Quantikine® ELISA

Human SPARC Immunoassay

Catalog Number DSP00

For the quantitative determination of human Secreted Protein, Acidic and Rich in Cysteine (SPARC) concentrations in cell culture supernates, serum, plasma, and human milk.

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

SPARC, an acronym for "Secreted Protein, Acidic and Rich in Cysteine", is also known as osteonectin or BM-40 (1). It is the founding member of a family of secreted matricellular proteins with similar domain structure. The 286 amino acid (aa), 43 kDa protein contains an N-terminal acidic region that binds calcium, a follistatin domain that contains Kazal-like sequences, and a C-terminal extracellular calcium (EC) binding domain with two EF-hand motifs (1-6). Crystal structure modeling shows that residues implicated in cell binding, inhibition of cell spreading, and disassembly of focal adhesions cluster on one face of SPARC, while collagen binding residues (R166, N173, L259, M262, and E263) and a glycosylation site (N115) are opposite this face (3, 4, 7, 8). Proteolytic cleavage within, or deletion of, helix C in the EC domain (aa 213-220) enhances binding to fibrillar collagens such as type I and IV by 10-fold (4, 7, 9). Mature human SPARC shows 92%, 92%, 97%, 99%, and 96% aa identity with mouse, rat, canine, bovine, and porcine SPARC, respectively.

SPARC is produced by fibroblasts, capillary endothelial cells, platelets, macrophages, adipocytes, and a variety of other cells during tissue morphogenesis and remodeling (5, 10-13). SPARC is abundantly expressed in bone, where it promotes osteoblast differentiation, inhibits adipogenesis, and inhibits osteoclast response to parathyroid hormone (6, 13-15). In obesity and diabetes, SPARC expression is correlated with leptin, inversely correlated with adiponectin, and associated with diabetic retinopathy and nephropathy (11, 13, 16). SPARC inhibits adipogenesis in adipose tissue, and deletion in mice increases their adiposity (11, 13, 17). SPARC can have both tumorigenic and tumor suppressing activities (12). It is highly expressed in many tumor types undergoing an endothelial to mesenchymal transition but downregulated by gene methylation in many tumors (12, 18, 19). Its expression in the tumor itself can decrease the likelihood of metastasis and confer sensitivity to chemotherapy and radiation, but expression by the surrounding macrophages and stroma can enhance angiogenesis and metastasis (12, 18-22).

Although SPARC shows context-specific effects, it generally inhibits cell adhesion, spreading and proliferation, and promotes collagen matrix formation (5, 6, 11, 23). SPARC-null mice show defects in skin collagen strength and premature cataract formation due to faulty collagen fibril assembly (24-26). Decreased SPARC binding to collagen is implicated in some forms of osteogenesis imperfecta, while increased SPARC-mediated collagen deposition is implicated in fibrosis, particularly in the lung (8, 24, 27). In idiopathic pulmonary fibrosis, lung fibroblast overexpression of SPARC may activate cell signaling pathways leading to impaired epithelial repair and resistance to apoptosis (27). SPARC aids cardiac repair after myocardial infarction, but also contributes to pressure overload-induced myocardial hypertrophy (28, 29). In addition to binding collagens, SPARC also binds fibroblast β1 integrins via its follistatin domain and increases the activity of the integrin-linked kinase (ILK), leading to enhanced fibronectin matrix assembly (17, 23, 30, 31). For endothelial cells, SPARC can disrupt focal adhesions and discourage angiogenesis by binding and sequestering PDGF and VEGF (5). However, an angiogenic SPARC peptide can be produced by metalloproteinase cleavage (10). For epithelial cells, SPARC or its EC fragment can inhibit proliferation by activating a TGF-β signaling pathway; conversely, TGF-β enhances SPARC expression (32). Stabilin-1, which is expressed on alternately activated macrophages, is reported to bind the SPARC EC domain and mediate endocytosis for degradation (33).

