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CanAg Glypican-3 EIA

REF 503-85

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Instructions for use 2022-06

Read highlighted changes

Enzyme immunometric assay kit

For 96 determinations

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

	Use by	Σ	Contains sufficient for <96> tests
LOT	Batch Code	2°C 8°C	Temperature limitation
	Date of manufacture		Consult Instructions for Use
REF	Catalogue number	CONT	Contents of kit
	Manufacturer	ORIG MOU	From mouse

EN

CanAg Glypican-3 EIA

INTENDED USE

The CanAg Glypican-3 EIA is a manual immunometric assay for the quantitative determination of Glypican-3 in human serum. This assay is not for use in diagnostic procedures.

SUMMARY AND EXPLANATION OF THE ASSAY

The CanAg Glypican-3 EIA is a solid-phase, non-competitive 2-step immunoassay based upon the direct sandwich technique using two mouse monoclonal antibodies directed against two epitopes in the protein core of Glypican-3. In the first step calibrators, controls and samples are incubated together with biotinylated Anti-Glypican-3 F(ab')2 from monoclonal antibody (MAb) in streptavidin coated microwells. In the second step, after washing, HRP labeled Anti-Glypican-3 MAb is added. After the incubation and washing, buffered Substrate/Chromogen reagent (hydrogen peroxide and 3, 3', 5, 5' tetra-methyl-benzidine) is added to each well and the enzyme reaction is allowed to proceed. During the enzyme reaction a blue color will develop if antigen is present. The intensity of the color is proportional to the amount of Glypican-3 present in the samples. The color intensity is determined in a microplate spectrophotometer at 450 nm after addition of Stop Solution.

Calibration curves are constructed for each assay by plotting absorbance value versus the concentration for each calibrator. The Glypican-3 concentrations of samples are then read from the calibration curve.

REAGENTS

- Each CanAg Glypican-3 EIA kit contains reagents for 96 tests.
- Store the kit at 2-8 °C. Do not freeze.
- Opened reagents are stable according to the table below provided they are not contaminated, stored in resealed original containers and handled as prescribed. Return to 2-8 °C immediately after use.
- Do not mix reagents from different kit lots.

Component	Quantity	Storage and stability after first use
Microplate		2-8 °C until expiry date stated on the label
MICROPLA	1 Plate	

12 x 8 wells coated with streptavidin. After opening, immediately return unused strips to the aluminium pouch, containing desiccant. Reseal carefully to keep dry.

Glypican-3 Calibrators

Glypican-3 Calibrator A		2-8 °C until expiry date stated on the vial
CAL Glypican-3 A	1 x 5 mL	

Phosphate buffered salt solution containing bovine serum albumin, an inert yellow dye, and a non-azide antimicrobial preservative. Ready for use. Should also be used for dilution of samples.

Glypican-3 Calibrator B-E	4 vials, lyophilized	Stability after reconstitution 4 weeks at 2-8 °C 4 months at ≤ -20 °C ≤ 5 freeze-thaw cycles
CAL Glypican-3 B	1 x 1 mL	Concentration stated on label
CAL Glypican-3 C	1 x 1 mL	Concentration stated on label
CAL Glypican-3 D	1 x 1 mL	Concentration stated on label
CAL Glypican-3 E	1 x 1 mL	Concentration stated on label

The lyophilized calibrators contain Glypican-3 antigen in a phosphate buffered salt solution containing bovine serum albumin, horse serum, an inert yellow dye, and a non-azide antimicrobial preservative.

Reconstitute with 1 mL of deionized or distilled water.

Component	Quantity	Storage and stability after first use
Glypican-3 Controls	2 vials, lyophilized	Stability after reconstitution 4 weeks at 2-8 °C 4 months at ≤ -20 °C ≤ 5 freeze-thaw cycles
CONTROL Glypican-3 1	1 x 1 mL	
CONTROL Glypican-3 2	1 x 1 mL	

The controls contain Glypican-3 antigen in a phosphate buffered salt solution containing bovine serum albumin, horse serum and a non-azide antimicrobial preservative. **Reconstitute with 1 mL of deionized or distilled water.**

Biotin Anti-Glypican-3		2-8 °C until expiry date stated on the label
BIOTIN Anti-Glypican-3	1 x 8 mL	

Biotin Anti-Glypican-3 monoclonal antibody from mouse, approximately 2 µg/mL. Contains phosphate buffered saline, bovine serum albumin, blocking agents, detergent, an inert red dye, and a non-azide antimicrobial preservative. Ready for use.

