Quantikine® ELISA

Human/Mouse/Rat Activin A Immunoassay

Catalog Number DAC00B

For the quantitative determination of human, mouse, or rat Activin A concentrations in cell culture supernates, serum, plasma, and saliva.

This package insert must be read in its entirety before using this product.
For research use only. Not for use in diagnostic procedures.

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INTRODUCTION

Activin and Inhibin, members of the TGF-β superfamily of cysteine knot cytokines, were originally purified from gonadal fluids as proteins that stimulated or inhibited, respectively, follicle stimulating hormone (FSH) release from the pituitary. They have since been shown to be involved in a wide range of biological processes including tissue morphogenesis and repair, fibrosis, inflammation, neural development, hematopoiesis, reproductive system function, and carcinogenesis (1-7).

Activin and Inhibin are produced as precursor proteins. Their amino terminal propeptides are proteolytically cleaved and facilitate formation of disulfide-linked dimers of the bioactive proteins (8-10). Activins are non-glycosylated homodimers or heterodimers of various β subunits (βA, βB, βC, and βE in mammals), while Inhibins are heterodimers of a unique α subunit and one of the β subunits. Activin A is a homodimer of two βA chains. The βA subunit can also heterodimerize with a βB or βC subunit to form Activin AB and Activin AC, respectively (11, 12). The 14 kDa mature human βA chain shares 100% amino acid sequence identity with bovine, feline, mouse, porcine, and rat βA. The βA chain is expressed by many cell types including fibroblasts and keratinocytes (13), vascular smooth muscle cells (14), epithelial and endothelial cells (14, 15), hepatocytes (16), osteoclasts and chondrocytes (17, 18), monocytes and macrophages (14, 19), neurons (20), somatic cells of the ovary and testes (21), and anterior pituitary gonadotrophs (22).

Activin A exerts its biological activities by binding to the type 2 serine/threonine kinase Activin RIIA which then noncovalently associates with the type 1 serine/threonine kinase Activin RIB/ALK-4 (7, 23, 24). Signaling through this receptor complex leads to Smad activation and regulation of activin-responsive gene transcription (7, 24). The bioactivity of Activin A is regulated by a variety of mechanisms (24). BAMBI, TGF-β RIII, and Cripto are cell-associated molecules that function as decoy receptors or limit the ability of Activin A to induce receptor complex assembly (25-27). The intracellular formation of Activin A can be prevented by the incorporation of the βA subunit into Activin AC or Inhibin A (3, 11, 12). The bioavailability of Activin A is restricted by its incorporation into inactive complexes with α_2-Macroglobulin, Follistatin, and FLRG (28-30).

The Quantikine® Human/Mouse/Rat Activin A Immunoassay is a 5.0 hour solid-phase ELISA designed to measure Activin A in human, mouse, or rat cell culture supernates, serum, plasma, and saliva. It contains CHO cell-expressed recombinant human/mouse/rat Activin A and has been shown to accurately quantitate the recombinant factor. Results obtained using natural human/mouse/rat Activin A showed linear curves that were parallel to the standard curves obtained using the Quantikine® kit standards. These results indicate that this kit can be used to determine relative mass values for naturally expressed human/mouse/rat Activin A.

PRINCIPLE OF THE ASSAY

This assay employs a 3-step quantitative sandwich enzyme immunoassay technique. The capture antibody is biotinylated and bound to streptavidin-coated plates. The plates are washed and assay diluent, standards, and samples are pipetted into the wells and any Activin A present is bound by the immobilized antibody. After washing away any unbound substances, an HRP-conjugate specific for the βA subunit is added to the wells. Following a wash to remove any unbound conjugate, a substrate solution is added to the wells and color develops in proportion to the amount of Activin A bound. The color development is stopped and the intensity of the color is measured.

LIMITATIONS OF THE PROCEDURE

- FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.
- The kit should not be used beyond the expiration date on the kit label.
- Do not mix or substitute reagents with those from other lots or sources.
- If samples generate values higher than the highest standard, further dilute the samples with calibrator diluent and repeat the assay.
- Any variation in diluent, operator, pipetting technique, washing technique, incubation time or temperature, and kit age can cause variation in binding.
- Variations in sample collection, processing, and storage may cause sample value differences.
- This assay is designed to eliminate interference by other factors present in biological samples. Until all factors have been tested in the Quantikine® Immunoassay, the possibility of interference cannot be excluded.

