GDF-9 ELISA

RUO

AL-176

INTENDED USE

The GDF-9 enzyme linked immunosorbent assay (ELISA) kit provides materials for the quantitative measurement of GDF-9 in human 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

BMP-15 and GDF-9 are synthesized as 249-295 amino acid N-terminal prodomains and 125-139 amino acid mature domains. The mature domains of GDF-9 and BMP-15 form 40 kDa and 34 kDa homodimers respectively and 37 kDa heterodimer [1,2]. Growth Differentiation Factor 9 (GDF9) and Bone Morphogenic Protein 9 (BMP15) are oocyte secreted factors and play key roles in promoting follicle growth, maturation and oocyte competence [1,3-5]. However, the action of BMP15 and GDF9 varied with respect to the species of origin and the stages of follicle development. Oocyte competence is an intrinsic developmental potential that allows a mature oocyte to become fertilized and develop to an embryo. Oocyte competence is achieved by long course of molecular, biochemical and morphological changes[4]. Recent study suggests AMH production is regulated by oocyte-secreted factors in primary human cumulus cells and the combination of GDF9 + BMP15 potently stimulates AMH expression[6]. Altered level and activity of GDF9 is implicated in premature ovarian failure and polycystic ovarian syndrome, implicating GDF9 in the etiology of these conditions [7-9].

PRINCIPLE OF THE TEST

The GDF-9 ELISA is a quantitative three-step sandwich type immunoassay. In the first step Calibrators, Controls and Unknown samples are added to GDF-9 antibody coated microtiter wells and incubated. After the first incubation and washing, the wells are incubated with biotinylated GDF-9 antibody. After the second incubation and washing, the wells are incubated with streptavidin horseradish peroxidase conjugate (SHRP). After the third incubation and washing step, the wells are incubated with substrate solution (TMB). After TMB incubation, an acidic stopping solution is added. In principle, the antibody-biotin conjugate binds to the solid phase antibody-antigen complex which in turn binds to the streptavidin-enzyme conjugate. The antibody-antigen-biotin conjugate-SHRP complex bound to the well is detected by enzyme-substrate reaction. 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 reference filter. The absorbance measured is directly proportional to the concentration of GDF-9 in the samples and calibrators.

MATERIALS SUPPLIED

CAL-176A **GDF-9 Calibrator A / Sample Diluent**

One vial, labeled A, 2.5 mL, containing concentration of 0 pg/mL GDF-9 in protein based buffer and Pro-Clean 400. Store at 2-8°C upon receipt until the expiration date.

CAL-176B - CAL-176F **GDF-9 Calibrators B thru F (Lyophilized)**

Five vials, labeled B-F, containing concentrations of approximately 50 – 5000 pg/mL GDF-9 in protein based buffer and Pro-Clean 400. Refer to **calibration card** for exact concentrations. Store unopened at 2 to 8°C until the expiration date. Reconstitute calibrators B-F with 1 mL deionized water. Solubilize, mix well and use after reconstitution

CTR-176-I & CTR-176-II **GDF-9 Controls I & II (Lyophilized)**

Two vials, labeled Levels I and II containing low and high GDF-9 concentrations in protein based buffer and Pro-Clean 400. Refer to **calibration card** for exact control ranges. 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.

PLT-176 **GDF-9 Antibody Coated Microtitration Strips**

One strip holder, containing 12 strips and 96 microtitration wells with GDF-9 antibody immobilized to the inside wall of each well. Store at 2-8°C until expiration date in the resealable pouch with a desiccant to protect from moisture.

ASB-176 **BMP-15/GDF-9 Assay Buffer A**

One bottle, 8 mL, containing a protein-based (BSA)-buffer with a non-mercury preservative. Store at 2-8°C until expiration date.

BCC-176 **GDF-9 Biotin Conjugate Concentrate**

One vial, 0.4 mL, containing detection antibody biotin in a protein-based buffer with a non-mercury preservative. Dilute prior to use in GDF-9 Conjugate diluent. Store at 2-8°C until expiration date.

CND-176 **BMP-15/GDF-9 Biotin Conjugate Diluent**

One bottle, 12 mL, containing a protein-based buffer with a non-mercury preservative. Store at 2-8°C until expiration date.

