Total β-hCG ELISA

AL-103

INTENDED USE

The Total β-hCG enzyme linked immunosorbent assay (ELISA) kit provides materials for the quantitative measurement of Total β-hCG in serum and other biological fluids. This kit is intended for laboratory research use only and is not for use in diagnostic or therapeutic procedures.

SUMMARY AND EXPLANATION

Human chorionic gonadotropin (hCG) is a two chain (alpha- and beta-subunits) glycoprotein hormone secreted by the chorionic tissue of the placenta and is normally found in urine and blood during pregnancy. Produced by the trophoblastic epithelium of the placenta, hCG acts to maintain the corpus luteum in the early stages of pregnancy.

Healthy, non-pregnant individuals have low (5 mIU/mL) to undetectable hCG concentrations in serum. During pregnancy, hCG concentrations increase to about 50 mIU/mL in the first week after conception and double every 1.5 to 3 days for the first six weeks. Levels continue to rise until the end of the first trimester, and then gradually fall to a lower level for the remainder of the pregnancy. After delivery, hCG returns to 5 mIU/mL and is usually undetectable several days postpartum.

The appearance of hCG soon after conception and its subsequent rise in concentration during early gestational growth make it an excellent marker for the early detection of pregnancy. Elevated serum hCG levels comparable to those observed in early pregnancy may also be associated with trophoblastic or nontrophoblastic neoplasms such as hydatidiform mole, choriocarinoma; therefore, the possibility of such diseases should be ruled out before a positive hCG result is considered for detection and monitoring of pregnancy.

PRINCIPLE OF THE TEST

The Total β-hCG ELISA is an enzymatically amplified two-step "sandwich" assay. In the assay, calibrators, controls, and unknown diluted samples are incubated in microtitration wells which have been coated with anti-Total β-hCG antibody. After incubation and washing, anti-Total β-hCG detection antibody labeled with enzyme horseradish peroxidase (HRP) is added to each well. After a second incubation and washing step, the substrate tetramethylbenzidine (TMB) is added to the wells. After incubation, an acidic stopping solution is added. The degree of enzymatic turnover of the substrate is determined by dual wavelength absorbance measurement at 450 nm as primary test filter and 630 nm as primary reference filter. The absorbance measured is directly proportional to the concentration of Total β-hCG in the samples.

MATERIALS SUPPLIED

CAL-103A– CAL-103F β-hCG Calibrator A-F

Six vials, labeled A-F containing approximate concentrations of 10, 30, 100, 300 and 1000 mIU/mL total β-hCG in a protein-based buffer with non-mercury preservative. Refer to **calibration card** for exact concentrations. Store unopened at 2 to 8°C until the expiration date. Reconstitute calibrators A-F with 1 mL deionized water. Solubilize, mix well and use after reconstitution. Aliquot and freeze for multiple use.

NOTE: *The hCG concentration in the Total β- hCG calibrators is traceable to World Health Organization preparation code 75/589.*

CTR-103-I & CTR-103-II β-hCG Controls

Two vials, labeled Levels I and II containing low and high total β- hCG concentrations in protein-based buffer with non-mercury preservative. Refer to **calibration card** for exact concentrations. Store unopened at 2 to 8°C until the expiration date. Reconstitute control Levels I and II with 1 mL deionized water. Solubilize, mix well, and use after reconstitution. Aliquot and freeze for multiple use.

PLT-103 β-hCG Antibody Coated Microtitration strips

Four strip-holder, containing 4x96 polystyrene microtitration wells with anti-beta-hCG antibody immobilized to the inside wall of each well. Store at 2 to 8°C until expiration date in the resealable pouch with a desiccant to protect from moisture.

ASB-103 hCG Assay Buffer

Four bottle, 22 mL each, containing a protein-based buffer with a non-mercury preservative. Store at 2 to 8°C until expiration date.

