KRIBIOLISA® Glatiramer Acetate (COPAXONE™) ELISA

REF : KOD1024

Ver.5.5

RUO

Enzyme Immunoassay for the Quantitative Determination of Glatiramer Acetate in cell culture supernatant and pharmaceutical preparations

RUO	For Research Use Only	REF	Catalog Number
	Store At	LOT	Batch Code
	Manufac d By		Biologic sk
	Expiry D		Consult rating Instructions

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REF KOD1024 Σ 96 tests

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Introduction:

Glatiramer Acetate (COPAXONE™) is a mixture of random-sized peptides that are composed of the four amino acids found in myelin basic protein, namely glutamic acid, lysine, alanine, and tyrosine. Myelin basic protein is the antigen in the myelin sheaths of the neurons that stimulates an autoimmune reaction in people with multiple sclerosis (MS), so the peptide may work as a decoy for the attacking immune cells.

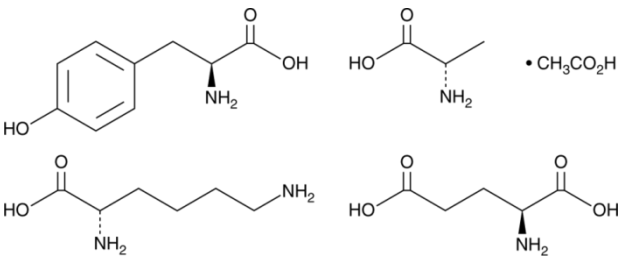
Trade Names:
Copaxone, Glatect, Glatopa

Generic Name:
Glatiramer

PubChem CID:
Not available

DrugBank Accession Number:
DB05259

Structure Depiction:

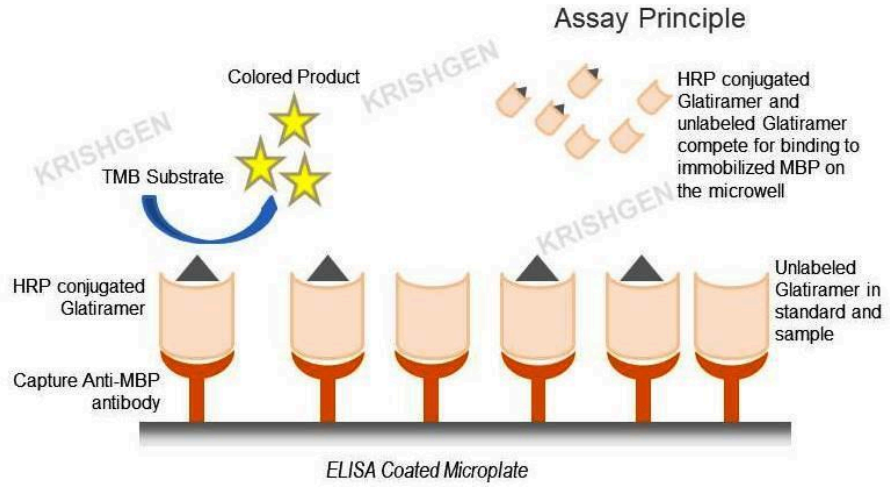


Intended Use:

The KRIBIOLISA® Glatiramer Acetate (COPAXONE™) ELISA kit is used for the quantitative estimation of Glatiramer Acetate in cell culture supernatant and pharmaceutical preparations.

Principle:

The Glatiramer Acetate ELISA is a competitive immunoassay for the determination of Glatiramer Acetate (GA). It is known that Glatiramer binds strongly to MBP-specific antigen. Hence using this principle the assay has been developed. A varying concentration of unlabeled standard or sample and constant concentration of GA:HRP conjugate will bind in sequence to the antibodies coated on the microplate. Upon washing, unbound GA:HRP Conjugate will be removed. Bound GA:HRP complex will produce a soluble blue colored product after the addition of 3, 3', 5, 5' Tetra Methyl Benzidine (TMB) Substrate. The enzyme reaction is stopped by dispensing of stop solution into the wells. The optical density (OD) of the solution at 450 nm is inversely proportional to the amount of bound Glatiramer Acetate present in the standards or samples.



