KRIBIOLISA™ Nilotinib ELISA

REF : KOD1012

Ver1.0

RUO

Enzyme Immunoassay for the quantitative determination of Nilotinib 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 KOD1012 **Σ 96 tests**

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Introduction:

Nilotinib (AMN107, trade name Tasigna), in the form of the hydrochloride monohydrate salt, is a small-molecule tyrosine kinase inhibitor approved for the treatment of imatinib-resistant chronic myelogenous leukemia. Structurally related to imatinib, it was developed based on the structure of the Abl-imatinib complex to address imatinib intolerance and resistance.

Intended Use:

This kit is used for quantitative estimation of Nilotinib in serum, plasma and cell culture supernatant.

Principle:

The Nilotinib ELISA is a competitive immunoassay for the determination of Nilotinib. A constant concentration of **Tracer**; varying concentrations of unlabeled standard and BCR-ABL Tyrosine Kinase are added in sequence to the uncoated microtitre plate wells and incubated. After 1st incubation, Anti- (BCR-ABL Tyrosine Kinase) Antibody is dispensed to all wells of uncoated Microtitre plate containing the reaction mixture and incubated. After 2nd incubation, all reaction mixtures are transferred to the respective wells of coated microtitre plate and incubated. After washing, Streptavidin HRP conjugate is added and incubated. Captured **Tracer** is subsequently bound by streptavidin HRP which produces a soluble colored product after addition of 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 Nilotinib molecule present in standards or samples.

Materials Provided:

1. Uncoated Microtiter Plate (12x8 wells) – 1
2. Microtiter Coated Plate (12x8 wells) – 1
3. BCR-ABL Tyrosine Kinase – 1 Vial
4. Anti- (BCR-ABL Tyrosine Kinase) Antibody - 1 Vial
5. Nilotinib Standard – 1 vial
6. Biotin Conjugate (**Tracer**) – 1 vial
7. Concentrated Streptavidin HRP conjugate – 1 vial
8. Wash Buffer (20X) – 25ml
9. Assay Diluent– 50ml
10. TMB Substrate – 12ml
11. Stop Solution – 12ml
12. Instruction Manual

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 50µl to 1000µl.
3. Deionized (DI) water.
4. Wash bottle or automated microplate washer.
5. Semi-log 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 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 -80ºC. Avoid repeated freeze-thaw cycles.

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 50 ml of 20X Wash Buffer in 950 ml of DI water.
4. **Standard:** The concentration of the standard in the stock solution is **1µM**. Prepare the standards as per the table given below using the provided standard Concentration and standard diluent.

400 nM	Standard No.1	80µl Original Standard (**1µM**) + 120µl of Assay Diluent
200 nM	Standard No.2	100µl Standard No.1 + 100µl Assay Diluent
100 nM	Standard No.3	100µl Standard No.2 + 100µl Assay Diluent
50 nM	Standard No.4	100µl Standard No.3 + 100µl Assay Diluent
25 nM	Standard No.5	100µl Standard No.4 + 100µl Assay Diluent
12.5 nM	Standard No.6	100µl Standard No.5 + 100µl Assay Diluent
6.25 nM	Standard No.7	100µl Standard No.6 + 100µl Assay Diluent
0 nM	Standard No.8	100µl Assay Diluent

Mix each tube thoroughly before the next transfer. Set up 7 points of diluted standard such as 400 nM, 200 nM, 100 nM, 50 nM, 25 nM, 12.5 nM and 6.25 nM.

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. If the concentration of the undiluted sample is less than the diluted sample, this may be indicative of the Hook Effect. To overcome Hook Effect samples to be assayed should be sufficiently diluted with our recommended diluent.
3. 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.
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.
9. Making serial dilution in the wells directly is not permitted.
10. **Prepare standard within 15 minutes before assay.** Please do not dissolve the reagents at 37ºC directly.
11. **Biotin Conjugate** and **Streptavidin-HRP** are sticky solutions; therefore, slowly pipette them to reduce the volume errors.
12. Please carefully reconstitute Standards or working **Biotin Conjugate** and **Streptavidin-HRP** according to the instruction, and avoid foaming and mix gently until the crystals are completely dissolved. To minimize imprecision caused by pipetting, use small volumes and ensure that pipettes are calibrated. It is recommended to suck more than 10µL for one pipetting.