ECR-103 hCG Antibody-Enzyme Conjugate Ready-to-Use

Four bottle, 12 mL each, containing anti beta-hCG antibody conjugated to HRP in a protein buffer with a non-mercury preservative. Store at 2 to 8°C until the expiration date.

TMB-100 TMB Chromogen Solution

Four bottle, 12 mL each, containing a solution of tetramethylbenzidine (TMB) in buffer with hydrogen peroxide. Store at 2 to 8°C until expiration date.

WSH-100 Wash Concentrate A

One bottle, 60 mL, containing buffered saline with a nonionic detergent. Store at 2 to 30°C until expiration date. Dilute 25-fold with deionized water prior to use.

SPD-106 Sample Diluent (10X)

One bottle, 60 mL, containing protein-based buffer with a nonionic detergent. Store at 2 to 30°C until expiration date. **Dilute 10-fold with deionized water prior to use.**

STP-100 Stopping Solution

Four bottle, 12 mL each, containing 0.2 M sulfuric acid. Store at 2 to 30°C until expiration date.

MATERIALS REQUIRED BUT NOT SUPPLIED

1. Microtitration plate reader capable of absorbance measurement at 450 nm, 405 nm, and 630 nm.
2. Microtitration orbital plate shaker.
3. Microtitration plate washer.
4. Semi-automated/manual precision pipette to deliver 2–250 µL.
5. Repeater pipette
6. Vortex mixer.
7. Deionized water.
8. Disposable 12 x 75 mm culture tubes.
9. Tight fitting 12 x 75 mm tube racks.

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Refer to package insert included with the product for exact specification

WARNINGS AND PRECAUTIONS

For *Research Use Only.*

The following precautions should be observed:

a) Follow good laboratory practice.

b) Use personal protective equipment. Wear lab coats and disposable gloves when handling immunoassay materials.

c) Handle and dispose of all reagents and material in compliance with applicable regulations.

WARNING: Potential Biohazardous Material

This reagent may contain some human source material (e.g., serum) or materials used in conjunction with human source materials. Handle all reagents and patient samples at a Biosafety Level 2, as recommended for any potentially infectious human material in the Centers for Disease Control/National Institutes of Health manual "Biosafety in Microbiological and Biomedical Laboratories," 5th Edition, 2007[1].

WARNING: Potential Chemical Hazard

Some reagents in this kit contain Pro-Clean 400 as a preservative. Pro-Clean 400 and peroxide in concentrated amounts are irritants to skin and mucous membranes.

For further information regarding hazardous substances in the kit, please refer to the MSDS, either at AnshLabs.com or by request.

SAMPLE COLLECTION AND PREPARATION

a) Serum is the recommended sample type.

b) Sample handling, processing, and storage requirements depend on the brand of blood collection tube that you use. Please reference the manufacturer's instructions for guidance. Each laboratory should determine the acceptability of its own blood collection tubes and serum separation products.

c) Samples may be stored at 4°C if assayed within 24 hours; otherwise, samples must be stored at -20°C or -80°C to avoid loss of bioactivity and contamination.

d) Avoid assaying lipemic, hemolyzed or icteric samples.

e) Avoid repeated freezing and thawing of samples. Thaw samples no more than 3 times.

f) For shipping, place specimens in leak proof containers in biohazard specimen bags with appropriate specimen identification and test requisition information in the outside pocket of the biohazard specimen bag. Follow DOT and IATA requirements when shipping specimens.[2]

PROCEDURAL NOTES

1. A thorough understanding of this package insert is necessary for successful use of the Total β-hCG ELISA assay. It is the responsibility of the customer to validate the assay for their use. Accurate results will only be obtained by using precise laboratory techniques and following the package insert.
2. A calibration curve must be included with each assay.
3. Bring all kit reagents to room temperature before use. Thoroughly mix the reagents before use by gentle inversion. Do not mix different lots of any kit component and do not use any component beyond the expiration date.
4. Use a clean disposable pipette tip for each reagent, calibrator, control, or sample. Avoid microbial contamination of reagents, contamination of the substrate solutions with the HRP conjugates. The enzyme used as the label is inactivated by oxygen, and is highly sensitive to microbial contamination, sodium azide, hypochlorous acid and aromatic chlorohydrocarbons often found in laboratory water supplies. Use deionized water.
5. Incomplete washing will adversely affect the outcome and assay precision. Care should be taken to add TMB into the wells accurately and efficiently to minimize potential assay drift due to variation in the TMB incubation time. Avoid exposure of the reagents to excessive heat or direct sunlight.