Tracer, HRP Anti-Glypican-3		2-8 °C until expiry date stated on the label
CONJ Anti-Glypican-3	1 x 0.75 mL	

Stock Solution of HRP Anti-Glypican-3 monoclonal antibody from mouse, approximately 21 µg/mL. Contains non-azide antimicrobial preservatives. Mix with Tracer Diluent before use.

Component	Quantity	Storage and stability after first use
Tracer Diluent Glypican-3		2-8 °C until expiry date stated on the label
DIL CONJ	1 x 15 mL	

Contains phosphate buffered saline, bovine serum albumin, blocking agents, detergent, an inert blue dye, and a non-azide antimicrobial preservative. Mix with Tracer, HRP Anti-Glypican-3 before use.

TMB HRP-Substrate		2-8 °C until expiry date stated on the label
SUBS TMB	1 x 12 mL	

Contains buffered hydrogen peroxide and 3, 3', 5, 5' tetra-methylbenzidine (TMB). Ready for use.

Stop Solution		2-8 °C until expiry date stated on the label
STOP	1 x 15 mL	

Contains 0.12 M hydrochloric acid. Ready for use.

Wash Concentrate		2-8 °C until expiry date stated on the label
WASHBUF 25X	1 x 50 mL	

A Tris-HCl buffered salt solution with Tween 20. Contains Germall II as preservative. To be diluted with distilled or deionized water 25 times before use.

Indications of instability

The TMB HRP-Substrate should be colourless. A blue colour indicates that the reagent has been contaminated and should be discarded.

WARNINGS AND PRECAUTIONS

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Not for use in
diagnostic procedures.

- Please refer to the U.S. Department of Health and Human Services (Bethesda, Md., USA) publication No. (CDC) 88–8395 on laboratory safety procedures or any other local or national regulation.
- Handle all serum specimens as potentially infectious.
- Follow local guidelines for disposal of all waste material.

CLP (1272/2008) HAZARD CLASSIFICATION

Information about CLP (1272/2008) HAZARD CLASSIFICATION can be found at the end of this document.

SPECIMEN COLLECTION AND HANDLING

The CanAg Glypican-3 EIA is intended for use with serum serum (RedTop and SST). Collect blood by venipuncture and follow the tube manufacturer's processing instructions for collection tubes. When serial specimens are being evaluated, the same type of specimen should be used throughout the study.

Store samples at -40 °C or colder and avoid repeated freeze-thawing. Bring frozen samples to room temperature and mix thoroughly before analysis. Samples that contain gross particulates should be centrifuged at 10.000 x g for 10 minutes prior to use to eliminate any particulate matter that may have developed from the thawing process. Place samples at 2-8°C immediately after use. Return samples to - 40 °C or colder for long-term storage.

PROCEDURE

Materials required but not supplied with the kit

1. **Microplate shaker**
   Shaking should be medium to vigorous, approximately 700-1100 oscillations/min.
2. **Microplate wash device**
   Automatic plate washer capable of performing **plate mode wash** with 1, 3 and 6 washing cycles. Use a minimal fill volume of 350 µL/well/wash cycle, preferably 800 µL.
3. **Microplate spectrophotometer**
   With a wavelength of 450 nm and an absorbance range of 0 to ≥3.0.
4. **Precision pipettes**
   With disposable plastic tips for dispensing microliter volumes. An 8-channel pipette or dispenser pipette with disposable plastic tips for delivery of 50 and 100 µL is recommended but not required.
   Pipettes for dispensing millilitre volumes.
5. **Distilled or deionized water**
   For reconstitution of Glypican-3 Calibrators, Glypican-3 Controls and for preparation of diluted Wash Solution.

Procedural notes

1. A thorough understanding of this package insert is necessary to ensure proper use of the CanAg Glypican-3 kit. The reagents supplied with the kit are intended for use as an integral unit. Do not mix identical reagents from kits having different lot numbers. Do not use kit reagents after the expiry date printed on the outside of the kit box.
2. Reagents and specimens should be allowed to reach room temperature (20-25 °C) prior to use. Frozen specimens must be thoroughly mixed after thawing. Avoid repeated freeze/thawing of specimens and prolonged storage at above -40 °C.
3. Before starting to pipette calibrators and specimens it is advisable to mark the strips to be able to clearly identify the samples during and after the assay.
4. The requirement for efficient and thorough washing for separation of bound and unbound antigen and reagents from the solid-phase bound antibody-antigen complexes is one of the most important steps in an EIA. **In order to ensure efficient washing make sure that <u>all</u> wells are completely filled to the top edge with wash solution during each wash cycle, that wash solution is dispensed at a good flow rate, that the aspira-**

tion of the wells between and after the wash cycles is complete and that the wells are empty. If there is liquid left, invert the plate and tap it carefully against absorbent paper.