The Quantikine® Human SPARC Immunoassay is a 4.5 hour solid-phase ELISA designed to measure human SPARC in cell culture supernates, serum, plasma, and human milk. It contains NS0-expressed recombinant human SPARC and has been shown to accurately quantitate the recombinant factor. Results obtained using natural human SPARC 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 natural human SPARC.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for human SPARC has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any SPARC present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for human SPARC is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of SPARC bound in the initial step. 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
Human SPARC Microplate	892353	96 well polystyrene microplate (12 strips of 8 wells) coated with a monoclonal antibody specific for human SPARC.	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.*
Human SPARC Conjugate	892354	21 mL of a polyclonal antibody specific for human SPARC conjugated to horseradish peroxidase with preservatives.	May be stored for up to 1 month at 2-8 °C.*
Human SPARC Standard	892355	Recombinant human SPARC in a buffer with preservatives; lyophilized. *Refer to the vial label for reconstitution volume.*	
Assay Diluent RD1-60	895328	11 mL of a buffered protein solution with blue dye and preservatives.	
Calibrator Diluent RD6-59	895071	21 mL of buffered animal serum 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	4 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.
- Horizontal orbital microplate shaker (0.12" orbit) capable of maintaining a speed of 500 ± 50 rpm.
- Refrigerator for incubation at 2-8 °C.
- Test tubes for dilution of standards and samples.
- Human SPARC Controls (optional; R&D Systems®, Catalog # QC160).

PRECAUTIONS

SPARC 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: *Animal serum used in the preparation of cell culture media may contain endogenous levels of SPARC. For best results, do not use animal serum for growth of cell cultures when assaying for SPARC 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 SPARC.*

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.

Plasma - Collect plasma on ice using EDTA or heparin as an anticoagulant. Centrifuge for 15 minutes at 2-8 °C at 1000 x g within 30 minutes of collection. An additional centrifugation step of the plasma at 10,000 x g for 10 minutes at 2-8 °C is recommended for complete platelet removal. Assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

SPARC is present in platelet granules and is released upon platelet activation. Therefore, to measure circulating levels of SPARC, platelet-free plasma should be collected for measurement. It should be noted that many protocols for plasma preparation, including procedures recommended by the Clinical Laboratory and Standards Institute (CLSI), result in incomplete removal of platelets from blood.

Note: *Citrate plasma has not been validated for use in this assay.*
Hemolyzed samples are not suitable for use in this assay.

Human Milk - Centrifuge for 15 minutes at 1000 x g at 2-8 °C. Collect the aqueous fraction and centrifuge twice more for a total of 3 times. Assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

SAMPLE PREPARATION

Cell culture supernate samples require at least an 8-fold dilution. A suggested 8-fold dilution is 40 µL of sample + 280 µL of Calibrator Diluent RD6-59.

Serum samples require at least a 40-fold dilution. A suggested 40-fold dilution is 10 µL of sample + 390 µL of Calibrator Diluent RD6-59.

Plasma samples require at least an 8-fold dilution. A suggested 8-fold dilution is 40 µL of sample + 280 µL of Calibrator Diluent RD6-59.

Human milk samples require at least a 2-fold dilution. A suggested 2-fold dilution is 150 µL of sample + 150 µL of Calibrator Diluent RD6-59.

REAGENT PREPARATION

The conjugate must be kept cold during use. Bring all other reagents to room temperature before use.

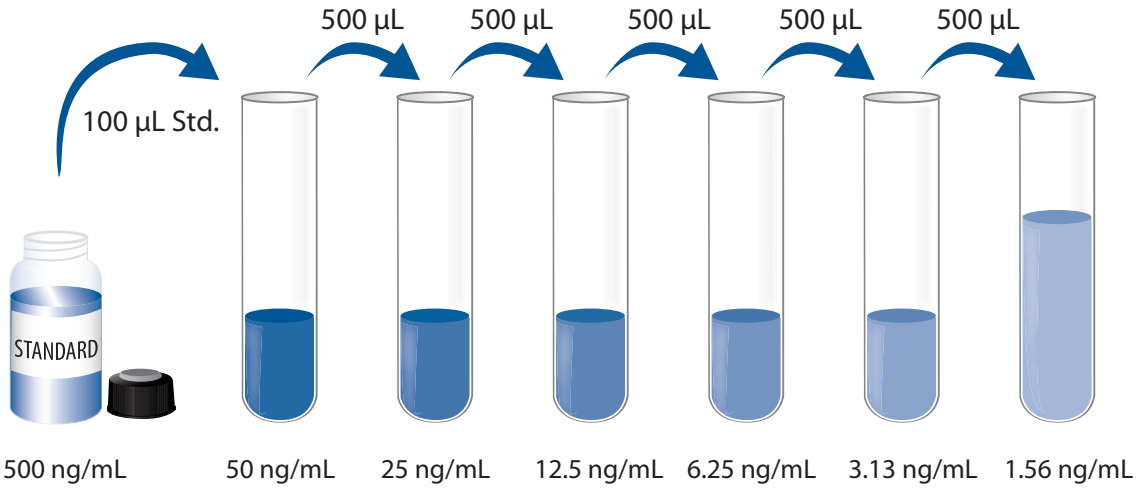
Note: *SPARC is detectable in saliva. It is recommended that a face mask and gloves be used to protect kit reagents from contamination.*