PREPARATION OF REAGENTS

1. **β-hCG Calibrators A-F and Controls I & II**: Tap and reconstitute β-hCG Calibrator A-F and Controls I & II each with 1 mL deionized water. Solubilize, mix well, and use after reconstitution.
2. **Sample Diluent**: Add **1 part of SPE-106** into **9 parts** of the deionized water, according to the number of wells used. For a one plate equivalent, pipet exactly **15 mL** of the **SPD-106** into **135 mL** of the deionized water, mix well and label as sample diluent ready to use.
3. **Wash Solution:** Dilute Wash Concentrate A 25-fold with deionized water. The wash solution is stable for one month at room temperature when stored in a tightly sealed bottle.
4. **Microtitration Wells:** Select the number of coated wells required for the assay. The remaining unused wells should be placed in the resealable pouch with a desiccant. The pouch must be resealed to protect from moisture.

SAMPLE PREPARATION

Dilution of pregnancy serum specimens should be performed on the same day prior to testing.

1. **Sample Diluent Preparation**: See preparation of reagents section.
2. For each unknown serum sample, label one 12 X 75 culture tube appropriately and add **1.5 mL** of the **sample diluent ready to use** to each tube.
3. Add **10 µL** of the serum specimens to the pre-labeled tube and vortex well.
4. Place the tubes in a tight-fitting tube rack and shake at a **slow speed (300-400 rpm)** at room temperature (23 ± 2°C) for **5-10 minutes**. Alternatively, vortex well prior to use.
5. The sample is now ready to be assayed.

ASSAY PROCEDURE

Allow all samples and reagents to reach room temperature. Mix reagents thoroughly by gentle inversion before use. After reconstitution of reagents, mix thoroughly, avoiding foaming. Calibrators, controls, and samples should be assayed in duplicate.

1. Mark the microtitration strips to be used.
2. Pipet **25 µL** of the **Calibrators, Controls and Unknown diluted samples** (see sample preparation section) to the appropriate wells.
3. Add **200 µL** of the **hCG Assay Buffer** to each well using a repeater pipette.
4. Incubate the wells, shaking at **600–800 rpm** on an orbital microplate shaker, for **30 mins** at room temperature (23 ± 2°C).
5. Aspirate and wash each well **5 times** with the wash solution **(350 µL/well)** using an automatic microplate washer.
6. Add **100 µL** of the **Antibody-enzyme conjugate ready-to-use** solution to each well using a repeater pipette.
7. Incubate the wells, shaking at **600–800 rpm** on an orbital microplate shaker, for **30 mins** at room temperature (23 ± 2°C).
8. Aspirate and wash each well **5 times** with the wash solution **(350 µL/well)** using an automatic microplate washer.
9. Add **100 µL** of the **TMB chromogen solution** to each well using a repeater pipette. Avoid exposure to direct sunlight.
10. Incubate the wells, shaking at **600–800 rpm** on an orbital microplate shaker, for **8-12 min** at room temperature (23 ± 2°C).
    NOTE: *Visually monitor the color development to optimize the incubation time.*
11. Add **100 µL** of the **Stopping solution** to each well using a repeater pipette. Read the absorbance of the solution in the wells within **20 minutes**, using a microplate reader set to **450 nm.**

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***NOTE**: Zero calibrator should be programmed as "**Blank**" while reading the optical density. If instrument has a wavelength correction, set the instrument to dual wavelength measurement at **450 nm** with background wavelength correction at **630 nm**.*

