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KRIBIOLISA® Neutralizing Antibodies to Imiglucerase (CEREZYME™) ELISA

REF : KBN4030

Ver 1.0

RUO

Enzyme Immunoassay for the Qualitative Detection of Neutralizing Antibodies against Imiglucerase in human serum or plasma

RUO	**For Research Use Only**	**REF**	**Catalog Number**
	**Store At**	**LOT**	**Batch Code**
	**Manufactured By**		**Biological Risk**
	**Expiry Date**		**Consult Operating Instructions**

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REF **KBN4030** **Σ** **96 tests**

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Introduction:

The KRIBIOLISA® ELISA kits are used for assessing the specific biomarker in samples analytes which may be serum and cell culture supernatant as validated with the kit. The kit employs a neutralizing ELISA technique which engages the blocking pathway to estimate the neutralizing antibodies. Imiglucerase is a recombinant analog of the lysosomal enzyme β-glucocerebrosidase indicated for enzyme replacement therapy in Gaucher disease.

Imiglucerase is a recombinant form of the lysosomal enzyme β-glucocerebrosidase that catalyzes the hydrolysis of glucocerebroside into glucose and ceramide within macrophage lysosomes, thereby correcting the underlying enzymatic deficiency in Gaucher disease. It is approved for enzyme replacement therapy in patients with Type 1 Gaucher disease and has demonstrated significant reductions in hepatosplenomegaly, improvement in hematological parameters, and stabilization or improvement of skeletal manifestations. However, a subset of patients may develop neutralizing anti-drug antibodies (NAbs) against Imiglucerase, which can inhibit enzymatic activity or interfere with receptor-mediated cellular uptake, potentially affecting therapeutic efficacy; therefore, monitoring of NAbs is recommended as part of immunogenicity assessment in patients with Gaucher disease (Barton et al. 1991; Grabowski et al. 1995; Weinreb et al. 2002; Mistry et al. 2017).

As with other therapeutic proteins, the development of anti-drug antibodies (ADAs) is a potential risk in patients treated with Imiglucerase. The formation of ADAs can alter the pharmacokinetic (PK) profile and potentially affect the safety and therapeutic response to enzyme replacement therapy. ADAs may be binding or neutralizing in nature, with neutralizing antibodies (NAbs) being of particular concern, as they may inhibit enzymatic activity or interfere with receptor-mediated cellular uptake, thereby reducing clinical efficacy. In some cases, the development of antibodies has also been associated with infusion-related reactions and hypersensitivity events in patients with Gaucher disease.

Given these risks, it is essential for drug development programs involving Imiglucerase or its biosimilars to include validated assays to detect the presence of ADAs and NAbs in patient serum. Regulatory agencies generally recommend cell-based or activity-based assays for NAb detection, as these methods reflect the functional inhibition of β-glucocerebrosidase enzymatic activity, which represents the therapeutic mechanism of action. Although cell-based or enzyme activity assays are considered biologically relevant, they may be associated with assay variability, limited drug tolerance, matrix interference, and increased operational complexity, thereby necessitating the development of robust and well-validated neutralizing antibody detection platforms for patients with Gaucher disease.

Alternatively, non-cell-based assays, such as competitive enzyme- or receptor-binding assays, can provide higher sensitivity, improved dynamic range, enhanced precision, and better matrix and drug tolerance for the detection of neutralizing antibodies against Imiglucerase. While cell-based or enzyme activity assays remain the regulatory preference due to their ability to reflect functional inhibition of β-glucocerebrosidase activity, both the FDA and EMA acknowledge that well-validated non-cell-based methods may be acceptable when scientifically justified. Ultimately, the choice of assay format is guided by the therapeutic mechanism of action, assay performance characteristics, and the immunogenicity risk profile of imiglucerase, with inhibition of enzymatic activity or receptor-mediated uptake serving as the primary determinant for neutralizing antibody assay design in patients with Gaucher disease (Mistry et al. 2017).

The KRIBIOLISA® Neutralizing Antibodies to Imiglucerase is a neutralizing ligand-binding assay that is specific for the detection of Imigluecarse NAbs in human serum. The kit is validated for assay precision, sensitivity, hook effect, selectivity, robustness, stability, and system suitability.

