Quantikine® ELISA

Human Glypican 3 Immunoassay

Catalog Number DGLY30

For the quantitative determination of human Glypican 3 concentrations in cell culture supernates, cell lysates, serum, and plasma.

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

Glypicans (GPCs) are a family of heparan sulfate proteoglycans that are attached to the cell surface by a glycosylphosphatidylinositol (GPI) anchor (1). Six members of this family have been identified in mammals (GPC1-GPC6) (2). All Glypican core proteins contain an N-terminal signal peptide, a large globular cysteine-rich domain (CRD) with 14 invariant cysteine residues, a stalk-like region containing the heparan sulfate attachment sites, and a C-terminal GPI attachment site (1, 3). Glypican proteins do not share strong amino acid (aa) sequence identity, but the conserved cysteine residues in their CRDs suggest similarity in their three-dimensional structure (3-5).

Human Glypican 3 is a 580 aa, approximately 70 kDa (predicted) protein that is expressed in the fetal lung, liver, and kidney (6, 7). Unlike most GPI-anchored proteins, Glypican 3 is found mostly outside cholesterol-sphingolipid-rich membrane domains called lipid rafts (8, 9). Glypican 3 is also detectable in the extracellular environment following release by Notum, a lipase that cleaves GPI anchors (10, 11). Glypican 3 has been shown to be involved in the regulation of Hedgehog and Wnt signaling pathways. It negatively regulates Hedgehog signaling by competing with Patched for Hedgehog binding (12, 13). Alternatively, Glypican 3 reportedly binds Frizzled to stimulate canonical Wnt signaling (14).

Glypican 3 appears to play an important role in hepatocellular carcinoma (HCC). It is expressed in HCC, but not normal hepatocytes (15, 16). Furthermore, expression of Glypican 3 promotes HCC growth *in vivo* and is associated with poor prognosis (17-20). Soluble Glypican 3 is also significantly increased in the serum of HCC patients, but is undetectable in healthy donors (15, 21). Mutations in GPC3 cause Simpson-Golabi-Behmel syndrome (SGBS), an X-linked condition characterized by pre- and postnatal overgrowth with visceral and skeletal abnormalities (6, 22).

The Quantikine Human Glypican 3 Immunoassay is a 4.5 hour solid phase ELISA designed to measure Glypican 3 levels in cell culture supernates, cell lysates, serum, and plasma. It contains NS0-expressed recombinant human Glypican 3 and antibodies raised against the recombinant protein. Results obtained for naturally occurring human Glypican 3 showed linear curves that were parallel to the standard curves obtained using the Quantikine Human Glypican 3 Immunoassay standards. These results indicate that this kit can be used to determine relative mass values for natural human Glypican 3.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for human Glypican 3 has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any Glypican 3 present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for human Glypican 3 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 Glypican 3 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, dilute the samples with Calibrator Diluent and repeat the assay.
- Any variation in standard 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 Glypican 3 Microplate	894805	96 well polystyrene microplate (12 strips of 8 wells) coated with a monoclonal antibody specific for human Glypican 3.	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 Glypican 3 Standard	894807	2 vials of recombinant human Glypican 3 in a buffered protein base with preservatives; lyophilized. *Refer to the vial label for reconstitution volume.*	Discard after use. Use a new standard for each assay.
Human Glypican 3 Conjugate	894806	21 mL of a polyclonal antibody specific for human Glypican 3 conjugated to horseradish peroxidase with preservatives.	May be stored for up to 1 month at 2-8 °C.*
Assay Diluent RD1-56	895102	12 mL of a buffered protein base with preservatives.	
Calibrator Diluent RD5P Concentrate	895151	21 mL of a concentrated buffered protein base with preservatives. *Use diluted 1:5 in this assay.*	
Wash Buffer Concentrate	895003	21 mL 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.
- 100 mL and 500 mL graduated cylinders.
- Horizontal orbital microplate shaker (0.12" orbit) capable of maintaining a speed of 500 ± 50 rpm
- Test tubes for dilution of standards and samples.
- Human Glypican 3 Controls (optional; R&D Systems, Catalog # QC203).

SUPPLIES REQUIRED FOR CELL LYSATE SAMPLES

- Cell Lysis Buffer 1 (R&D Systems, Catalog # 890713).
- PBS

PRECAUTIONS

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

Some components in this kit contain ProClin® 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. Please refer to the MSDS 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.

