the preparation is unusable and must be discarded.
Dispense the Revelation Solution within 15 minutes following the washing of the microtiterplate.
During incubation with Revelation Solution, avoid direct sunlight on the microtiterplate.

B. Procedure

1. Select the required number of strips for the run. The unused strips should be resealed in the bag with a desiccant and stored at 2-8°C.
2. Secure the strips into the holding frame.
3. Pipette 50 µl of each Calibrator, Control and Sample into the appropriate wells.
4. Pipette 50 µl of anti-FSH-HRP conjugate into all the wells.
5. Incubate for 30 minutes at room temperature on a horizontal shaker set at 700 rpm ± 100 rpm.
6. Aspirate the liquid from each well.
7. Wash the plate 3 times by:
   - Dispensing 0.4 ml of Wash Solution into each well
   - Aspirating the content of each well
8. Pipette 200 µl of the freshly prepared revelation solution into each well within 15 minutes following the washing step.
9. Incubate the microtiterplate for 15 minutes at room temperature on a horizontal shaker set at 700 rpm ± 100 rpm, avoid direct sunlight.
10. Pipette 50 µl of Stop Solution into each well.
11. Read the absorbencies at 450 nm and 490 nm (reference filter 630 nm or 650 nm) within 1 hour and calculate the results as described in section XI.

XI. CALCULATION OF RESULTS

A. Polychromatic Reading:

1. In this case, the ELISA-AID™ software will do the data processing.
2. The plate is first read at 450 nm against a reference filter set at 650 nm (or 630 nm).
3. A second reading is performed at 490 nm against the same reference filter.
4. The ELISA-AID™ Software will drive the reader automatically and will integrate both readings into a polychromatic model. This technique can generate OD's up to 10.
5. The principle of polychromatic data processing is as follows:
   - Xi = OD at 450 nm
   - Yi = OD at 490 nm
   - Using a standard unweighted linear regression, the parameters A & B are calculated : Y = A*X – B
   - If Xi < 3 OD units, then X calculated = Xi
   - If Xi > 3 OD units, then X calculated = (Yi-B)/A
   - A 4-parameter logistic curve fitting is used to build up the calibration curve.
   - The FSH concentration in samples is determined by interpolation on the calibration curve.

B. Bichromatic Reading

1. Read the plate at 450 nm against a reference filter set at 650 nm (or 630 nm).
2. Calculate the mean of duplicate determinations.
3. On semi-logarithmic or linear graph paper plot the OD values (ordinate) for each calibrator against the corresponding concentration of FSH (abscissa) and draw a calibration curve through the calibrator points by connecting the plotted points with straight lines.
4. Read the concentration for each control and sample by interpolation on the calibration curve.
5. Computer assisted data reduction will simplify these calculations. If automatic result processing is used, a 4-parameter logistic function curve fitting is recommended.

XII. TYPICAL DATA

The following data are for illustration only and should never be used instead of the real time calibration curve.

FSH-ELISA		OD units Polychromatic model
Calibrator	0 mIU/ml	0.011
	1 mIU/ml	0.085
	5 mIU/ml	0.374
	15 mIU/ml	1.222
	50 mIU/ml	2.908
	150 mIU/ml	4.575

XIII. PERFORMANCE AND LIMITATIONS

A. Detection Limit

Twenty zero calibrators were assayed along with a set of other calibrators. The detection limit, defined as the apparent concentration two standard deviations above the average OD at zero binding, was 0.15 mIU/ml.

B. Specificity

Cross-reactive hormones (FSH, TSH & hCG) were added to the zero calibrator and to a high value calibrator (30 mIU/ml). The apparent FSH response was measured.

added Hormone	FSH CAL 0 mIU/ml	FSH CAL 30 mIU/ml mIU/ml
-	0.0	30.3
LH 250 mIU/ml	0.0	26.2
TSH 250 µIU/ml	0.07	28.5
hCG 86 IU/ml	0.35	25.0

C. Precision

INTRA ASSAY				INTER ASSAY			
Serum	N	<X> ± SD (mIU/ml)	CV (%)	Serum	N	<X> ± SD (mIU/ml)	CV (%)
A	23	0.8 ± 0.04	5.6	A	20	7.7 ± 0.3	3.6
B	23	4.0 ± 0.3	6.8	B	20	17.9 ± 0.9	3.9
C	22	53.0 ± 3.5	6.7				

SD : Standard Deviation; CV: Coefficient of variation

D. Accuracy

RECOVERY TEST

Sample	Added FSH (mIU/ml)	Recovered FSH (mIU/ml)	Recovery (%)
Serum	57.3	59.6	104
	39.2	39.6	101
	30.2	31.7	105
	24.8	24	97

DILUTION TEST

Sample	Dilution	Theoretical Concent. (mIU/ml)	Measured Concent. (mIU/ml)
FSH-1	1/1	-	69.6
	1/2	34.8	27.2
	1/5	13.9	13.0
	1/10	7.0	7.2
	1/20	3.5	3.8
	1/50	1.4	1.3
	1/100	0.7	0.9
FSH-2	1/1	-	66.0
	1/2	33.0	29.2
	1/5	13.2	13.2
	1/10	6.6	7.3
	1/20	3.3	3.5

Samples were diluted with zero calibrator.