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.

Human SPARC Standard - **Refer to the vial label for reconstitution volume.** Reconstitute the Human SPARC Standard with deionized or distilled water. This reconstitution produces a stock solution of 500 ng/mL. Mix the standard to ensure complete reconstitution and allow the standard to sit for a minimum of 15 minutes. Mix well prior to making dilutions.

Pipette 900 µL of Calibrator Diluent RD6-59 into the 50 ng/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 50 ng/mL standard serves as the high standard. Calibrator Diluent RD6-59 serves as the zero standard (0 ng/mL).



ASSAY PROCEDURE

The conjugate must be kept cold during use. Bring all other reagents and samples to room temperature before use. It is recommended that all standards, controls, and samples be assayed in duplicate.

Note: *SPARC is detectable 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, and samples as directed in the previous sections.
2. Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, and reseal.
3. Add 100 µL of Assay Diluent RD1-60 to each well.
4. Add 100 µL of standard, control, or sample* per well. 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 towels.
6. Add 200 µL of **cold** Human SPARC Conjugate to each well. Cover with a new adhesive strip. Incubate for **1 hour at 2-8 °C without shaking.**
7. Repeat the aspiration/wash as in step 5.
8. Add 200 µL of Substrate Solution to each well. **Protect from light.** Incubate for 30 minutes at room temperature **on the benchtop.**
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 require dilution. See Sample Preparation section.

CALCULATION OF RESULTS

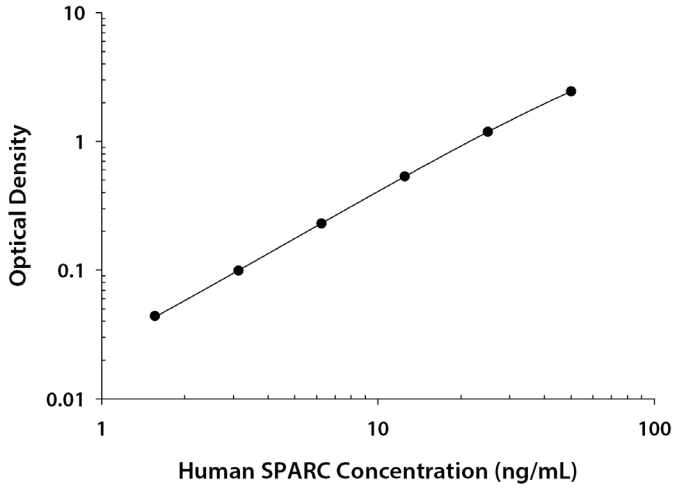
Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density (O.D.).

Create a standard curve by reducing the data using computer software capable of generating a four parameter logistic (4-PL) 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 the graph. The data may be linearized by plotting the log of the human SPARC concentrations versus the log of the O.D. and the best fit line can be determined by regression analysis. This procedure will produce an adequate but less precise fit of the data.

Since 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.