RESULTS

***NOTE:** The results in this package insert were calculated by plotting the **log optical density (OD) data on the y-axis and log β-hCG concentration on X-axis** using a cubic regression curve-fit. Alternatively, log vs. log quadratic regression curve-fit can be used. Other data reduction methods may give slightly different results.*

1. Optimum results can be obtained at incubation temperature of **23 ± 2°C**.
2. Calculate the mean absorbance for each calibrator, control or sample. Using data reduction software set the program to use log-log scaling with cubic regression curve fitting to plot the mean absorbance readings for each of the calibrators along the y-axis versus the Total β-hCG concentrations in mIU/mL on the x-axis.
3. Determine the hCG concentrations of the controls and samples from the calibration curve by matching their mean absorbance readings with the corresponding hCG concentrations.
4. Any sample reading higher than the highest calibrator should be appropriately diluted using Sample Diluent ready to use and re-assayed.
5. Any sample reading lower than the analytical sensitivity should be reported as such.
6. Multiply the measured concentrations by the dilution factor of the sample preparation method (150x). Divide the result by 1000 to obtain IU/mL.

LIMITATIONS

- The reagents supplied in this kit are optimized to measure Total β-hCG levels in serum specimens.
- For assays employing antibodies, the possibility exists for interference by heterophile antibodies in the sample. Samples from individuals which have been regularly exposed to animals or have received immunotherapy or diagnostic procedures utilizing immunoglobulins or immunoglobulin fragments may produce antibodies, e.g. HAMA, that interferes with immunoassays. Additionally, other heterophile antibodies such as human anti-goat antibodies may be present in samples.[3]
- If there is evidence of microbial contamination or excessive turbidity in a reagent, discard the vial.

QUALITY CONTROL

- Each laboratory should establish mean values and acceptable ranges to assure proper performance.
- Total β-hCG ELISA controls or other commercial controls should fall within established confidence limits.
- The confidence limits for Total β- hCG ELISA controls are printed on the calibration card.
- A full calibration curve, along with low- and high-level controls, should be included in each assay.
- The TMB chromogen solution should be colorless. Development of a blue color may indicate reagent contamination or instability.

REPRESENTATIVE CALIBRATION CURVE DATA

Well Number	Well Contents Calibrators	Mean OD	Conc. (mIU/mL)
A1, A2	A	0.008 (**Blank**)	0
B1, B2	B	0.031	9.9
C1, C2	C	0.10	35.6
D1, D2	D	0.39	147.0
E1, E2	E	1.17	464.0
F1, F2	F	3.05	1389.0

***CAUTION:** The above data must not be employed in lieu of data obtained by the user in the laboratory.*

ANALYTICAL CHARACTERISTICS

Note: *All analytical data in this IFU is reported as observed concentration and is not corrected for the dilution factor except method comparison and expected value.*

Analytical Sensitivity:

The analytical sensitivity in the assay as calculated by the interpolation of mean plus two standard deviation of 16 replicates of calibrator A and calibrator B (9.9 mIU/mL) is 0.94 mIU/mL.

Imprecision:

Reproducibility of the Total β-hCG assay was determined in a study using three serum samples (diluted 1:150 independently prior to assay). The study included a total of six runs, three replicates per run (n=18). Representative data are presented in the following table.

Sample	Mean (mIU/mL)	Within run		Between run	
		SD	CV	SD	CV
Low	71.1	2.8	4.0%	3.7	5.1%
Mid	216.9	13.4	6.2%	6.9	3.2%
High	770.3	81.8	10.6%	25.3	3.3%

Linearity:

Multiple dilutions of calibrator F and two serum samples containing various hCG levels were diluted with sample diluent (SPD-106, Ready-to-Use). The % recovery on individual samples is represented in the following table.