TECHNICAL HINTS

- When mixing or reconstituting protein solutions, always avoid foaming.
- To avoid cross-contamination, change pipette tips between additions of each standard level, between sample additions, and between reagent additions. Also, use separate reservoirs for each reagent.
- To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary.
- When using an automated plate washer, adding a 30 second soak period following the addition of Wash Buffer, and/or rotating the plate 180 degrees between wash steps may improve assay precision.
- Substrate Solution should remain colorless until added to the plate. Keep Substrate Solution protected from light. Substrate Solution should change from colorless to gradations of blue.
- Stop Solution should be added to the plate in the same order as the Substrate Solution. The color developed in the wells will turn from blue to yellow upon addition of the Stop Solution. Wells that are green in color indicate that the Stop Solution has not mixed thoroughly with the Substrate Solution.

MATERIALS PROVIDED & STORAGE CONDITIONS

Store the unopened kit at 2-8 °C. Do not use past kit expiration date.

PART	PART #	DESCRIPTION	STORAGE OF OPENED/ RECONSTITUTED MATERIAL
Streptavidin Microplate	890649	96 well polystyrene microplate (12 strips of 8 wells) coated with Streptavidin.	Return unused wells to the foil pouch containing the desiccant pack. Reseal along entire edge of the zip-seal. May be stored for up to 1 month at 2-8 °C.*
Activin A Biotinylated Antibody	893686	21 mL of a monoclonal antibody specific for Activin A conjugated to biotin with preservatives.	May be stored for up to 1 month at 2-8 °C.*
Activin Conjugate	890708	21 mL of a monoclonal antibody specific for Activin A conjugated to horseradish peroxidase with preservatives.	
Activin A Standard	890709	Recombinant human Activin A in a buffered protein base with preservatives; lyophilized. *Refer to the vial label for reconstitution volume.*	
Assay Diluent RD1-98	895596	11 mL of a buffered protein base with blue dye and preservative.	
Calibrator Diluent RD5-54	895598	21 mL of a buffered protein base with preservatives.	
Wash Buffer Concentrate	895003	2 vials (21 mL/vial) of a 25-fold concentrated solution of buffered surfactant with preservative. *May turn yellow over time.*	
Color Reagent A	895000	12 mL of stabilized hydrogen peroxide.	
Color Reagent B	895001	12 mL of stabilized chromogen (tetramethylbenzidine).	
Stop Solution	895032	6 mL of 2 N sulfuric acid.	
Plate Sealers	N/A	8 adhesive strips.	

* Provided this is within the expiration date of the kit.

OTHER SUPPLIES REQUIRED

- Microplate reader capable of measuring absorbance at 450 nm, with the correction wavelength set at 540 nm or 570 nm
- Pipettes and pipette tips
- Deionized or distilled water
- Squirt bottle, manifold dispenser, or automated microplate washer
- 1000 mL graduated cylinder
- Collection device for saliva samples that has no protein binding or filtering capabilities such as Salivette® or equivalent
- Horizontal orbital microplate shaker (0.12" orbit) capable of maintaining a speed of 500 ± 50 rpm
- Test tubes for serial dilution of standards
- Human/Mouse/Rat Activin A Controls (optional; R&D Systems®, Catalog # QC33)

PRECAUTIONS

Activin A is detectable in saliva. Take precautionary measures to prevent contamination of kit reagents while running this assay.

The Stop Solution provided with this kit is an acid solution.

Some components in this kit contain a preservative which may cause an allergic skin reaction. Avoid breathing mist.

Color Reagent B may cause skin, eye, and respiratory irritation. Avoid breathing fumes.

Wear protective gloves, clothing, eye, and face protection. Wash hands thoroughly after handling. Refer to the SDS on our website prior to use.

SAMPLE COLLECTION & STORAGE

The sample collection and storage conditions listed below are intended as general guidelines. Sample stability has not been evaluated.