5. Automatic strip washer: Follow the manufacturer's instructions for cleaning and maintenance diligently and wash the required number of wash cycles prior to and after each incubation step. Select plate mode wash as strip mode wash (where one strip is washed all cycles before continuation to the next strip) may cause a drift over the plate. It is highly recommended to use overflow wash with a dispensing volume of 800 µL. The aspiration/ wash device should not be left standing with the Wash Solution for long periods, as the needles may get clogged resulting in poor liquid delivery and aspiration.

6. The TMB HRP-Substrate is very sensitive for contamination.
   For optimal stability of the TMB HRP-Substrate, pour the required amount from the vial into a clean reservoir or preferably a disposable plastic tray to avoid contamination of the reagent. Be sure to use clean disposable plastic pipette tips (or dispenser pipette tip).

7. Be sure to use clean disposable plastic pipette tips and a proper precision pipetting technique when handling samples and reagents. Do not allow the pipette tip to touch the surface of the liquid in order to avoid carry-over. A diligent pipetting technique is of particular importance when handling the samples and the TMB HRP-Substrate solution.

Preparation of reagents	Stability of prepared reagent
Glypican-3 Calibrator B-E	4 weeks at 2-8 °C 4 months at ≤ -20 °C ≤ 5 freeze-thaw cycles

Add exactly 1.0 mL of distilled or deionised water to each vial. Allow to stand for at least 10 minutes to reconstitute. Mix before use. NOTE: The concentration of the calibrators is stated on the labels and should be used for calculation of results.

Glypican-3 Controls	4 weeks at 2-8 °C 4 months at ≤ -20 °C ≤ 5 freeze-thaw cycles

Add exactly 1.0 mL of distilled or deionised water to each vial. Allow to stand for at least 10 minutes to reconstitute. Mix before use. NOTE: The ranges of the controls are stated on the labels.

Wash Solution	2 weeks at 2-25° C in a sealed container

Pour the 50 mL Wash Concentrate into a clean container and dilute 25-fold by adding 1200 mL of distilled or deionized water to give a buffered Wash Solution.

Protocol Sheet

Glypican-3 EIA REF 503-85

Prepare components before use, see section: Preparation of reagents.
Use wash and incubation conditions according to instructions.

Step	Vial/Plate	Procedure
Prepare Wash Solution	WASHBUF 25X	Dilute 50 mL of Wash Concentrate with 1200 mL of distilled or deionized water
Prepare Tracer Working Solution	CONJ Anti-Glypican-3 DIL CONJ	Mix 50 µL of Tracer, HRP Anti-Glypican-3 with 1 mL of Tracer Diluent per strip (see table on page 12)
Prewash	MICROPLA	Wash each well once with Wash Solution
Add Calibrators, Controls and specimens	CAL Glypican-3 CONTROL Glypican-3	100 µL in each well Calibrators A, B, C, D, E Controls 1, 2
Add Biotin Anti-Glypican-3	BIOTIN Anti-Glypican-3	50 µL in each well
Incubate	MICROPLA	2 hours shaking at room temperature
Wash	MICROPLA	Wash each well 3 times with Wash Solution

Add Tracer working solution	CONJ Anti-Glypican-3	100 µL in each well
Incubate	MICROPLA	30 minutes shaking at room temperature
Wash	MICROPLA	Wash each well 6 times with Wash Solution
Add TMB HRP-Substrate	SUBS TMB	100 µL in each well
Incubate	MICROPLA	30 min shaking at room temperature
Add Stop Solution	STOP	100 µL in each well
Incubate	MICROPLA	1 minute shaking at room temperature
Read absorbance	MICROPLA	Read at 450 nm within 5 minutes

Preparation of reagents	Stability of prepared reagent
Tracer Working Solution	1 week at 2-8 °C in a sealed container

Prepare the required quantity of Tracer Working Solution by mixing 50 µL of Tracer, HRP Anti-Glypican-3 with 1 mL of Tracer Diluent per strip (see table below).

