KRIBIOLISA™ Alemtuzumab ELISA

REF : KBI1012

Ver 4.0

RUO

Enzyme Immunoassay for the quantitative determination of Alemtuzumab in serum, plasma and cell culture supernatant

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 **KBI1012** **96 tests**

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Introduction:

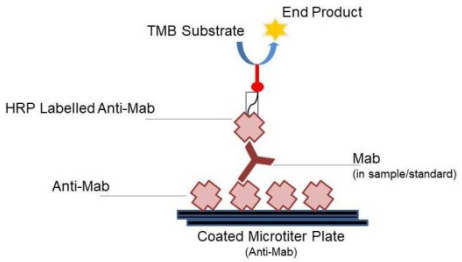
Alemtuzumab is a drug used in the treatment of chronic lymphocytic leukemia (CLL), cutaneous T-cell lymphoma (CTCL) and T-cell lymphoma under the trade names Campath, MabCampath and Campath-1H, and in the treatment of multiple sclerosis as Lemtrada. It is also used in some conditioning regimens for bone marrow transplantation, kidney transplantation and islet cell transplantation. It is a monoclonal antibody that binds to CD52, a protein present on the surface of mature lymphocytes, but not on the stem cells from which these lymphocytes are derived. After treatment with alemtuzumab, these CD52-bearing lymphocytes are targeted for destruction. MabCampath was withdrawn from the markets in the US and Europe in 2012 to prepare for a higher-priced relaunch of Lemtrada aimed at multiple sclerosis.

Intended Use:

The KRIBIOLISA™ Alemtuzumab ELISA is used as an analytical tool for quantitative determination of Alemtuzumab in serum, plasma and cell culture supernatant.

Principle:

The method employs the quantitative sandwich enzyme immunoassay technique. Antibodies to Alemtuzumab are pre-coated onto microwells. Samples and standards are pipetted into microwells and human Alemtuzumab present in the sample are bound by the capture antibody. Then, a HRP (horseradish peroxidase) conjugated anti-Alemtuzumab antibody is pipetted and incubated. After washing microwells in order to remove any non-specific binding, the ready to use substrate solution (TMB) is added to microwells and color develops proportionally to the amount of Alemtuzumab in the sample. Color development is then stopped by addition of stop solution. Absorbance is measured at 450 nm.



Materials Provided:

1. Anti-Alemtuzumab Coated Microtiter Plate (12x8 wells) – 1 no
2. Alemtuzumab Standard, (0.5 ml/vial) – 0, 10, 20, 40, 80, 160, 320 and 640 ng/ml
3. Anti-Alemtuzumab:HRP Conjugate – 12 ml
4. Assay Diluent – 6 ml
5. Sample Diluent – 50 ml
6. (20X) Wash Buffer – 25 ml
7. TMB Substrate – 12 ml
8. Stop Solution – 12 ml
9. Instruction Manual

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. All reagents should be stored at 2ºC to 8ºC for stability.
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:

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. If samples are to be used for several assays, initially aliquot samples and keep at - 20°C.

For Cell Culture Supernatant **–** If necessary, centrifuge to remove debris prior to analysis. Samples can be stored at -20°C or -80^{0}C. Avoid repeated freeze-thaw cycles.

For Serum - Samples have to be diluted 1:100 (v/v), e.g. 1 ul sample + 99 ul sample diluent prior to assay. The samples may be kept at 2 - 8°C for up to three days. Long-term storage requires -20°C.

For Plasma - Samples have to be diluted 1:1000 (v/v), e.g. 1 ul sample + 999 ul sample diluent prior to assay. The samples may be kept at 2 - 8°C for up to three days. Long-term storage requires -20°C.

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 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. High Dose Hook Effect may be observed in samples with very high concentrations of Alemtuzumab. High Dose Hook Effect is due to excess of antibody for very high concentrations of Alemtuzumab present in the sample. High Dose Hook effect is most likely encountered from samples early in the purification process. If Hook Effect is possible, the samples to be assayed should be diluted with a compatible diluent. Thus if the Alemtuzumab concentration of the undiluted sample is less than the diluted sample, this may be indicative of the Hook Effect.
3. Avoid assay of Samples containing sodium azide (NaN_3), as it could destroy the HRP activity resulting in under-estimation of the amount of Alemtuzumab.
4. It is recommended that all Standards and Samples be assayed in duplicates.
5. Maintain a repetitive timing sequence from well to well for all the steps to ensure that the incubation timings are same for each well.

6. 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.
7. The plates should be read within 30 minutes after adding the Stop Solution.
8. Make a work list in order to identify the location of Standards and Samples.

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 37°C

2. Pipette out **50 ul** of **Assay Diluent** in each well.

3. Add **100 ul** of **Standards** or **diluted Samples** into the respective wells.

4. Cover the plate and incubate for 60 minutes at 37°C

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. Pipette without delay in the same order **100 ul** of **Anti-Alemtuzumab:HRP Conjugate** into each well.

7. Cover the plate and incubate for 60 minutes at 37°C

8. 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.