Cell Culture Supernates - Remove particulates by centrifugation and assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Note: *Culture media containing biotin, such as RPMI and McCoy's media, are not suitable for use in this assay. Animal serum used in the preparation of cell culture media may contain endogenous levels of Activin A. For best results, do not use animal serum for growth of cell cultures when assaying for Activin A production. If animal serum is used as a supplement in the media, precautions should be taken to prepare the appropriate control and run the control in the immunoassay to determine the baseline concentration of Activin A.*

Human Serum - Use a serum separator tube (SST) and allow samples to clot for 30 minutes at room temperature before centrifugation for 15 minutes at 1000 x g. Remove serum and assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Mouse Serum - Allow blood samples to clot for 2 hours at room temperature before centrifuging for 20 minutes at 2000 x g. Remove serum and assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Rat Serum - Allow blood samples to clot for 2 hours at room temperature before centrifugation for 30 minutes at 1000 x g. Remove serum and assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Plasma - Collect plasma using EDTA or heparin as an anticoagulant. Centrifuge for 15 minutes at 1000 x g within 30 minutes of collection. Assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Note: *Citrate plasma has not been validated for use in this assay.*
Grossly hemolyzed or lipemic samples are not suitable for use in this assay.

Saliva - Collect saliva using a collection device such as a Salivette or equivalent. Assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Note: *Saliva collector must not have any protein binding or filtering capabilities.*

SAMPLE PREPARATION

Serum, plasma, and saliva samples require at least a 2-fold dilution. A suggested 2-fold dilution is 150 µL of sample + 150 µL of Calibrator Diluent RD5-54.

REAGENT PREPARATION

Bring all reagents to room temperature before use.

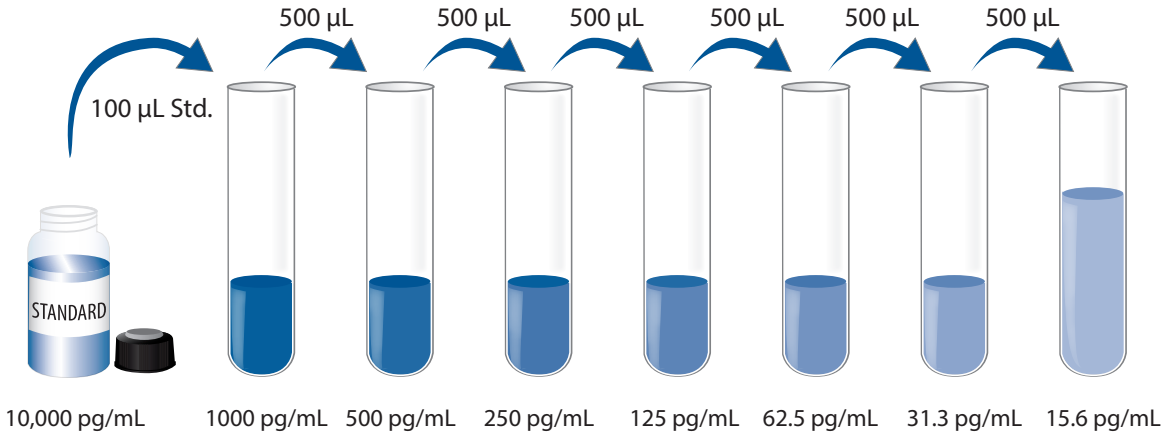
Note: *High concentrations of Activin A are found in saliva. It is recommended that a face mask and gloves be used to protect kit reagents from contamination.*

Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Add 40 mL of Wash Buffer Concentrate to 960 mL of deionized or distilled water to prepare 1000 mL of Wash Buffer.

Substrate Solution - Color Reagents A and B should be mixed together in equal volumes within 15 minutes of use. Protect from light. 200 µL of the resultant mixture is required per well.

Activin A Standard - **Refer to the vial label for reconstitution volume.** Reconstitute the Activin A Standard with deionized or distilled water. This reconstitution produces a stock solution of 10,000 pg/mL. Allow the standard to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions.

