

GDF-9/BMP-15 Complex ELISA

RUO

AL-181-r

INTENDED USE

The GDF-9/BMP-15 Complex Enzyme-Linked Immunosorbent Assay (ELISA) kit provides materials for the quantitative measurement of GDF-9/BMP-15 Complex in follicular fluid. The kit is intended for **research use only**.

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. 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 expression⁶. Altered level and activity of GDF9 is implicated in premature ovarian failure and polycystic ovarian syndrome, implicating GDF9 in the etiology of these conditions⁷⁻⁹.

PRINCIPLE OF THE TEST

The GDF-9/BMP-15 Complex ELISA is a quantitative three-step sandwich type immunoassay. In the first step Calibrators, Controls and unknown samples are added to anti-GDF-9/BMP-15 Complex antibody coated microtiter wells and incubated. After first incubation and washing step, the wells are incubated with biotin labelled antibody conjugate. After a second incubation and washing step, the wells are incubated with streptavidin horseradish peroxidase conjugate (SHRP) solution. 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/BMP-15 Complex in the samples and calibrators.

MATERIALS SUPPLIED

CAL-181A - CAL-181F GDF-9/BMP-15 Complex Calibrators A thru F (Lyophilized)

Six vials, labeled A-F, containing concentrations of approximately 0-5200 AU/mL GDF-9/BMP-15 Complex 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 **1.0 mL** deionized water. Solubilize for **10 minutes**, mix well, and use after reconstitution.

CTR-181-I & CTR-181-II GDF-9/BMP-15 Complex Controls I & II (Lyophilized)

Two vials, labeled Levels I and II containing low and high GDF-9/BMP-15 Complex in a protein-based buffer with non-mercury preservative. 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 **1.0 mL** deionized water. Solubilize for **10 minutes**, mix well, and use after reconstitution.

PLT-181 GDF-9/BMP-15 Complex Antibody Coated Microtitration Strips

One strip holder, containing 12 strips and 96 microtitration wells with GDF-9/BMP-15 Complex 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-181 GDF-9/BMP-15 Complex Assay Buffer

One bottle, 10 mL, containing a protein based (BSA)-buffer with a non-mercury preservative. Store at 2-8°C until expiration date.

CND-179 BMP-15 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.

BCC-181 GDF-9/BMP-15 Complex Biotin Conjugate Concentrate

One vial, 0.4 mL, containing a solution of anti-GDF-9/BMP-15 Complex antibody biotin concentrate in a protein-based buffer with a non-mercury preservative. Dilute prior to use in BMP-15 Conjugate diluent. Store at 2-8°C until expiration date.

NOTE: *The dilution of this reagent should be made 15-30 minutes prior to use in the assay.*

SAR-181 GDF-9/BMP-15 Complex Streptavidin-Enzyme Conjugate Ready-to-Use (RTU)

One bottle, 12 mL, containing streptavidin-HRP (horseradish peroxidase) in a protein-based buffer with 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. Microtitration plate orbital shaker.
3. Microtitration plate 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.**

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.
- d) If external package is damaged, inspect the components inside for any other damage. Do not use if the components are damaged.

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, 2007¹⁰.

WARNING: Potential Chemical Hazard

Some reagents in this kit contain Pro-Clean 400 and Sodium azide¹¹ as a preservative. Pro-Clean 400 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.

PROCEDURAL NOTES

1. A thorough understanding of this package insert is necessary for successful use of the GDF-9/BMP-15 Complex ELISA. It is the responsibility of the customer to validate the assay for their purposes. 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 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 enzyme 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/BMP-15 Complex Calibrators A-F and Controls I & II:** Tap and reconstitute BMP-15 Calibrators A-F and Controls I & II with **1.0 mL** deionized water. Solubilize for **10 minutes**, mix well, and use after reconstitution.
2. **Wash Solution:** Dilute wash concentrate 25-fold with deionized water. The wash solution is stable for one month at room temperature when stored in a tightly sealed bottle.
3. **GDF-9/BMP-15 Complex Antibody-Biotin Conjugate Solution:** The GDF-9/BMP-15 Complex Antibody Biotin Conjugate Concentrate should be diluted at a ratio of 1-part conjugate to 50 parts of BMP-15 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 BMP-15 Conjugate Diluent.
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.

ASSAY PROCEDURE

Allow all specimens and reagents to reach room temperature and mix thoroughly by gentle inversion before use. Calibrators, controls, and unknowns should be assayed in duplicate.