Sample ID	Dilution factor (1 in X)	Expected Value in mIU/mL	Observed Value in mIU/mL	%Recovery	Average %Recovery
Calibrator F	Neat	1389.0			95%
	1:2	694.5	733.7	106%	
	1:4	347.3	360.0	104%	
	1:8	173.6	168.6	97%	
	1:16	86.8	75.4	87%	
	1:32	43.4	35.4	82%	
1	1:75	299.7			95%
	1:150	149.8	151.5	101%	
	1:300	74.9	74.9	100%	
	1:600	37.5	31.0	83%	
2	1:75	256.7			91%
	1:150	128.3	124.3	97%	
	1:300	64.2	58.4	91%	
	1:600	32.1	27.5	86%	

Analytical Specificity:

This monoclonal antibody pair used in the assay detects Total β-hCG. Other related molecules at the concentration in the table below did not show any significant cross-reaction. Specificity to other species has not been determined.

Sample	Cross-reactant	Concentration	% Cross-reactivity
1	Estriol	50 ng/mL	ND
2	r PAPP-A	50 ng/mL	ND
3	ht PAPP-A	50 ng/mL	ND
4	Inhibin A	50 ng/mL	ND
5	Inhibin B	50 ng/mL	ND
6	Activin A	50 ng/mL	ND
7	Activin B	50 ng/mL	ND
8	FSTL-3	50 ng/mL	ND
9	Native AFP (>99% purity)	50 ng/mL	<0.4
10	Alpha 2M	50 ng/mL	ND
11	Beta hCG	50 ng/mL	ND
12	Prolactin	250 ng/mL	ND
14	FSH	500 mIU/mL	ND
15	LH	165 mIU/mL	ND

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ND = Non-Detectable

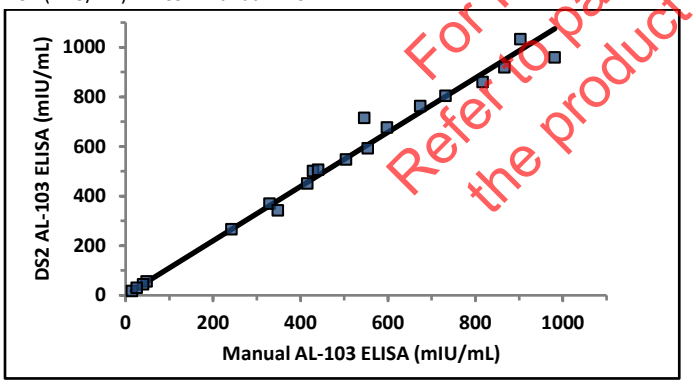
Interference:

When hemoglobin, bilirubin and triglycerides were added at a greater than two folds of their physiological concentration to control sample, Total β- hCG concentration were within ± 15% of the control as represented in the following table. This study was based on CLSI-EP7-P.

Interferent	Interferent Dose	Sample Total β hCG (mIU/mL)	Dosed Sample Total β hCG (mIU/mL)	Total β hCG Difference (mIU/mL)	% Difference to Reference
Hemoglobin	1 mg/mL	214.8	207.6	-7.2	-3.4
	0.5 mg/mL	260.6	249.5	11.8	-4.3
	0.1 mg/mL	166.9	160.7	-6.2	-3.7
Hemoglobin	1 mg/mL	232.8	246.0	4.6	5.7
	0.5 mg/mL	165.2	170.3	5.1	3.1
	0.1 mg/mL	177.1	188.3	10.0	6.3
Biotin	1200 ng/mL	241.6	223.8	-17.8	-7.4
	600 ng/mL	245.7	229.8	-16.0	-6.5
	200 ng/mL	179.3	179.1	-0.1	-0.1
Biotin	1200 ng/mL	240.6	243.3	2.7	1.1
	600 ng/mL	170.3	169.2	-1.1	-0.7
	200 ng/mL	181.3	190.2	8.9	4.9
Intralipids	20 mg/mL	244.9	274.9	30.0	12.3
	10 mg/mL	296.1	299.2	3.1	1.1
	5 mg/mL	203.5	200.0	-3.5	-1.7
Intralipids	20 mg/mL	270.2	295.4	25.2	9.3
	10 mg/mL	205.1	194.2	-10.8	-5.3
	5 mg/mL	208.1	216.4	8.3	4.0
Bilirubin	0.66 mg/mL	157.7	146.5	-11.2	-7.1
	0.2 mg/mL	118.2	121.6	3.4	2.9
Bilirubin	0.66 mg/mL	210.8	236.5	25.7	12.2
	0.2 mg/mL	165.6	185.9	20.3	12.2