Materials Provided:

Part	Description	Qty
Anti-MBP polyclonal antibody Coated Microtiter Plate	96 well polystyrene microplate (12 strips of 8 wells) coated with Anti-MBP polyclonal antibody Coated Microtiter Plate	1 x 96 wells

Glatiramer Acetate Standard	Glatiramer Acetate in a buffered protein base with preservative 0.02% methylisothiazolone and 0.02% bromonitrodioxane – (lyophilized, concentrated 10,000 ng/ml)	2 vials
Part	**Description**	**Qty**
Glatiramer Acetate:HRP Conjugate concentrated	Glatiramer Acetate conjugated to Horseradish Peroxidase Concentrated (1 mg/ml)	2 vials
Detection Diluent	Buffer with protein stabilizer and preservatives 0.02% methylisothiazolone and 0.02% bromonitrodioxane	12 ml
Assay Diluent	Buffer with protein stabilizer and preservatives 0.02% methylisothiazolone and 0.02% bromonitrodioxane	50 ml
(1X) Standard Diluent	Buffer with protein stabilizer and preservatives 0.02% methylisothiazolone and 0.02% bromonitrodioxane	10 ml
(20X) Wash Buffer	20-fold concentrated solution of buffered surfactant with preservative thiomersal < 0.01%. May turn yellow over time.	25 ml
TMB Substrate	Stabilized TMB Chromogen	12 ml
Stop Solution	0.73M Phosphoric Acid	12 ml
Instruction Manual		1 no

Materials to be provided by the End-User:

1. Microplate Reader able to measure absorbance at 450 nm.
2. Adjustable pipettes to measure volumes ranging from 50 ul to 1000 ul.
3. Deionized (DI) water.
4. Wash bottle or automated microplate washer.
5. Graph paper or software for data analysis.
6. Tubes to prepare standard/sample dilutions.
7. Timer.
8. Absorbent paper.
9. Incubator.

Handling/Storage:

1. All reagents should be stored at 2-8ºC for stability. Glatiramer Acetate:HRP Conjugate concentrated should be stored at -20ºC. It is recommended to make 2 ul aliquots incase the wells are being used more than once. On use, remove and bring to RT and prepare as per instructed dilution using Detection Diluent. Diluted conjugate should be discarded after use.
   Note: Glatiramer Acetate:HRP Conjugate is inherently unstable, hence more than 1-2 freeze-thaws should be avoided to ensure reproducible results.
2. All the reagents and wash solutions should be used within 12 months from manufacturing date.
3. Before using, bring all components to room temperature (18-25ºC). Upon assay completion ensure all components of the kit are returned to appropriate storage conditions.
4. The Substrate is light-sensitive and should be protected from direct sunlight or UV sources.

Health Hazard Warnings:

1. Reagents that contain preservatives may be harmful if ingested, inhaled or absorbed through the skin.
2. For Research Use Only.



Sample Preparation and Storage:

For Cell Culture Supernatant - If necessary, centrifuge to remove debris prior to analysis. Samples can be stored at -20°C or -80°C. Avoid repeated freeze-thaw cycles.

For Pharmaceutical Preparations - If necessary, centrifuge to remove debris / un-dissolved matter prior to analysis. Dissolve in Assay Diluent to the desired concentration to avoid matrix or dilutional linearity effect. Samples may be stored at recommended temperature.

Preparation before Use:

Allow samples to reach room temperature prior to assay. Take care to agitate patient samples gently in order to ensure homogeneity.

Reagent Preparation (all reagents should be diluted immediately prior to use):

1. Label any aliquots made with the kit Lot No and Expiration date and store it at appropriate conditions mentioned.
2. Bring all reagents to Room Temperature before use.
3. To make **(1X) Wash Buffer**; dilute **25 ml of 20X Wash Buffer in 475 ml of DI water**.
4. **Standards Preparation**: Reconstitute the concentrated Standard lyophilized vial with 1 ml of (1X) Standard Diluent to obtain 10,000 ng/ml. Keep the vial for 15 mins with gentle agitation and then run the assay procedure. Use the Standard Diluent (1X) as the zero standard. Below table shows the calculation for the standard range.

Standard Concentration (ng/ml)	Standard No.	Dilution Particulars
10,000 ng/ml	Lyophilized standard	Lyophilized Standard + 1ml of Standard Diluent (1X)
1000 ng/ml	Standard No.8	100 ul Reconstituted Standard + 900 ul Standard Diluent (1X)
500 ng/ml	Standard No.7	500 ul Standard No.8 + 500 ul Standard Diluent (1X)
250 ng/ml	Standard No.6	500 ul Standard No.7 + 500 ul Standard Diluent (1X)
125 ng/ml	Standard No.5	500 ul Standard No.6 + 500 ul Standard Diluent (1X)
62.5 ng/ml	Standard No.4	500 ul Standard No.5 + 500 ul Standard Diluent (1X)
31.25 ng/ml	Standard No.3	500 ul Standard No.4 + 500 ul Standard Diluent (1X)
15.6 ng/ml	Standard No.2	500 ul Standard No.3 + 500 ul Standard Diluent (1X)
7.8 ng/ml	Standard No.1	500 ul Standard No.2 + 500 ul Standard Diluent (1X)
0 ng/ml	Standard No.0	Standard Diluent (1X) Only

Mix each tube thoroughly before the next transfer. Use the standards for experiment within one hour of preparation of standard. Discard standard after use.