The strength of the KRIBIOLISA® Neutralizing Antibodies to Imiglucerase is that it delivers a direct functional readout. The kit is not just detecting antibodies but showing functional inhibition of the drug's binding to Recombinant Human IGF2R Protein. It is also highly specific since Imiglucerase is the only ligand interacting with the immobilized Recombinant Human IGF2R protein (cation-independent mannose-6-phosphate receptor) in the assay system; therefore, any reduction in signal specifically reflects the presence of neutralizing antibodies against imiglucerase that inhibit its receptor binding and subsequent functional activity in patients with Gaucher disease.

Intended Use:

The KRIBIOLISA® Neutralizing Antibodies to Imiglucerase ELISA kit is used as an analytical tool for the qualitative detection of neutralizing antibodies against Imiglucerase in serum or plasma.

Principle:

The method employs a neutralizing ELISA technique. The first step involves the antibody–protein interaction between the neutralizing antibody and Imiglucerase conjugated with HRP. The second step involves the binding of free Imiglucerase conjugated with HRP to Recombinant Human IGF2R Protein immobilized on the microplate.

Samples and controls are pipetted into an uncoated microtitre plate and incubated with Imiglucerase:HRP conjugate (in-house conjugated anti-hGBAa purified Mouse Monoclonal IgG). Neutralizing antibodies to Imiglucerase present in the samples/positive control will bind to the Imiglucerase:HRP conjugate. This mixture of bound and unbound Imiglucerase is then transferred and incubated in a second plate coated with Recombinant Human IGF2R Protein.

The unbound Imiglucerase:HRP conjugate present will bind to the immobilized Recombinant Human IGF2R Protein. After washing, TMB substrate is added to the microwells. Following incubation, color develops inversely proportional to the amount of neutralizing antibodies present in the sample, as these antibodies inhibit Imiglucerase from binding to the immobilized Recombinant Human IGF2R Protein. The color development is stopped by the addition of stop solution, and absorbance is measured at 450 nm for quantitative interpretation in the context of Gaucher disease.

Materials Provided:

Part	Description	Qty
Recombinant Human IGF2R Protein Coated Microtiter Plate	96 well polystyrene microplate (12 strips of 8 wells) coated with recombinant Human IGF2R Protein.	1 x 96 wells
Uncoated Microtiter Plate	96 well polystyrene uncoated microplate (12 strips of 8 wells)	1 x 96 wells
Neutralizing Imiglucerase Antibody:Positive Contol	Neutralizing Antibody IgG Positive Control, buffered protein base with preservative 0.02% Methylisothiazolinone and 0.02% Bromonitrodioxane (lyophilized).	2 vials
Imiglucerase:HRP concentrated	Imiglucerase conjugated to HRP, concentrated.	2 vial
Imiglucerase:HRP Diluent	Buffer with protein stabilizer and preservatives 0.02% Methylisothiazolinone and 0.02% Bromonitrodioxane	12 ml
(1X) Sample Diluent	Buffered protein base with preservative 0.02% Methylisothiazolinone and 0.02% Bromonitrodioxane.	50 ml
(1X) Control Diluent	Buffered protein base with preservative thiomersol 0.02% Methylisothiazolinone and 0.02% Bromonitrodioxane with 1:100 dilution human serum.	10 ml
(20X) Wash Buffer	20-fold concentrated solution of buffered surfactant with preservative thiomersol < 0.01%. May turn yellow over time.	25 ml
TMB Substrate	Stabilized Chromogen.	12 ml
Stop Solution	0.73M Phosphoric Acid.	12 ml
Instruction Manual		1 no

Materials to be provided by the End-User:

1. Microtiter Plate Reader able to measure absorbance at 450 nm.
2. Adjustable pipettes and multichannel pipettor to measure volumes ranging from 25 ul to 1000 ul.
3. Deionized (DI) water.
4. Wash bottle or automated microplate washer.
5. Graph paper or software for data analysis.
6. Timer.
7. Absorbent Paper.

Handling/Storage:

1. It is advisable to aliquot and store the Imiglucerase:HRP concentrated at -20°C upon receipt. Rest of the kit components should be stored at 2-8°C. Immediately discard any excess Working Imiglucerase:HRP after running your assay.
2. Before using, bring all components to room temperature (18-25°C). Upon assay completion return all components to appropriate storage conditions.
3. 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.

Sample Preparation and Storage:

Specimens should be clear and non-hemolyzed. Samples should be run at a number of dilutions to ensure accurate quantitation.