Pipette 900 µL of Calibrator Diluent RD5-54 into the 1000 pg/mL tube. Pipette 500 µL into the remaining tubes. Use the stock solution to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The 1000 pg/mL standard serves as the high standard. Calibrator Diluent RD5-54 serves as the zero standard (0 pg/mL).



MICROPLATE PREPARATION

1. Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, and reseal.
2. Pipette 200 µL of the Activin A Biotinylated Antibody into all wells. Securely cover and incubate for 15 minutes at room temperature on a horizontal orbital microplate shaker (0.12" orbit) set at 500 ± 50 rpm.
3. Aspirate each well and wash, repeating the process for a total of two washes. Wash by filling each well with Wash Buffer (400 µL) using a squirt bottle, manifold dispenser, or autowasher. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.
4. Proceed to the Assay Procedure immediately after wash. Do not allow the wells to dry.

ASSAY PROCEDURE

Bring all reagents and samples to room temperature before use. It is recommended that all standards, controls, and samples be assayed in duplicate.

Note: *High concentrations of Activin A are found in saliva. It is recommended that a face mask and gloves be used to protect kit reagents from contamination.*

1. Prepare all reagents, working standards, microplate, and samples as directed in the previous sections.
2. Add 100 µL of Assay Diluent RD1-98 to each well.
3. Add 100 µL of standard, control, or sample* to each well. A plate layout is provided to record standards and samples assayed.
4. Cover with the adhesive strip provided. Incubate for 3 hours at room temperature on a horizontal orbital microplate shaker (0.12″ orbit) set at 500 ± 50 rpm.
5. Aspirate each well and wash, repeating the process five times for a total of six washes. Wash by filling each well with Wash Buffer (400 µL) using a squirt bottle, manifold dispenser, or autowasher. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper toweling.
6. Add 200 µL of Activin A Conjugate to each well. Cover with a new adhesive strip. Incubate for 1 hour at room temperature on the shaker.
7. Repeat the aspiration/wash as in step 5.
8. Add 200 µL of Substrate Solution to each well. Incubate for 30 minutes at room temperature **on the benchtop. Protect from light.**
9. Add 50 µL of Stop Solution to each well. The color in the wells should change from blue to yellow. If the color in the wells is green or the color change does not appear uniform, gently tap the plate to ensure thorough mixing.
10. Determine the optical density of each well within 30 minutes, using a microplate reader set to 450 nm. If wavelength correction is available, set to 540 nm or 570 nm. If wavelength correction is not available, subtract readings at 540 nm or 570 nm from the readings at 450 nm. This subtraction will correct for optical imperfections in the plate. Readings made directly at 450 nm without correction may be higher and less accurate.

*Samples may require dilution. See Sample Preparation section.

CALCULATION OF RESULTS

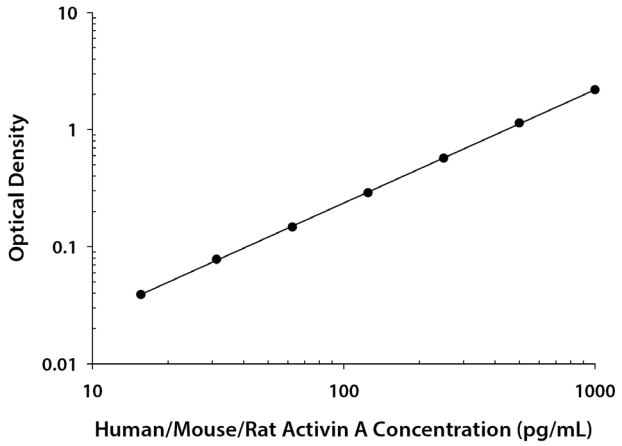
Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density (O.D.).

Create a standard curve by reducing the data using computer software capable of generating a log/log curve-fit. As an alternative, construct a standard curve by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis and draw a best fit curve through the points on a log/log graph. The data may be linearized by plotting the log of the human/mouse/rat Activin A concentrations versus the log of the O.D. on a linear scale, and the best fit line can be determined by regression analysis.

If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

TYPICAL DATA

This standard curve is provided for demonstration only. A standard curve should be generated for each set of samples assayed.