SAR-176 **GDF-9 Streptavidin-Enzyme Conjugate—Ready-to-Use (RTU)**

One amber bottle, 12 mL, containing streptavidin-HRP (horseradish peroxidase) in a protein-based buffer and a non-mercury preservative. Store undiluted at 2-8°C until expiration date.

TMB-100 **TMB Chromogen Solution**

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

STP-100 **Stopping Solution**

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

WSH-100 **Wash Concentrate A**

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

MATERIALS REQUIRED BUT NOT PROVIDED

1. Microplate reader capable of absorbance measurement at 450 nm, 405 nm and 630 nm.
2. Microplate orbital shaker.
3. Microplate washer.
4. Semi-automated/manual precision pipette to deliver 10–250 µL.
5. Vortex mixer.
6. Deionized water.
7. Disposable 12 x 75 mm culture tubes.
8. Tight fitting 12 x 75 mm tube racks.

WARNINGS AND PRECAUTIONS

For Research Use Only. Not for use in diagnostic procedures.

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.[10]

WARNING: Potential Chemical Hazard

Some reagents in this kit contain Pro-Clean 400 and Sodium azide[11] as a preservative. Pro-Clean 400 and Sodium Azide 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.[12]

PROCEDURAL NOTES

1. A thorough understanding of this package insert is necessary for successful use of the GDF-9 ELISA assay. It is the user's responsibility to validate the assay for their purpose. 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 (23 ± 2°C) before use. Thoroughly mix the reagents before use by gentle inversion. Do not mix various 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. To minimize potential assay drift due to variation in the substrate incubation time, care should be taken to add the substrate solution into the wells. Avoid exposure of the reagents to excessive heat or direct sunlight during storage and incubation.

PREPARATION OF REAGENTS

1. **GDF-9 Calibrators B-F and GDF-9 Controls I & II**: Tap and reconstitute GDF-9 Calibrator B-F and GDF-9 Controls I & II each with **1 mL** deionized water. Solubilize, mix well and use after reconstitution.
2. **Calibrator B/2 preparation** :150 µL of CAL-B + 150 µL of Calibrator A/ sample Diluent.
3. **Wash Solution:** Dilute wash concentrate 25-fold with deionized water. The wash solution is stable for one month at room temperature (23 ± 2°C) 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.
5. **GDF-9 Antibody-Biotin Conjugate Solution:** The GDF-9 Antibody-Biotin Conjugate Concentrate should be diluted at a ratio of 1-part conjugate to 50 parts of BMP-15/GDF-9 Conjugate Diluent, according to the number of wells used. If an entire plate is to be used pipet exactly 220 µL of the Concentrate in to 11 mL of the diluent.

ASSAY PROCEDURE

Allow all specimens and reagents to reach room temperature (23 ± 2°C) and mix thoroughly by gentle inversion before use. Calibrators, controls, and unknowns should be assayed in duplicate.

NOTE: All serum samples reading higher than the highest calibrator should be mixed and diluted in the 0 pg/mL reconstituted Calibrator A prior to assay.

1. Reconstitute GDF-9 Calibrators B-F and GDF-9 Controls I & II each with **1 mL** deionized water. Solubilize for **10 minutes**. Mix well.
2. Label the microtitration strips to be used.
3. Pipette **50 µL** of the Calibrators B/2, B-F, Controls and Unknowns to the appropriate wells.
4. Add **50 µL** of the BMP-15/GDF-9 Assay Buffer to each well using a repeater pipette.
5. Incubate the plate, shaking at a fast speed (**600-800 rpm**) on an orbital microplate shaker, for **3 hours** at room temperature (23 ± 2°C).
6. During the last **20-30 minutes** of incubation, prepare the GDF-9 Antibody-Biotin Conjugate Solution by diluting the GDF-9 Biotin Conjugate Concentrate in BMP-15/GDF-9 Conjugate Diluent as described under the Preparation of the Reagents section of this package insert.
7. Aspirate and wash each strip **5 times** with Washing Solution **(350 µL/per well**) using an automatic microplate washer.
8. Add **100 µL** of the Antibody-Biotin Conjugate Solution to each well using a repeater pipette.
9. Incubate the plate, shaking at a fast speed (**600-800 rpm**) on an orbital microplate shaker, for **1 hour** at room temperature (23 ± 2°C).