Blood is taken by venipuncture. Serum is separated after clotting by centrifugation. Plasma can be used, too. Lipaemic, hemolytic or contaminated samples should not be run. Repeated freezing and thawing should be avoided.

Samples should be diluted 1:100 (v/v) for optimal recovery, (for example 1 ul sample + 99 ul sample diluent) prior to assay. In cases where matrix interferences is under or over observed, the samples may be diluted less or more respectively with Sample Diluent accordingly.

The samples may be kept at 2-8°C for up to three days. For long-term storage please store at -20°C.
Note: *Grossly hemolyzed samples are not suitable for use in this assay.*

Preparation before Use:

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

In cases where matrix interferences is under or over observed, the samples may be diluted with Sample Diluent accordingly.

The samples may be kept at 2 - 8°C for up to three days. For long-term storage please store at -20°C.

Note: *Grossly hemolyzed samples are not suitable for use in this assay.*

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. **Preparation of Working Neutralizing Imiglucerase Positive Control** - Refer to the Reagent Preparation sheet attached with the IFU and COA (enclosed in the kit).
4. **Control Diluent is the Neutralizing Imiglucerase Negative Control.**
5. **Preparation of Working Imiglucerase:HRP** - Refer to the Reagent Preparation sheet attached with the IFU and COA (enclosed in the kit).
6. To make **Wash Buffer (1X)**; dilute **25 ml** of **20X Wash Buffer** in **475 ml of DI water**.

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 resulting in under-estimation of the amount of bound Neutralizing antibodies to Imiglucerase.
3. It is recommended that the Standards and Samples be assayed in duplicates.
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.

Assay Procedure:

1) Neutralization Reaction

1. Pipette **100 ul** of **Working Positive / Negative Control** in duplicate to the respective wells in the uncoated microplate.

2. Pipette **100 ul** of the diluted **Samples solution** into the respective wells in the uncoated microplate.

3. Pipette **100 ul** of the **Working Imiglucerase:HRP** into all the wells in the uncoated microplate, and mix well.

4. Seal the plate and **incubate** for **60 minutes** at **Room Temperature (18-25°C).**

2) Binding Reaction

1. Pipette **100 ul** of the **Positive / Negative Control Solution Complex** into the respective wells of the Recombinant Human IGF2R Protein coated microplate from the neutralization reaction plate.

2. Pipette **100 ul** of the diluted **Samples Solution Complex** into the respective wells of the Recombinant Human IGF2R Protein coated microplate from the neutralization reaction plate.

3. Seal plate and **incubate** for **60 minutes** at **Room Temperature (18-25°C)**.

4. 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. All the washes should be performed similarly.

5. Pipette **100 ul** of **TMB Substrate** solution to all the wells.

6. Incubate in the dark for **30 minutes** at **Room Temperature**.

7. Stop reaction by adding **100 ul** of **Stop Solution** to each well.

8. Read Absorbance at 450 nm within 15 minutes of stopping reaction.

Qualitative Interpretation:

Calculation for Cut Off Values -
Read the sample and positive control wells on microtitre plate reader at 450 nm.

The OD (Optical Density) of NC (Negative Control) in duplicate should be used for calculating the mean and standard deviation. This is the $Negative_{mean}$.

The Cut-Off for Negative Samples is $Negative_{mean}$ + 2*Standard Deviation.

Formula:
Negative Sample Value = OD > **(Negative$_{mean}$ +** ***2*SD)***

Typical example –

Sample Type	*Absorbance #1*	*Absorbance #2*	*Mean*
Negative	*1.511*	*1.555*	*1.533*

Therefore, Cut-Off = Mean + 2*SD
= 1.533 + (2*0.044)
= 1.533 + 0.088
= 1.621

This means samples which have OD450 values > than 1.62 (Cut-Off Value) are negative for Neutralizing antibodies to Imiglucerase. Samples which have OD450 values < than 1.62 (Cut-Off Value) are positive for Neutralizing antibodies to Imiglucerase.