(pg/mL)	O.D.	Average	Corrected
0	0.051 0.051	0.051	—
15.6	0.089 0.090	0.090	0.039
31.3	0.127 0.130	0.129	0.078
62.5	0.196 0.199	0.198	0.147
125	0.330 0.350	0.340	0.289
250	0.601 0.639	0.620	0.569
500	1.180 1.199	1.190	1.139
1000	2.207 2.258	2.233	2.182

PRECISION

Intra-Assay Precision (Precision within an assay)
Three samples of known concentration were tested twenty times on one plate to assess intra-assay precision.

Inter-Assay Precision (Precision between assays)
Three samples of known concentration were tested in forty separate assays to assess inter-assay precision. Assays were performed by at least three technicians using two lots of components.

	Intra-Assay Precision			Inter-Assay Precision		
Sample	1	2	3	1	2	3
n	20	20	20	40	40	40
Mean (pg/mL)	101	295	498	106	308	529
Standard deviation	4.2	12.5	21.9	8.4	15.5	24.8
CV (%)	4.2	4.2	4.4	7.9	5.0	4.7

RECOVERY

The recovery of human/mouse/rat Activin A spiked to three different levels in human samples throughout the range of the assay was evaluated.

Sample Type	Average % Recovery	Range
Cell culture media (n=4)	105	93-114%
Serum* (n=4)	109	100-115%
EDTA plasma* (n=4)	110	95-115%
Heparin plasma* (n=4)	107	99-113%

*Samples were diluted prior to assay.

LINEARITY

To assess the linearity of the assay, human samples containing and/or spiked with high concentrations of human/mouse/rat Activin A were diluted with the calibrator diluent to produce samples with values within the dynamic range of the assay.

		Cell culture supernates* (n=4)	Serum* (n=4)	EDTA plasma* (n=4)	Heparin plasma* (n=4)	Saliva* (n=4)
1:2	Average % of Expected	99	107	102	109	103
	Range (%)	93-106	105-111	101-104	104-112	99-109
1:4	Average % of Expected	97	108	104	112	91
	Range (%)	90-103	101-115	100-111	109-114	82-98
1:8	Average % of Expected	96	110	107	112	93
	Range (%)	89-108	107-119	102-116	103-118	———
1:16	Average % of Expected	102	101	98	105	———
	Range (%)	96-108	94-116	88-114	88-120	———

*Samples were diluted prior to assay.

SENSITIVITY

Forty assays were evaluated and the minimum detectable dose (MDD) of human/mouse/rat Activin A ranged from 0.75-7.85 pg/mL. The mean MDD was 3.67 pg/mL.

The MDD was determined by adding two standard deviations to the mean O.D. value of twenty zero standard replicates and calculating the corresponding concentration.

CALIBRATION

This immunoassay is calibrated against a highly purified CHO cell-expressed recombinant human/mouse/rat Activin A produced at R&D Systems®.

The NIBSC/WHO Activin A First Reference Reagent Preparation (91/626), which is intended as a potency standard, was evaluated in this kit. The dose response curve of this standard parallels the Quantikine® Activin A standard curve. To convert sample values obtained with the Quantikine® Human/Mouse/Rat Activin A assay to approximate NIBSC 91/626 nominally assigned mass values, use the equation below.

NIBSC (91/626) approximate value (mU/mL) = 1.185 x Quantikine® Activin A value (pg/mL)

SAMPLE VALUES

Serum/Plasma/Saliva - Samples were evaluated for the presence of human/mouse/rat Activin A in this assay. No medical histories were available for the donors used in this study.