5. **Working Glatiramer Acetate:HRP Conjugate - Refer to the Reagent Preparation sheet attached with the IFU and COA (enclosed in the kit).**

Procedural Notes:

1. In order to achieve good assay reproducibility and sensitivity, proper washing of the plates to remove excess un-reacted reagents is essential.
2. Avoid assay of Samples containing Sodium Azide (NaN_3), as it could destroy the HRP activity of the conjugate resulting in under-estimation of the antibodies.
3. It is recommended that all Standards and Samples be assayed in duplicates or triplicates.
4. Maintain a repetitive timing sequence from well to well for all the steps to ensure that the incubation timings are same for each well.
5. If the Substrate has a distinct blue color prior to use it may have been contaminated and use of such substrate can lead to compromisation of the sensitivity of the assay.
6. The plates should be read within 30 minutes after adding the Stop Solution.
7. Make a work list in order to identify the location of Standards and Samples.
8. Making serial dilution in the wells directly is not permitted.
9. Prepare the Standard within 15 minutes prior to running the assay.
10. Please carefully dilute Standards according to the instruction, and avoid foaming. To minimize imprecision caused by pipetting, use small volumes and ensure that pipettes are calibrated.
11. If crystals have formed in the Wash Solution (20X) concentrate, warm to room temperature and mix gently until the crystals are completely dissolved.
12. Contaminated water or container for reagent preparation will influence the detection results.

Assay Procedure:

1. It is strongly recommended that all Standards and Samples be run in duplicates or triplicates. A standard curve is required for each assay. All steps must be performed at Room Temperature.

2. Pipette out **100 ul of Standards and samples** to the respective wells.

3. Add **100 ul Working Glatiramer Acetate:HRP Conjugate** to each well.

4. Cover the plate and incubate for 90 mins at Room Temperature.
5. Aspirate and wash plate 4 times with **Wash Buffer (1X)** and blot residual buffer by firmly tapping plate upside down on absorbent paper. Wipe of any liquid from the bottom outside of the microtiter wells as any residue can interfere in the reading step.

6. Add **100 ul** of **TMB Substrate** in each well.

7. Incubate the plate at RT for 30 minutes in dark. DO NOT SHAKE or else it may result in higher backgrounds and worse precision. Positive wells should turn bluish in color.

8. Pipette out **100 ul** of **Stop Solution**. Wells should turn from blue to yellow in color.

9. Read the absorbance at 450 nm with a microplate within 10-15 minutes after addition of Stop solution.

Calculation of Results:

Determine the Mean Absorbance for each set of duplicate or triplicate Standards and Samples. Calculate percent conjugate bound (%B) for each standard and sample relative to the maximum binding (B0, Zero standard) wells as follows:

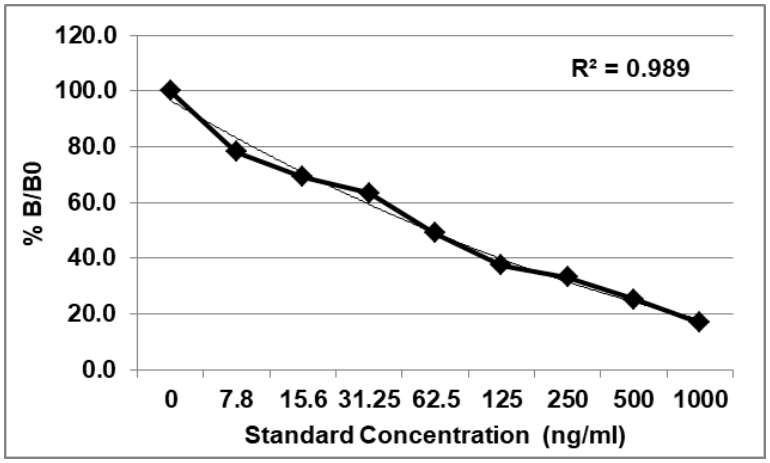
%B/B0 = Mean Absorbance (Standard / Sample) / Mean Absorbance (Zero '0' Standard) x 100

Using a lin-lin graph paper, plot the B/Bo (%) for each standard point (y axis) versus the concentration (x axis). Draw a best-fit line (logit-log or 4-PL) through the points. To determine the concentration of your samples, find the B/Bo (%) value on the y axis. Read the corresponding value on the x axis which is the concentration of your Glatiramer Acetate in the sample. If samples were diluted, multiply by the appropriate dilution factor.