Interpretation of Results:

Positive NAb- Imiglucerase Samples *	**< Cut-Off ***
Negative NAb- Imiglucerase Samples *	**=>Cut-Off***

** The cut-off value is based on validation using recombinant antibodies in the assay. Users may set up their own cut-off values based on different patient serum panels from different co-existent diseases, geographic locations or ethnic backgrounds.*

Explanation of Results Interpetation:

i) If Neutralizing Antibody is present in the sample, it will bind to Imiglucerase (conjugated to HRP). Imiglucerase -Nab complex will not be able to bind to Recombinant Human IGF2R Protein coated well. This in turn will lead to a lower absorbance value as less free Imiglucerase is available.
ii) If Neutralizing Antibody is not present in the sample, it will not bind to Imiglucerase (conjugated to HRP). Free Imiglucerase (conjugated to HRP) will then bind to Recombinant Human IGF2R Protein coated well. This in turn will lead to a higher absorbance value as more Imiglucerase (conjugated to HRP) is available.

Limitation of the Procedure:

This ELISA test is designed for qualitative detection of the neutralizing antibodies to Imiglucerase only. The Kit sensitivity is measured in terms of unit ng/ml of Neutralizing Antibodies to Imiglucerase and concentrations, if any of such antibodies being present lower than the kit sensitivity will not be detected with this kit.

Performance Characteristics:

This kit has been validated as per EMA/FDA guidelines in line with ICH Code for Harmonization of Biological Assays. Please note that this validation is performed in our laboratory and will not necessarily be duplicated in your laboratory with the same results.

The 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. Note Imiglucerase used in the kit is a research grade biosimilar.

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.

Assay Range:
The kit provided measures and interprets results as Qualitative.
However, the kit has been quantified to measure sensitivity using recombinant neutralizing antibodies to Imiglucerase.
Limit of Quantification: It is defined as the lowest concentration of an analyte that can be determined with an acceptable repeatability and the LOQ was found to be ~125 ng/ml.

Limit Of Detection: It is defined as the lowest detectable concentration corresponding to a signal of Mean of '0' standard plus 2*SD
10 replicates of '0' standards were evaluated and the LOD was found to be less than ~125 ng/ml.

Precision:
Intra-Assay: ~CV<17%
Inter-Assay: ~CV<20%

Specificity of the Immobilized Recombinant Human IGF2R Protein:
The immobilised human IGF2R is a recombinant protein expressed in HEK293 cells using a DNA sequence encoding the extracellular domain of human IGF2R (Insulin-like Growth Factor 2 Receptor). Its binding activity was verified using a functional ELISA. Immobilised human IGF2-hFc (2 µg/mL; 100 µL per well) demonstrated specific and dose-dependent binding to recombinant human IGF2R.

Specificity of Imiglucerase:
Imiglucerase is a recombinant analogue of the human lysosomal enzyme β-glucocerebrosidase, produced in Chinese hamster ovary (CHO) cells with an approximate molecular weight of 60 kDa.

Specificity of the Neutralizing Antibody (Positive Control):
The human Imiglucerase Neutralizing Antibody is a paratope-specific, inhibitory anti-idiotypic antibody that specifically recognises and binds to the active site or functional epitope of imiglucerase. It selectively binds the free recombinant beta-glucocerebrosidase molecule, thereby blocking its enzymatic activity toward glucocerebroside substrate. This neutralising antibody does not cross-react with unrelated recombinant enzymes and is intended for use as a positive control in assays designed to detect anti-drug antibodies against imiglucerase.

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.

References:

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Typical Example of a Work List

Well #	Contents	Absorbance at 450nm	Mean Absorbance
1A 2A	Negative Control Negative Control		
1B 2B	Positive Control Positive Control		
1C 2C	Sample Sample		
1D 2D	Sample Sample		
1E 2E	Sample Sample		
1F 2F	Sample Sample		
1G 2G	Sample Sample		
1H 2H	Sample Sample		

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SYMBOLS KEY

COATED MTP	Recombinant Human IGF2R Protein Coated Microtiter Plate (12x8 wells)
UNCOATED MTP	Uncoated Microtiter Plate (12x8 wells)
PC	Neutralizing Imiglucerase antibody:Positive Contol
DET HRP	Imiglucerase:HRP concentrated
HRP DIL	Imiglucerase:HRP Diluent
1X CNTRL DIL	(1X) Control Diluent
1X SAMP DIL	(1X) Sample Diluent
20X WASH BUF	(20X) Wash Buffer
SUB TMB	TMB Substrate
SOLN STOP	Stop Solution
	Consult Instructions for Use
REF	Catalog Number
	Expiration Date
	Storage Temperature