E. Time delay between last calibrator and sample dispensing

As shown hereafter, assay results remain accurate even when a sample is dispensed 40 minutes after the calibrators have been added to the coated wells.

TIME DELAY					
	T0	10 min	20 min	30 min	40 min
1052	3.9	3.9	3.5	3.7	3.4
1540	21.9	22.2	22.4	22.0	19.4
1338	100.6	105.0	102.5	94.4	99.5

F. Hook effect

A sample spiked with FSH up to 2000 mIU/ml gives higher OD's than the last calibrator point.

XIV. INTERNAL QUALITY CONTROL

- If the results obtained for Control 1 and/or Control 2 are not within the range specified on the vial label, the results cannot be used unless a satisfactory explanation for the discrepancy has been given.
- If desirable, each laboratory can make its own pools of control samples, which should be kept frozen in aliquots. Controls that contain azide will interfere with the enzymatic reaction and cannot be used.
- Acceptance criteria for the difference between the duplicate results of the samples should rely on Good Laboratory Practises
- It is recommended that Controls be routinely assayed as unknown samples to measure assay variability. The performance of the assay should be monitored with quality control charts of the controls.
- It is good practise to check visually the curve fit selected by the computer.

XV. REFERENCE INTERVALS

These values are given only for guidance; each laboratory should establish its own normal range of values.
The range is expressed as 2.5% to 97.5% percentiles.

Identification	Number of subjects	Mean (mIU/ml)	Range (mIU/ml)
Children			
. Newborn to onset of puberty			
- Girls	21	2.6	0.6 – 6.7
- Boys	15	0.9	0.4 – 1.8
. Puberty			
- Girls	6	4.1	2.3 – 5.2
- Boys	6	3.4	1.3 – 7.6
Adult males	85	4.8	0.8 – 16.6
Women			
. Ovulatory cycles			
- Follicular phase (day –10 to -4)	18	5.8	3.4 – 7.7
- Ovulatory peak (day 0)	19	11.9	5.6 – 18.8
- Luteal phase (day +4 to +10)	19	3.3	1.4 – 6.1
. Postmenopausal	60	45.4	4.8 – 78.4

XVI. PRECAUTIONS AND WARNINGS

Safety
For *in vitro* diagnostic use only.
The human blood components included in this kit have been tested by European approved and/or FDA approved methods and found negative for HBsAg, anti-HCV, anti-HIV-1 and 2. No known method can offer complete assurance that human blood derivatives will not transmit hepatitis, AIDS or other infections. Therefore, handling of reagents, serum or plasma specimens should be in accordance with local safety procedures.
All animal products and derivatives have been collected from healthy animals. Bovine components originate from countries where BSE has not been reported. Nevertheless, components containing animal substances should be treated as potentially infectious.
Avoid any skin contact with all reagents, Stop Solution contains H_2SO_4, the chromogen contains TMB in Dimethylformamide, Substrate buffer contains H_2O_2. In case of contact, wash thoroughly with water.
Do not smoke, drink, eat or apply cosmetics in the working area. Do not pipette by mouth. Use protective clothing and disposable gloves.

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XVIII. SUMMARY OF THE PROTOCOL

	CALIBRATORS (µl)	SAMPLE(S) CONTROLS (µl)
Calibrators (0-5) Samples, Controls Anti-FSH-HRP conjugate	50 - 50	- 50 50
Incubate for 30 min at room temperature with continuous shaking at 700 rpm. Aspirate the contents of each well. Wash 3 times with 400 µl of Wash Solution and aspirate.		
Revelation Solution	200	200
Incubate for 15 min at room temperature with continuous shaking at 700 rpm.		
Stop Solution	50	50
Read on a microtiterplate reader and record the absorbance of each well at 450 nm (versus 630 or 650 nm) and 490 nm (versus 630 or 650 nm)		

Bio-Line Catalogue Nr : BL-22-E	Version : 040702-BL	Revision nr : 040220/1