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

10. Incubate the plate at 37°C 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.

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

12. Read the absorbance at 450 nm with a microplate reader.

Calculation of Results:

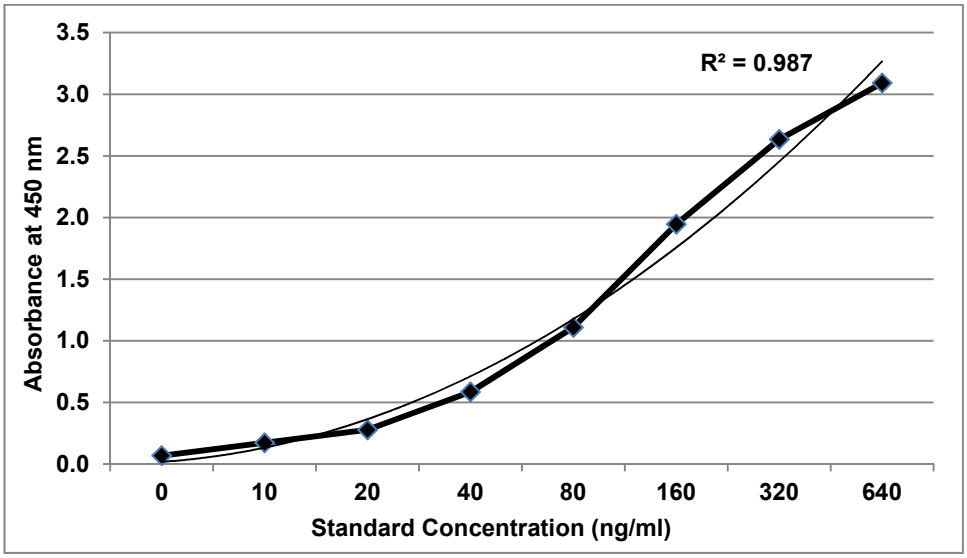
Determine the Mean Absorbance for each set of duplicate or triplicate Standards and Samples. Using Graph paper, plot the average value (absorbance 450nm) of each standard on the Y-axis versus the corresponding concentration of the standards on the X-axis. Draw the best fit curve through the standard points. To determine the unknown Alemtuzumab concentrations, find the unknown's Mean Absorbance value on the Y-axis and draw a horizontal line to the standard curve. At the point of intersection, draw a vertical line to the X-axis and read the Alemtuzumab Concentration. If samples were diluted, multiply by the appropriate dilution factor. Software which is able to generate a cubic spline curve-fit is best recommended for automated results.

Note:
It is recommended to repeat the assay at a different dilution factor in the following cases:
- If the sample absorbance value is below the first standard.
- If the absorbance value is equivalent or higher than the 640 ng/ml standard.

Typical Data

Standard provided (ng/ml)	Abs A	Abs B	Mean Abs	Interpolated Concentration	% Interpolated Concentration against Actual Concentration
0	0.069	0.067	0.068	--	--
10	0.169	0.177	0.173	11.3	112.8
20	0.302	0.255	0.278	19.6	98.2
40	0.620	0.549	0.585	40.9	102.2
80	1.172	1.046	1.109	78.1	97.6
160	1.972	1.920	1.946	163.1	101.9
320	2.619	2.648	2.633	315.5	98.6
640	3.091	3.090	3.090	644.5	100.7

Typical Graph



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 of the Kit:

This kit has been validated as per EMA/FDA guidelines in line with ICH Code for Harmonization of Biological Assays.

Sensitivity:

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 2.5 ng/ml

Linearity:

Standards provided in the kit will be used for measuring the linearity range of Alemtuzumab present in matrix.

Human Serum Dilution	Standards provided (ng/ml)	Abs	Interepolated Concentration	% Interpolated Concentration against Actual Concentration
Serum 1:10 dilution	0	0.069	--	--
	640	3.271	Abs* beyond Std Range	*
Serum 1:100 dilution	0	0.059	--	--
	640	3.016	551.7	86.2
Serum 1:1000 dilution	0	0.060	--	--
	640	2.944	484.5	75.7
Serum 1:2000 dilution	0	0.058	--	--
	640	2.834	408.4	63.8
Serum 1:5000 dilution	0	0.057	--	--
	640	3.107	670.6	104.8

Abs* absorbance was beyond the absorbance value of the graph, hence interpolation of the concentration not possible

Human Plasma Dilution	Standards provided (ng/ml)	Abs	Interepolated Concentration	% Interpolated Concentration against Actual Concentration
Plasma 1:10 dilution	0	0.051	--	--
	640	3.184	825.2	128.9
Plasma 1:100 dilution	0	0.059	--	--
	640	2.923	467.9	73.1
Plasma 1:500 dilution	0	0.060	--	--
	640	2.920	465.6	72.8
Plasma 1:1000 dilution	0	0.066	--	--
	640	2.998	533.2	83.3
Plasma 1:2000 dilution	0	0.058	-- --	--
	640	2.994	529.3	82.7
Plasma 1:5000 dilution	0	0.062		--
	640	2.899	450.3	70.4

Precision:

Precision is defined as the percent coefficient of variation (%CV) i.e. standard deviation divided by the mean and multiplied by 100. Assay precision was determined by both intra (n=5 assays) and inter assay (n=5 assays) reproducibility on two pools with low (10ng/ml), medium (80ng/ml) and high (640ng/ml) concentrations. While actual precision may vary from laboratory to laboratory and technician to technician, it is recommended that all operators achieve precision below these design goals before reporting results.