1. Mark the microtitration strips to be used.
2. Pipette **25 μL** of the **Calibrator, Controls and Unknowns** to the appropriate wells.
3. Add **75 μL** of the **GDF-9/BMP-15 Complex Assay Buffer** to each well using a repeater pipette.
4. Incubate the wells, shaking at a fast speed (**600-800 rpm**) on an orbital microplate shaker, for **3 hours** at room temperature ($23 \pm 2^\circ\text{C}$).
5. With 30-40 minutes remaining of incubation time, prepare the GDF-9/BMP-15 Complex Antibody-Biotin Conjugate Solution by diluting the GDF-9/BMP-15 Complex Biotin Conjugate Concentrate in BMP-15 Conjugate Diluent as described under the Preparation of the Reagents section of this insert.
6. Aspirate and wash each well **5 times** with Wash Solution (**350 μL /per well**) using an automatic microplate washer.
7. Add **100 μL** of the **Antibody-Biotin Conjugate solution** to each well using a repeater pipette.
8. Incubate the wells, shaking at a fast speed (**600-800 rpm**) on an orbital microplate shaker, for **1 hour** at room temperature ($23 \pm 2^\circ\text{C}$).
9. Aspirate and wash each well 5 times with the Wash Solution (**350 μL /per well**) using an automatic microplate washer.
10. Add **100 μL** of the **Streptavidin-Enzyme Conjugate-RTU** to each well using a repeater pipette.
11. Incubate the wells, shaking at a fast speed (**600-800 rpm**) on an orbital microplate shaker, for **30 minutes** at room temperature ($23 \pm 2^\circ\text{C}$).
12. Aspirate and wash each well **5 times** with the Wash Solution (**350 μL /per well**) using an automatic microplate washer.
13. Add **100 μL** of the **TMB chromogen solution** to each well using a repeater pipette. Avoid exposure to direct sunlight.
14. Incubate the wells, shaking at **600–800 rpm** on an orbital microplate shaker, for **8-12 minutes** at room temperature ($23 \pm 2^\circ\text{C}$).
NOTE: Visually monitor the color development to optimize the incubation time.
15. 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**.
*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 GDF-9/BMP-15 Complex 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.

- Optimum results can be obtained at incubation temperature of $23 \pm 2^\circ\text{C}$.
- Calculate the mean absorbance for each calibrator, Control, or Unknown. Plot the log of the mean absorbance readings for each of the Calibrators along the y-axis versus log of the GDF-9/BMP-15 Complex concentrations in AU/mL along the x-axis, using a cubic regression curve-fit.
- Determine the GDF-9/BMP-15 Complex concentrations of the Controls and unknowns from the calibration curve by matching their mean absorbance readings with the corresponding GDF-9/BMP-15 Complex concentrations.
- Any sample reading higher than the highest Calibrator should be appropriately diluted with the sample diluent and re-assayed. Multiply the value by a dilution factor.
- Any sample reading lower than the analytical sensitivity should be reported as such.

LIMITATIONS

The reagents supplied in this kit are optimized to measure GDF-9/BMP-15 Complex levels in follicular fluid. 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 samples¹².

QUALITY CONTROL

- Each laboratory should establish mean values and acceptable ranges to assure proper performance.
- GDF-9/BMP-15 Complex controls or other commercial controls should fall within established confidence limits.
- The confidence limits for GDF-9/BMP-15 Complex controls are printed on the **Calibration card**.
- A full calibration curve-, 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	Mean OD	Conc. (AU/mL)
	Calibrators		
A1, A2	A	0.07	0
B1, B2	B	0.09	30
C1, C2	C	0.19	203
D1, D2	D	0.60	824
E1, E2	E	1.37	2142
F1, F2	F	2.94	5200

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

ANALYTICAL CHARACTERISTICS

All analytical characteristics are stated AU/mL.

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 (0 AU/mL) and calibrator B (30 AU/mL) is 8.68 AU/mL.

Imprecision:

Reproducibility of the GDF-9/BMP-15 Complex ELISA assay was determined in a study using two controls. The study included a total of 5 assays, two replicates of each per assay (n=10). Representative data were calculated based on NCCLS EP5-A guidelines and are presented in the following table.

SUMMARY:			Within run		Between run		Total	
Sample	Sample #	Mean	SD	CV	SD	CV	SD	CV
CI	1	528.1	17.5	3.3%	0.0	0.0%	17.5	3.3%
CII	2	1569.0	55.8	3.6%	68.3	4.4%	88.2	5.6%

Linearity:

Multiple dilutions of CAL-181F were diluted in sample diluent (CAL-181A) and three samples containing various GDF-9/BMP-15 complex levels diluted in sample containing lower concentration of GDF-9/BMP-15 complex. The % recovery on CAL-F and individual samples is represented in the following table.

Sample ID	Dilution factor	Expected Conc. (AU/mL)	Observed Conc. (AU/mL)	%Recovery	Average %Recovery
F Dilution	NEAT	5200.0			100%
	2	2600.0	2419.1	93%	
	4	1300.0	1229.6	95%	
	8	650.0	637.5	98%	
	16	325.0	337.1	104%	
	32	162.5	175.8	108%	
S1	2	342.1	320.7	94%	95%
	4	187.9	166.6	89%	
	8	110.9	113.8	103%	
S2	2	396.9	340.7	86%	91%
	4	270.2	244.4	90%	
	8	206.9	200.6	97%	
S3	2	678.1	621.1	92%	91%
	4	356.0	301.7	85%	
	8	194.9	190.5	98%	

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