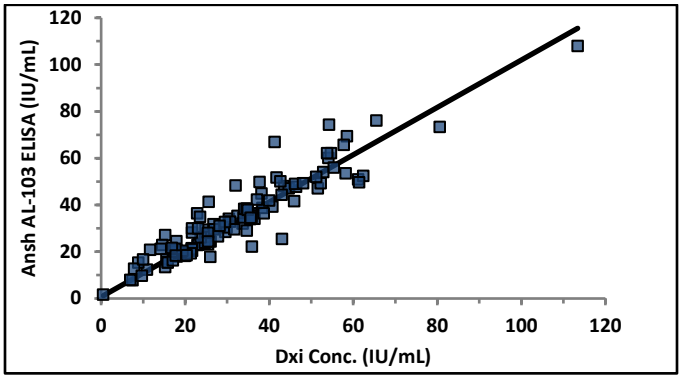
Manual vs Instrument Comparison:

The Ansh Labs Total β-hCG ELISA manual assay has been compared to DS2 Total β-hCG ELISA using 20 pregnant female serum samples.
Passing Bablok analysis of the results yielded the following Regression:
DS2 (mIU/mL) = 1.094 Manual ELISA + 1.114



Method Comparison:

The Total β-hCG ELISA has been compared to commercially available Total β-hCG kit (Method A) using 120 pregnant female serum samples in the range of 0.52-113.4 IU/mL.
Passing Bablok analysis of the results yielded the following Regression:
Intact hCG ELISA (AL-103) =1.01 (Method A) + 0.7
(r=0.94; P<0.0001)



Expected Value:

Serum samples were analyzed using Ansh Labs Total β-hCG ELISA kit. The expected ranges for hCG were calculated using 95% non-parametric estimation using Analyse-It® for Microsoft Excel as shown in table below.

Gestational Age	No. of specimens (n)	Median Concentration (IU/mL)	hCG (IU/mL) 95% CI
10 weeks	20	93.4	80.8 to 102.0
11 weeks	20	92.9	65.0 to 112.0
12 weeks	20	69.3	58.3 to 92.0
13 weeks	20	68.1	58.2 to 81.7
15 weeks	229	37.4	33.7 to 42.8
16 weeks	259	31.4	28.6 to 34
17 weeks	158	26.0	22.8 to 29.2
18 weeks	108	22.9	20.7 to 27.8
19 weeks	48	18.2	14.6 to 22.1
20 weeks	26	16.5	13.9 to 19.6
21 weeks	12	19.5	9.9 to 24.5
22 weeks	9	19.2	6.4 to 30.7

Note: *It is recommended that each laboratory should determine the reference range(s) for its own patient population. The results of this assay should be used in conjunction with other relevant and applicable clinical information.*

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REFERENCES

1. HHS Publication, 4th ed., April 1999. Biosafety in Microbiological and Biomedical Laboratories. Available http://www.cdc.gov/od/ohs/biosfty/ bmbl4/bmbl4toc.htm
2. National Committee for Clinical Laboratory Standards. Blood collection on filter paper for neonatal programs- 2nd ed.; approved standard. NCCLS publications LA4-A2. Villanova, PA: NCCLS, 1992.
3. Kricka L. Interferences in immunoassays – still a threat. Clin Chem 2000; 46: 1037–1038.

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