Pool	Intra Assay %CV	Inter Assay %CV
Low	<10%	<10%
Medium	<5%	<5%
High	<5%	<5%

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/w) 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:

Alemtuzumab compared with chlorambucil as first-line therapy for chronic lymphocytic leukemia P Hillmen, AB Skotnicki, T Robak, B Jaksic… - Journal of Clinical …, 2007 - ascopubs.org

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Phase II trial of subcutaneous anti-CD52 monoclonal antibody alemtuzumab (Campath-1H) as first-line treatment for patients with B-cell chronic lymphocytic leukemia … J Lundin, E Kimby, M Björkholm, PA Broliden… - Blood, 2002 - Am Soc Hematology

Alemtuzumab versus interferon beta 1a as first-line treatment for patients with relapsing-remitting multiple sclerosis: a randomised controlled phase 3 trial. JA Cohen, AJ Coles, DL Arnold, C Confavreux, EJ Fox… - The Lancet, 2012 - Elsevier

Alemtuzumab for patients with relapsing multiple sclerosis after disease-modifying therapy: a randomised controlled phase 3 trial. AJ Coles, CL Twyman, DL Arnold, JA Cohen… - The Lancet, 2012 - Elsevier.

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Subcutaneous alemtuzumab in fludarabine-refractory chronic lymphocytic leukemia: clinical results and prognostic marker analyses from the CLL2H study of the …S Stilgenbauer, T Zenz, D Winkler, A Bühler… - Journal of Clinical …, 2009 - ascopubs.org

Eradication of minimal residual disease in B-cell chronic lymphocytic leukemia after alemtuzumab therapy is associated with prolonged survival ... P Moreton, B Kennedy, G Lucas, M Leach… - Journal of Clinical …, 2005 - journal.9med.net

Results from a human renal allograft tolerance trial evaluating the humanized CD52-specific monoclonal antibody alemtuzumab (CAMPATH-1H). AD Kirk, DA Hale, RB Mannon, DE Kleiner… - …, 2003 - journals.lww.com

SCHEMATIC ASSAY PROCEDURE

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

Components (2-8ºC) → 30 mins RT Before Use → 

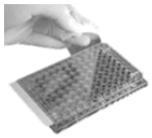
→ Thaw at Room Temperature (18-24ºC) → USE NOW

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

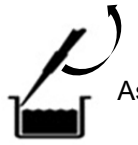
Kit Components → AVOID → COOL (2-8ºC) → THAW (22±4ºC) → THAW (22±4ºC) → COOL (2-8ºC) → AVOID → NO CHANGE IN RESULTS

3.  Pipette **50 ul Assay Diluent** into each well.

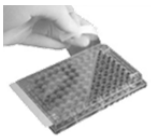
4.  Pipette **100 ul Standards** / **diluted Samples** into the respective wells.

5. Cover plate  and incubate for 

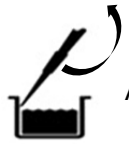
at 37°C.

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

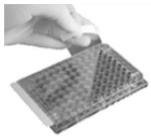
7.  Pipette **100 ul Anti Alemtuzumab:HRP** into each well.

8. Cover plate  and incubate for 

at 37°C.

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

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

11. Cover plate  and incubate for 

at 37°C.

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

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

of stopping reaction.

Typical Example of a Work List

Well #	Contents	Absorbance at 450nm	Mean Absorbance	ng/ml Alemtuzumab equivalent
1A 2A	zero std zero std			
1B 2B	10 ng/ml 10 ng/ml			
1C 2C	20 ng/ml 20 ng/ml			
1D 2D	40 ng/ml 40 ng/ml			
1E 2E	80 ng/ml 80 ng/ml			
1F 2F	160 ng/ml 160 ng/ml			
1G 2G	320 ng/ml 320 ng/ml			
1H 2H	640 ng/ml 640 ng/ml			
3A 4A	Sample			
3B 4B	Sample			

LIMITED WARRANTY

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THANK YOU FOR USING KRISHGEN PRODUCT!

SYMBOLS KEY

MTP	**Anti-Alemtuzumab Microtiter Plate (12X8 wells)**
STD	**Alemtuzumab Standard**
HRP CONJ	**Conjugate Horseradish Peroxidase**
ASY DIL	**Assay Diluent**
SAMP DIL	**Sample Diluent**
20X WASH BUF	**(20X) Wash Buffer**
SUB TMB	**TMB Substrate**
SOLN STOP	**Stop Solution**
	**Consult Instructions for Use**
REF	**Catalogue Number**
	**Expiration Date**
	**Storage Temperature**