Human Samples	Mean (pg/mL)	Range (pg/mL)	Standard Devation (pg/mL)
Serum (n=35)	352	142-753	129
EDTA plasma (n=35)	319	115-665	124
Heparin plasma (n=35)	316	111-695	126

Human Samples	Mean of Detectable (pg/mL)	% Detectable	Range (pg/mL)
Saliva (n=13)	192	85	ND-428

Mouse Samples	Mean (pg/mL)	Range (pg/mL)	Standard Devation (pg/mL)
Serum (n=20)	197	78.1-352	93.1
EDTA plasma (n=20)	254	136-471	84.2
Heparin plasma (n=20)	188	130-272	46.0

Rat Samples	Mean (pg/mL)	Range (pg/mL)	Standard Devation (pg/mL)
Serum (n=8)	306	109-1466	469
EDTA plasma (n=9)	214	97.7-312	63.9
Heparin plasma (n=7)	276	210-342	42.9

Cell Culture Supernates:

Note: *Cell culture supernates were not corrected for the presence of fetal bovine serum.*

IMR-90 human fetal lung fibroblasts were cultured in MEM with 10% fetal bovine serum until confluent. An aliquot was removed, assayed for human/mouse/rat Activin A, and measured 5608 pg/mL.

WS-1 human fetal skin fibroblasts were cultured in MEM with 10% fetal bovine serum, NEAA, and 2 mM L-glutamine until confluent. Cells were then stimulated for an additional 24 hours with 100 ng/mL LPS, 10 ng/mL recombinant human IL-1β, or 10 ng/mL recombinant human TNF-α. Aliquots were removed and assayed for levels of human/mouse/rat Activin A.

Stimulant	Day 1 (pg/mL)
LPS	8489
IL-1β	21,033
TNF-α	21,098

CMT-93 mouse rectal carcinoma cells were cultured in MEM with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin sulfate until confluent. An aliquot was removed, assayed for human/mouse/rat Activin A, and measured 655 pg/mL.

SAMPLE VALUES *CONTINUED*

ST-2 mouse bone marrow-derived stromal cells were cultured in DMEM with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin sulfate. An aliquot was removed, assayed for human/mouse/rat Activin A, and measured 3156 pg/mL.

LL/2 mouse Lewis lung carcinoma cells were cultured in DMEM with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin sulfate. An aliquot was removed, assayed for human/mouse/rat Activin A, and measured 44.3 pg/mL.

C6 rat glioma cells were cultured in DMEM with 10% fetal bovine serum and 2 mM L-glutamine until confluent. An aliquot was removed, assayed for human/mouse/rat Activin A, and measured 1559 pg/mL.

C58(NT)D rat thymic lymphoma cells were cultured in DMEM with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, and 4.5 g/L glucose until confluent. An aliquot was removed, assayed for human/mouse/rat Activin A, and measured 21.0 pg/mL.

SPECIFICITY

This assay recognizes natural and recombinant human/mouse/rat Activin A.

The factors listed below were prepared at 50 ng/mL in calibrator diluent and assayed for cross-reactivity. Preparations of the following factors at 50 ng/mL in a mid-range recombinant human/mouse/rat Activin A control were assayed for interference. No significant cross-reactivity or interference was observed.

Recombinant human:

Activin AC Heterodimer
Activin B
Activin C
Activin RIA
Activin RIB
Activin RIIA
Activin RIIB
ALK-1
BAMBI
BMPRII
BMP-1/PCP
BMP-2
BMP-3
BMP-3b/GDF-10
BMP-4
BMP-5
BMP-6
BMP-8b
BMP-10
BMP-15
Cripto
DAN
Endoglin
Follistatin 288
Follistatin 300
Follistatin 315
Inhibin B
Lefty A
MIS
Osteoactivin
LAP
TGF-α
TGF-β1
TGF-β1.2
TGF-β2
TGF-β3
TGF-β RII
TGF-β RIII/betaglycan

Recombinant mouse:

Activin C
Activin RIB
Activin RIIB
ALK-1
BAMBI
BMPRIA
BMPRIB
BMP-3b/GDF-10
Cripto
DAN
Endoglin
Lefty
MIS
Noggin
Osteoactivin

Recombinant rat:

Agrin
ALK-7
MIS

Other recombinants:

zebrafish BMP-2
porcine TGF-β2
amphibian TGF-β5

Natural proteins:

porcine TGF-β1

Recombinant human Inhibin A cross-reacts approximately 0.2% in this assay.

Recombinant human Activin AB cross-reacts approximately 0.45% in this assay.

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PLATE LAYOUT

Use this plate layout to record standards and samples assayed.