Typical Data (representative only)

Standard Concentration (ng/ml)	Mean Absorbance	Interpolated Concentration	% Interpolated Concentration against Actual Concentration	%B/B0
0	2.507	--	--	100.0
7.8	1.958	7.9	101.2	78.1
15.6	1.734	16.8	107.5	69.2
31.25	1.591	25.5	81.6	63.5
62.5	1.229	68.2	109.1	49.0
125	0.943	150.1	120.1	37.6
250	0.834	213.0	85.2	33.3
500	0.634	434.3	86.9	25.3
1000	0.427	1192.7	119.3	17.0

Typical Graph (representative only)



Quality Control:

It is recommended that for each laboratory assay appropriate quality control samples in each run to be used to ensure that all reagents and procedures are correct.

Performance Characteristics:

Please note that this validation is performed in our laboratory and will not necessarily be duplicated in your laboratory. This data has been generated to enable the user to get a preview of the assay and the characteristics of the kit and is generic in nature. We recommend that the user performs at the minimum; the spike and recovery assay and the dilutional linearity assay to assure quality results. For a more comprehensive validation, the user may run the protocols as suggested by us herein below to develop the parameters for quality control to be used with the kit.

Detection Range:
7.8 ng/ml - 1000 ng/ml.

Sensitivity:
The minimum detectable dose of Glatiramer Acetate is <50 ng/ml.

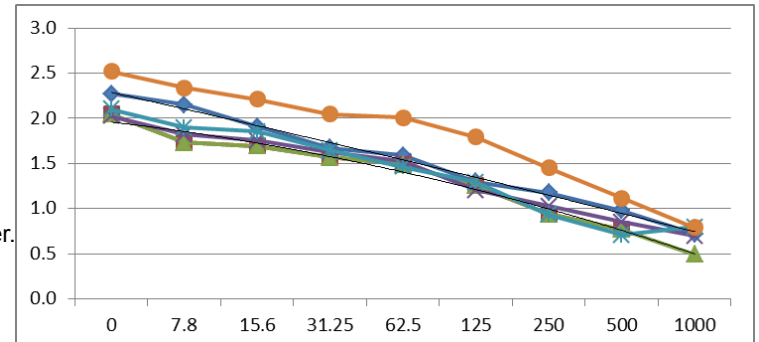
Precision:
Intra-Assay Precision: 3 samples with low, middle and high level human Glatiramer Acetate were tested 20 times on one plate, respectively.
Inter-Assay Precision: 3 samples with low, middle and high level human Glatiramer Acetate were tested on 3 different plates, 8 replicates in each plate.

CV (%) = SD/mean X 100
Intra-Assay: CV<15%
Inter-Assay: CV<18%

Stability Profile of GA:HRP Conjugate (conc):
2 kits were run with the GA:HRP Conjugate (conc) kept at 37ºC to run an accelerated stability study to see the stability of the GA:HRP Conjugate (conc). The GA:HRP Conjugate (conc) was freshly diluted as per IFU prior to each run. All components used were common through and kept at 2-8ºC as per prescribed storage instructions. The GA:HRP Conjugate (conc) was stable till the expiry date.

Standards	Day 0	Day 1	Std Dev %	Day 4	Std Dev %	Day 7	Std Dev %	Day 11	Std Dev %
		26 days		104 days		182 days		286 days	
0	2.270	2.046	15.8	2.025	17.3	2.101	12.0	2.517	17.5
7.8	2.151	1.734	29.5	1.821	23.3	1.896	18.0	2.339	13.3
15.6	1.910	1.693	15.4	1.752	11.2	1.850	4.2	2.211	21.3
31.25	1.674	1.568	7.5	1.625	3.4	1.644	2.1	2.048	26.5
62.5	1.588	1.489	7.0	1.520	4.8	1.460	9.1	2.007	29.6
125	1.299	1.251	3.4	1.205	6.7	1.291	0.6	1.795	35.1
250	1.173	0.936	16.7	1.025	10.5	0.932	17.0	1.452	19.7
500	0.979	0.767	15.0	0.852	9.0	0.710	19.1	1.116	9.7
1000	0.719	0.495	15.8	0.695	1.7	0.795	5.4	0.789	4.9

Stability Profile Graph for Accelerated Study done on GA:HRP Conjugate (conc)



Safety Precautions:

- **This kit is For Research Use only**. Follow the working instructions carefully.
- The expiration dates stated on the kit are to be observed. The same relates to the stability stated for reagents.
- Do not use or mix reagents from different lots.
- Do not use reagents from other manufacturers.
- Avoid time shift during pipetting of reagents.
- All reagents should be kept in the original shipping container.
- Some of the reagents contain small amount of sodium azide (< 0.1 % w/v) as preservative. They must not be swallowed or allowed to come into contact with skin or mucosa.
- Source materials maybe derived from **human body fluids** or organs used in the preparation of this kit were tested and found negative for HBsAg and HIV as well as for HCV antibodies. However, no known test guarantees the absence of such viral agents. Therefore, handle all components and all patient samples as if potentially hazardous.
- Since the kit contains potentially hazardous materials, the following precautions should be observed
  - Do not smoke, eat or drink while handling kit material.
  - Always use protective gloves.
  - Never pipette material by mouth.
  - Wipe up spills promptly, washing the affected surface thoroughly with a decontaminant.
- In any case GLP should be applied with all general and individual regulations to the use of this kit.

Reference:

Antibodies to glatiramer acetate do not interfere with its biological functions and therapeutic efficacy… Teitelbaum D, Brenner T, Abramsky O, Aharoni R… Mult Scler…2003…SAGE

Blocking effects of serum reactive antibodies induced by glatiramer acetate treatment in multiple sclerosis… Hassan H. Salama Jian Hong Ying C. Q. Zang Azza El-Mongui Jingwu Zhang… Brain…2003…Oxward academic

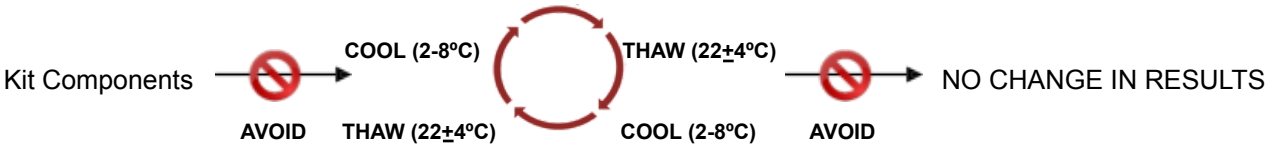
The Effect of Glatiramer Acetate Therapy on Functional Properties of B Cells From Patients With Relapsing-Remitting Multiple Sclerosis…Sara J. Ireland, Alyssa A. Guzman, Dina E. O'Brien…JAMA Neurol..2014

SCHEMATIC ASSAY PROCEDURE

1. Remove all components, 30 minutes before adding into the assay plate.



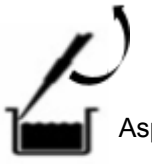
2. Avoid repeated cool-thaw of the components as there will be a loss of activity and this can affect the results.



3.  Pipette **100 ul prepared Standard** and **Sample** into respective well.

4.  Pipette **100 ul Working GA:HRP Conjugate** into the respective wells.

5. Cover plate  and incubate for  at Room Temperature.

6.  Aspirate and wash wells **4 times** with **Wash Buffer (1X).**

7.  Pipette **100 ul TMB Substrate** into each well.

8. Cover plate  and incubate for  at Room Temperature.



9. Pipette **100 ul Stop Solution** into each well.

10. Read absorbance at 450 nm with a  microplate reader within 

of stopping reaction.

Typical Example of a Work List

Well #	Contents	Absorbance at 450 nm	Mean Absorbance	ng/ml Glatiramer Acetate equivalent
1A 2A	zero standard zero standard			
1B 2B	7.8 ng/ml 7.8 ng/ml			
1C 2C	15.6 ng/ml 15.6 ng/ml			
1D 2D	31.25 ng/ml 31.25 ng/ml			
1E 2E	62.5 ng/ml 62.5 ng/ml			
1F 2F	125 ng/ml 125 ng/ml			
1G 2G	250 ng/ml 250 ng/ml			
1H 2H	500 ng/ml 500 ng/ml			
3A 4A	1000 ng/ml 1000 ng/ml			
3B 4B	Sample Sample			
3C 4C	Sample Sample			

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SYMBOLS KEY

	A MTP polyclonal antibody Coated Microtiter P 8 wells)
	G STD r Acetate Standard, Lyophilized
	HRP CONJ etate:HRP Conjugate (concentrated)
	DETN DIL uent
	ASY DIL
	1X STD DIL Diluent
	20X WASH BUF fer
	SUB TMB te
	SOLN STOP
	Consult Instructions for Use
	C REF umber
	Expiration Date
	Storage Temperature