Cell Lysates - Cells must be lysed prior to assay as directed in the Cell Lysis Procedure.

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 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 icteric samples are not suitable for use in this assay.

CELL LYSIS PROCEDURE

Use the following procedure for the preparation of cell lysate samples.

1. Wash cells three times in cold PBS.
2. Resuspend cells at 1 x 10^7 cells/mL in Cell Lysis Buffer 1.
3. Incubate with gentle agitation for up to 60 minutes at room temperature.
4. Centrifuge at 8000 x g for 10 minutes to remove cell debris.
5. Assay immediately or aliquot the lysis supernates and store at ≤ -70 °C until ready for use.

SAMPLE PREPARATION

Cell lysate samples require at least a 5-fold dilution. A suggested 5-fold dilution is 60 µL of sample + 240 µL of Calibrator Diluent RD5P (diluted 1:5)*.

*See Reagent Preparation section.

REAGENT PREPARATION

Bring all reagents to room temperature before use.

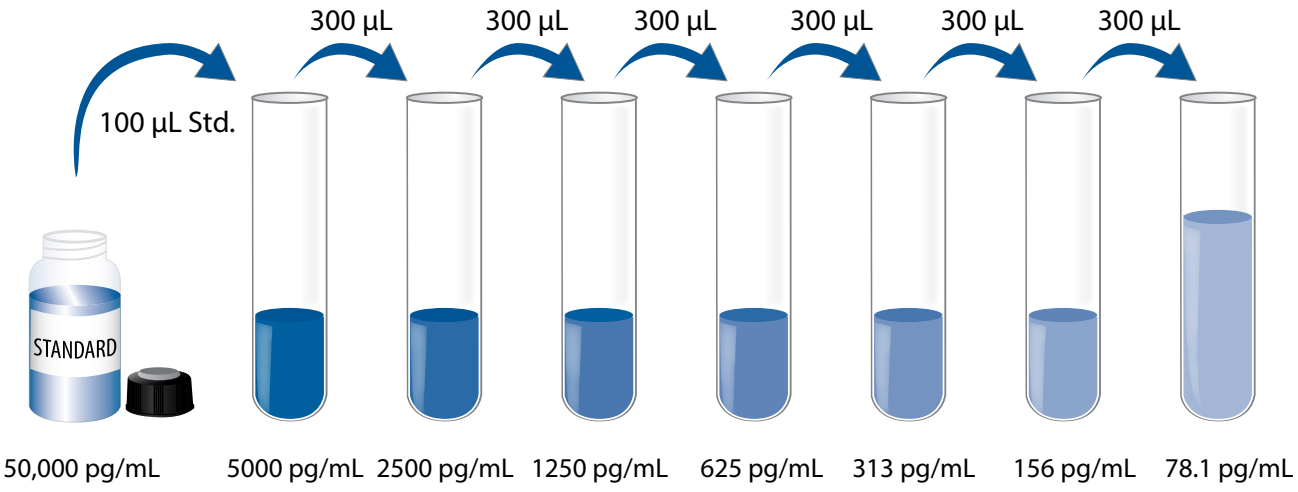
Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Add 20 mL of Wash Buffer Concentrate to deionized or distilled water to prepare 500 mL of Wash Buffer.

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.

Calibrator Diluent RD5P (diluted 1:5) - Add 20 mL of Calibrator Diluent RD5P Concentrate to 80 mL of deionized or distilled water to prepare 100 mL of Calibrator Diluent RD5P (diluted 1:5).

Human Glypican 3 Standard - **Refer to the vial label for reconstitution volume.** Reconstitute the Human Glypican 3 Standard with deionized or distilled water. This reconstitution produces a stock solution of 50,000 pg/mL. Mix the standard to ensure complete reconstitution and allow the standard to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions.

Pipette 900 µL of Calibrator Diluent RD5P (diluted 1:5) into the 5000 pg/mL tube. Pipette 300 µL into the remaining tubes. Use the stock solution to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The 5000 pg/mL standard serves as the high standard. Calibrator Diluent RD5P (diluted 1:5) serves as the zero standard (0 pg/mL).



ASSAY PROCEDURE

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

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 RD1-56 to each well.
4. Add 100 µL of Standard, control, or sample* per well. Cover with the adhesive strip provided. Incubate for 2 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 three times for a total of four 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 Human Glypican 3 Conjugate to each well. Cover with a new adhesive strip. Incubate for 2 hours 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 the Sample Preparation section.