No. of strips	Tracer, HRP Anti-Glypican-3 (µL)	Tracer Diluent (mL)
1	50	1
2	100	2
3	150	3
4	200	4
5	250	5
6	300	6
7	350	7
8	400	8
9	450	9
10	500	10
11	550	11
12	600	12

Be sure to use a clean plastic or glass bottle for preparation of Tracer Working Solution.
NOTE: The Tracer Working Solution is only stable for 1 week at 2-8 °C. Do not prepare more Tracer Working Solution than will be used within this period.
Avoid direct sunlight and make sure that it is stored properly.

ASSAY PROCEDURE

Perform each determination in duplicate for both calibrators, controls and unknown samples. A calibration curve should be run with each assay. All reagents and samples must be brought to room temperature (20-25 °C) before use.

1. Start preparing Calibrators B-E, Controls, Wash Solution and Tracer Working Solution. It is important to use clean containers. Follow the instructions carefully.
2. Transfer the required number of microplate strips to a strip frame. (Immediately return the remaining strips to the aluminium pouch containing desiccant and reseal carefully). Wash each strip once with the Wash Solution. **Do not wash more strips than can be handled within 30 min.**
3. Pipette 100 µL of each of the Glypican-3 Calibrators (CAL A, B, C, D and E), Glypican-3 Controls (C1, C2) and unknown specimens (Unk) into the strip wells according to the following scheme:

	1	2	3	4	5	6	7 etc.
A	Cal A	Cal E	Unk 2				
B	Cal A	Cal E	Unk 2				
C	Cal B	C1	Etc.				
D	Cal B	C1					
E	Cal C	C2					
F	Cal C	C2					
G	Cal D	Unk 1					
H	Cal D	Unk 1					

4. Add 50 µL of Biotin Anti-Glypican-3 solution to each well using a 50 µL precision pipette (or an 8-channel 50 µL precision pipette). Do not allow the pipette tip to touch the surface of the liquid in order to avoid carry-over.
5. Incubate the plate for 2 hours (± 10 min) in a temperature-controlled environment, (20-25 °C), using a microplate shaker at 700-1100 oscillations /min. **Note**: It is important to use the recommended shake speed as lower shake speed may result in wrong concentrations of samples.
6. Aspirate and wash each strip 3 times, using the wash procedure described in Procedural notes.
7. Add 100 µL of Tracer working solution to each well using a 100 µL precision pipette (or an 8-channel 100 µL precision pipette).
8. Incubate the plate for 30 min (± 5 min) in a temperature-controlled environment, (20-25 °C), using a microplate shaker at 700-1100 oscillations/min. Note: It is important to use the recommended shake speed as lower shake speed may result in wrong concentrations of samples.
9. Aspirate and wash each strip 6 times, using the wash procedure described in Procedural notes.
10. Add 100 µL of TMB HRP-Substrate to each well. The time between addition of substrate to the first and last well should not exceed 5 min.
11. Incubate for 30 min (± 5 min) at room temperature (20-25 °C) using a microplate shaker at 700-1100 oscillations/min.
    Avoid exposure to direct sunlight.
12. Add 100 µL of Stop Solution to each well. Mix for 1 minute by using a microplate shaker and read absorbance at 450 nm in a microplate spectrophotometer within 5 minutes after addition of Stop Solution.

Measurement range

The CanAg Glypican-3 EIA measures concentrations between at least 0.38 and 2380 pg/mL. The lot specific concentration of calibrator E is stated on the vial. If Glypican-3 concentrations are above calibrator E, dilute the samples as described below in section Calculation of Results.

Quality control

Glypican-3 Control 1 and 2 should be used for validation of the assay series. Ranges of expected results are indicated on the vial labels. If values outside of the specified range are obtained, a complete check of reagents and reader performance should be made and the analysis repeated. Each laboratory may also prepare its own serum pools at different levels, which can be used as internal controls in order to assure the precision of the assay.

Reference material

Since no common reference material is available for Glypican-3 antigen, Glypican-3 EIA Calibrator values are assigned against a set of Fujirebio diagnostics in-house reference standards with traceability to amino acid analysis of human recombinant Glypican-3.