(ng/mL)	O.D.	Average	Corrected
0	0.031 0.031	0.031	—
1.56	0.074 0.076	0.075	0.044
3.13	0.128 0.131	0.130	0.099
6.25	0.258 0.264	0.261	0.230
12.5	0.559 0.571	0.565	0.534
25	1.195 1.238	1.217	1.186
50	2.450 2.504	2.477	2.446

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 twenty 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	20	20	20
Mean (ng/mL)	7.34	14.3	29.9	6.65	13.5	28.3
Standard deviation	0.129	0.282	0.733	0.541	0.989	2.40
CV (%)	1.8	2.0	2.5	8.1	7.3	8.5

RECOVERY

The recovery of human SPARC spiked to levels throughout the range of the assay in various matrices was evaluated. Samples were diluted prior to this assay.

Sample Type	Average % Recovery	Range
Cell culture media (n=4)	102	86-111%
Platelet-poor EDTA plasma (n=4)	104	93-111%
Platelet-poor heparin plasma (n=4)	91	82-100%

LINEARITY

To assess the linearity of the assay, samples containing and/or spiked with high concentrations of human SPARC were serially diluted with calibrator diluent to produce samples with values within the dynamic range of the assay. Samples were diluted prior to this assay.

		Cell culture supernates (n=4)	Serum (n=4)	Platelet-poor EDTA plasma (n=4)	Platelet-poor Heparin plasma (n=4)	Human milk (n=4)
1:2	Average % of Expected	102	100	97	100	92
	Range (%)	101-103	94-104	92-105	94-104	89-95
1:4	Average % of Expected	98	98	92	101	89
	Range (%)	95-101	89-106	88-95	93-108	86-92
1:8	Average % of Expected	94	96	94	105	———
	Range (%)	91-97	88-101	89-98	97-110	———
1:16	Average % of Expected	90	94	95	107	———
	Range (%)	88-92	91-97	90-99	99-113	———

SENSITIVITY

Twenty-nine assays were evaluated and the minimum detectable dose (MDD) of human SPARC ranged from 0.044-0.269 ng/mL. The mean MDD was 0.099 ng/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 NS0-expressed recombinant human SPARC produced at R&D Systems®.

SAMPLE VALUES

Serum/Plasma/Human Milk - Samples from apparently healthy volunteers were evaluated for the presence of human SPARC in this assay. No medical histories were available for the donors used in this study.

Sample Type	Mean (ng/mL)	Range (ng/mL)	Standard Deviation (ng/mL)
Serum (n=35)	913	322-1420	189
Platelet-poor EDTA plasma (n=35)	35.8	20.4-96.8	13.8
Platelet-poor heparin plasma (n=35)	57.8	20.2-123	25.2
Human milk (n=8)	11.7	5.81-18.5	4.77

Cell Culture Supernates:
Human peripheral blood leukocytes (PBL) were cultured in RPMI 1640 and supplemented with 10% fetal bovine serum. Cells were then cultured unstimulated or stimulated with 10 µg/mL PHA for 1 or 6 days. Aliquots of the cell culture supernates were removed and assayed for levels of human SPARC.

Sample Type	Day 1 (ng/mL)	Day 6 (ng/mL)
PBL unstimulated*	30.0	38.6
PBL stimulated w/PHA*	30.9	35.0

MG-63 human osteosarcoma cells were cultured in MEM/NEAA and supplemented with 10% fetal bovine serum. An aliquot of the cell culture supernate was removed, assayed for human SPARC, and measured 54.0 ng/mL.*

IMR-90 human lung fibroblast cells were cultured in MEM and supplemented with 10% fetal bovine serum. An aliquot of the cell culture supernate was removed, assayed for human SPARC, and measured 238 ng/mL.*

**SPARC values reported have not been corrected for endogenous SPARC contained in the cell culture media supplemented with 10% FBS.*

SPECIFICITY

This assay recognizes natural and recombinant human SPARC.

The factors listed below were prepared at 500 ng/mL in calibrator diluent and assayed for cross-reactivity. Preparations of the following factors at 500 ng/mL in a mid-range recombinant human SPARC control were assayed for interference. No significant cross-reactivity or interference was observed.

Recombinant human:

Glypican-3
PDGF-AA
PDGF-AB
PDGF-BB
SPARC-like 1
Stabilin-1
VCAM-1
$VEGF_{111}$
$VEGF_{121}$
$VEGF_{165}$

Recombinant mouse:

SPARC
SPARC-like 1

Natural proteins:

human PDGF

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