CALCULATION OF RESULTS

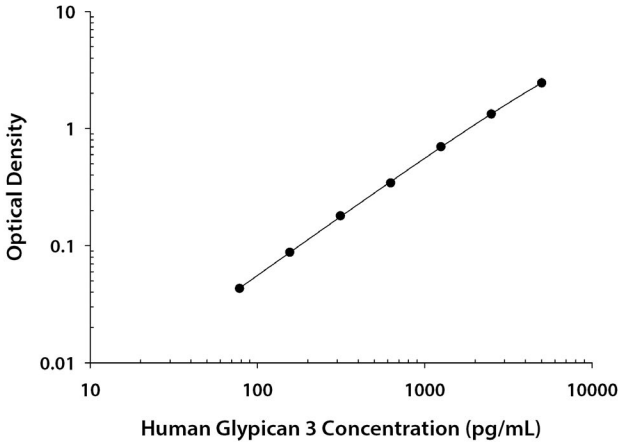
Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density (O.D.).

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 Glypican 3 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.

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	0.041 0.043	0.042	—
78.1	0.081 0.088	0.085	0.043
156	0.129 0.130	0.130	0.088
313	0.217 0.226	0.222	0.180
625	0.380 0.391	0.386	0.344
1250	0.736 0.748	0.742	0.700
2500	1.369 1.379	1.374	1.332
5000	2.490 2.505	2.498	2.456

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 (pg/mL)	412	1208	2387	466	1231	2359
Standard deviation	12.0	48.0	79.6	38.4	75.3	143
CV (%)	2.9	4.0	3.3	8.2	6.1	6.1

RECOVERY

The recovery of human Glypican 3 spiked to levels throughout the range of the assay in various matrices was evaluated.

Sample Type	Average % Recovery	Range
Cell culture media (n=4)	99	88-112%
Serum (n=4)	89	82-97%
EDTA plasma (n=4)	88	81-95%
Heparin plasma (n=4)	89	81-96%

LINEARITY

To assess the linearity of the assay, samples containing and/or spiked with high concentrations of human Glypican 3 were diluted with Calibrator Diluent to produce samples with values within the dynamic range of the assay.

		Cell culture media (n=4)	Cell lysate* (n=1)	Serum (n=4)	EDTA plasma (n=4)	Heparin plasma (n=4)
1:2	Average % of Expected	101	96	98	97	95
	Range (%)	95-107	———	94-102	93-101	90-104
1:4	Average % of Expected	102	98	103	101	100
	Range (%)	98-106	———	99-108	99-102	95-108
1:8	Average % of Expected	101	99	104	104	103
	Range (%)	99-104	———	99-112	103-105	99-111
1:16	Average % of Expected	103	107	107	106	105
	Range (%)	102-104	———	99-113	100-110	100-114

*Samples were diluted prior to assay.

SENSITIVITY

Twenty-seven assays were evaluated and the minimum detectable dose (MDD) of human Glypican 3 ranged from 3.07-20.6 pg/mL. The mean MDD was 9.18 pg/mL.

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

CALIBRATION

This immunoassay is calibrated against highly purified NS0-derived recombinant human Glypican 3 produced at R&D Systems.

SAMPLE VALUES

Serum/Plasma - Thirty-five samples from apparently healthy volunteers were evaluated for the presence of human Glypican 3 in this assay. No detectable levels were observed. No medical histories were available for the donors used in this study.

Cell Culture Supernates/Cell Lysates:
Hep3B human hepatocellular carcinoma cells were cultured in DMEM:F12 supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin sulfate and grown until confluent. Aliquots of the cell culture supernates were removed and assayed for human Glypican 3, and measured 4305 pg/mL.

HepG2 human hepatocellular carcinoma cells were cultured in DMEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin sulfate and grown until confluent. Aliquots of the cell culture supernates were removed and assayed for human Glypican 3. Cells were lysed and assayed for human Glypican 3.

Cell Line	Cell Culture Supernate (pg/mL)	Cell Lysate (pg/mg)
HepG2	175,185	125,309

SPECIFICITY

This assay recognizes natural and recombinant human Glypican 3.

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 Glypican 3 control were assayed for interference. No significant cross-reactivity or interference was observed.

Recombinant human:

DPPIV/CD26
FGF basic
Glypican 1
Glypican 2
Glypican 5
Glypican 6

Recombinant mouse Glypican 3 cross-reacts approximately 0.5% in this assay.

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