CALCULATION OF RESULTS

A microplate spectrophotometer with built-in data calculation program is recommended, refer to the manual for the spectrophotometer and create a program using the concentration stated of each of the Glypican-3 Calibrators. For automatic calculation of Glypican-3 results it is recommended to use either of the following methods:

- Quadratic (Polynomial of degree 2) curve fit method (preferred). Calibrator A should be included in the curve with the value 0 pg/mL.
- Cubic spline curve fit method. Calibrator A should be included in the curve with the value 0 pg/mL.
- 4-Parameter curve fit method. Calibrator A should be included in the curve with the value 0 pg/mL.

Note: Linear regression should not be used.

For manual evaluation, a calibration curve is constructed by plotting the absorbance (A 450 nm) values obtained for each Glypican-3 calibrator against the corresponding Glypican-3 concentration (in pg/mL), see example in figure below. The unknown Glypican-3 concentrations can then be read from the calibration curve using the mean absorbance value of each patient specimen.

If Glypican-3 concentrations are above the concentration of Calibrator E, it is recommended to dilute samples 1/2 in Cal A and reanalyse to obtain the accurate Glypican-3 concentration. Prepare the dilution directly before use. Do not exceed 1/2 dilution in Cal A.

Example: 1/2 dilution = 150 µL of specimen + 150 µL of Cal A
The Glypican-3 concentration of the undiluted sample is calculated as:

[Glypican-3]Undiluted sample = 2 x [Glypican-3]Diluted sample in Cal A

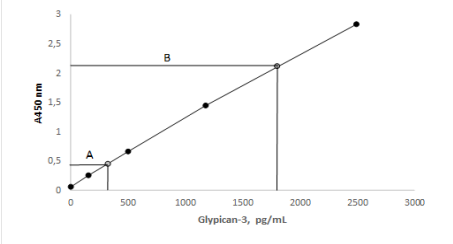
For samples with concentrations >4800 pg/mL, it is recommended to dilute samples 1/10 in normal human serum and reanalyse to obtain the accurate Glypican-3 concentration. Prepare the dilution directly before use. Do not exceed 1/10 dilution in normal human serum.

Example: 1/10 dilution = 50 µL of specimen + 450 µL of normal human serum. The Glypican-3 concentration of the undiluted sample is calculated as:

[Glypican-3]Undiluted sample = 10 x [Glypican-3]Diluted sample – 9 x [Glypican-3]Normal serum

Example of results

Specimen	**Calibrator Values** pg/mL	**Mean abs value** A450 nm	**Glypican-3** pg/mL
CAL | Glypican-3 | A	0	0.059	
CAL | Glypican-3 | B	155	0.248	
CAL | Glypican-3 | C	504	0.654	
CAL | Glypican-3 | D	1180	1.442	
CAL | Glypican-3 | E	2490	2.830	
Specimen A		0.447	323
Specimen B		2.118	1803



Example (do not use this curve or table above to determine actual assay results).

LIMITATIONS OF THE PROCEDURE

Anti-reagent antibodies (human anti-mouse antibody (HAMA) or heterophilic antibodies) in the serum sample may occasionally interfere with the assay, even though specific blocking agents are included in the buffers.

The assay must be performed in a temperature controlled environment, 20-25 °C.

Elevated levels of Glypican-3 are normally detected in samples from pregnant women. (1)

Biotin interferes with the assay giving falsely low results. This should be taken into consideration for subjects with high biotin intake. (2)

CLP (1272/2008) HAZARD CLASSIFICATION

The following warnings and precautions apply to SUBS | TMB

Hazard pictograms:

Signal word:	Danger
Hazard Statement:	Repr. 1B: H360D May damage the unborn child.
Prevention statement:	P202 Do not handle until all safety precautions have been read and understood.
Prevention:	P280 Wear protective gloves / protective clothing / eye protection / face protection.
Precautionary statement response:	P308+P313 IF exposed or concerned get medical advice/attention.
Precautionary statement distposal:	P501 Dispose of contents / container to an approved hazardous / special waste disposal facility in accordance with local and national regulations.

Restricted to professional users.

Hazardous substances: 2- Pyrrolidone

Other hazards

None of the mixtures in the kit contains any substances considered to meet the criteria classifying them as PBT and/or vPvB.

REFERENCES

1. The maternal plasma proteome changes as a function of gestational age in normal pregnancy: a longitudinal study. R Romero et. al. .Am J Obstet Gynecol. (2017) 217(1):67.e1-67.e21.
2. Population pharmacokinetics of exogenous biotin and the relationship between biotin serum levels and in vitro immunoassay interference, P. Grimsey et. al., Int. J. Pharmacokinet. (2017) 2 (4), 247-256.

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