10. Aspirate and wash each strip **5 times** with the Wash Solution **(350 µL/per well)** using an automatic microplate washer.
11. Add **100 µL** of the Streptavidin-Enzyme Conjugate-RTU to each well using a repeater pipette.
12. Incubate the plate, shaking at a fast speed (**600-800 rpm**) on an orbital microplate shaker, for **30 minutes** at room temperature (23 ± 2°C).
13. Aspirate and wash each strip **5 times** with the Wash Solution **(350 µL/per well**) using an automatic microplate washer.
14. Add **100 µL** of the TMB chromogen solution to each well using a precision pipette. Avoid exposure to direct sunlight.
15. Incubate the wells, shaking at **600–800 rpm** on an orbital microplate shaker, for **15-18 minutes** at room temperature (23 ± 2°C).
    ***NOTE**: Visually monitor the color development to optimize the incubation time.*
16. Add **100 µL** of the stopping solution to each well using a precision pipette. Read the absorbance of the solution in the wells within **20 minutes**, using a microplate reader set to **450 nm.**
    ***NOTE**: While reading the absorbance of the microtitration well, it is necessary to program the zero calibrator as a "Blank".*

RESULTS

***NOTE:** The results in this package insert were calculated by plotting the data on a log vs. log scale using a cubic regression curve-fit. 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 OD for each calibrator, Control, or Unknown.
3. Plot the log of the mean OD readings for each of the Calibrators along the y-axis versus log of the GDF-9 concentrations in pg/mL along the x-axis, using a cubic regression curve-fit.
4. Determine the GDF-9 concentrations of the Controls and unknowns from the calibration curve by matching their mean OD readings with the corresponding GDF-9 concentrations.
5. Any sample reading higher than the highest Calibrator should be appropriately diluted with the 0 pg/mL (CAL A) and re-assayed.
6. Any sample reading lower than the analytical sensitivity should be reported as such.
7. Multiply the value by a dilution factor, if required.

LIMITATIONS

The reagents supplied in this kit are optimized to measure GDF-9 levels in human serum. If there is evidence of microbial contamination or excessive turbidity in a reagent, discard the vial. For assays employing antibodies, the possibility exists for interference by heterophile antibodies in the samples.[13]

QUALITY CONTROL

- Each laboratory should establish mean values and acceptable ranges to assure proper performance.
- GDF-9 ELISA controls or other commercial controls should fall within established confidence limits.
- The confidence limits for GDF-9 controls are printed on the **Calibration Card**.
- A full calibration curve, low and high-level controls, should be included in each assay.
- TMB should be colorless. Development of any color may indicate reagent contamination or instability.
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REPRESENTATIVE CALIBRATION CURVE DATA

Well Number	Well Contents	Mean OD	Conc. (pg/mL)
A1, A2	Calibrators A	(Blank) 0.064	0
B1, B2	B	0.043	48
C1, C2	C	0.17	222
D1, D2	D	0.55	775
E1, E2	E	1.62	2550
F1, F2	F	3.43	5800

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

ANALYTICAL CHARACTERISTICS

Analytical Sensitivity:

The analytical sensitivity in the assay as calculated by the interpolation of mean plus two standard deviations of 16 replicates of calibrator A (0 pg/mL) and calibrator B (48 pg/mL) is 3 pg/mL.

Linearity:

Calibrator F was diluted with calibrator A. The % recovery on individual samples is represented in the following table.

Sample ID	Dilution factor	Expected Value in pg/mL	Observed Value in pg/mL	% Recovery	Average % Recovery
Cal F	NEAT VALUE	5880.0	NA	NA	108%
	2	2900.0	3118.9	108%	
	4	1450.0	1612.2	111%	
	8	725.0	770.4	106%	
	16	362.5	391.2	108%	
	32	181.3	193.7	107%	

Imprecision:

Reproducibility of the GDF-9 ELISA assay was determined in a study using two controls. The study included 9 assays, with a total of 23 replicates per control. Representative data were calculated and is presented in the following table.

Sample	Mean Conc. (pg/mL)	SD	Total %CV
1	392.0	17.2	4.3%
2	3902.0	270.4	6.9%

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Research Use Only

Not for use *in-vitro* diagnostic procedures.

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Ansh Labs
445 Medical Center Blvd.
Webster, TX 77598-4217, U.S.A.