13. If crystals have formed in the Wash Solution concentrate (20X), warm to room temperature and mix gently until the crystals are completely dissolved.
14. Contaminated water or container for reagent preparation will influence the detection result.

Assay Procedure:

1. It is strongly recommended that all Controls 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 **50µl of Tracer, 50µl of Standard and 50µl of BCR-ABL Tyrosine Kinase** to all marked wells in the uncoated microtitre plate.
3. Cover the plate and incubate for 120 minutes at 37°C.
4. Add **50µl Anti- (BCR-ABL Tyrosine Kinase) Antibody** in to respective wells containing the reaction mixture.
5. Cover the plate and incubate for 60 minutes at 37°C.
6. Transfer **150 µl of all reaction mixtures** to the marked wells in the coated microtitre plate.
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. Pipette in the same order **100 µl of Streptavidin-HRP Conjugate** into each well.
10. Cover plate and Incubate at 37˚C for 1.0 hour.
11. 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.
12. Add **100 µl** of **TMB Substrate** in each well.
13. Incubate the plate at 37°C for 30 - 60 minutes in dark. DO NOT SHAKE or else it may result in higher backgrounds and worse precision. Positive wells should turn bluish in color.
14. Pipette out **100 µl** of **Stop Solution**. Wells should turn from blue to yellow in color.
15. 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 Semi-Log 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 Nilotinib 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 Nilotinib 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.

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: 6.25 nM - 400 nM. The standard curve concentrations are 400 nM, 200 nM, 100 nM, 50 nM, 25 nM, 12.5 nM and 6.25 nM.

Sensitivity:

The minimum detectable dose of Nilotinib is typically less than 1 nM. The sensitivity of this assay, or Lower Limit of Detection (LLD) was defined as the lowest protein concentration that could be differentiated from zero. It was determined by subtracting two standard deviations to the mean optical density value of twenty zero standard replicates and calculating the corresponding concentration.

Precision:

Intra-assay Precision: 3 samples with low, middle and high level human Nilotinib were tested 20 times on one plate, respectively.
Inter-assay Precision: 3 samples with low, middle and high level human Nilotinib were tested on 3 different plates, 8 replicates in each plate.
CV (%) = SD/mean X 100
Intra-Assay: CV<10%
Inter-Assay: CV<12%

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:

Extended kinase profile and properties of the protein kinase inhibitor nilotinib.Manley, P.W., Drueckes, P., Fendrich, G., Furet, P. Liebetanz, J., Martiny-Baron, G., Mestan, J., Trappe, J., Wartmann, M. & Fabbro D. (2010) Biochim. Biophys. Acta

Activity-based kinase profiling of approved tyrosine kinase inhibitors…Daisuke Kitagawa,* Koichi Yokota Masaki Gouda…Genes to Cells (2013)…. the Molecular Biology Society of Japan….Wiley

A systematic interaction map of validated kinase inhibitors with Ser/Thr kinases. Fedorov, O., Marsden, B., Pogacic, V., Rellos, P., Muller, S.,Bullock, A.N., Schwaller, J., Sundstrom, M. & Knapp, S. Epub 2007…Proc. Natl Acad. Sci. USA

Nilotinib: a phenylamino- pyrimidine derivative with activity against BCR-ABL, KIT and PDGFR kinases. Quintas-Cardama, A. & Cortes, J. (2008)…Future Oncol.

SCHEMATIC ASSAY PROCEDURE

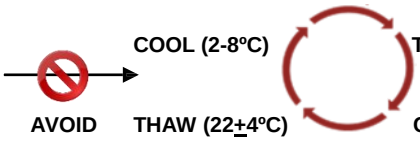
1. Remove all components, 30 minutes before adding into the assay plate.

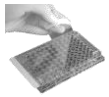
Components (2-8ºC) → (30 mins, RT Before Use) → 

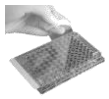
Thaw at Room Temperature (18-24ºC) → USE NOW

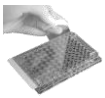
2. Avoid repeated cool-thaw of the components as there will be a loss of activity and this can affect the results.

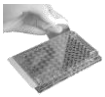
Kit Components → AVOID → COOL (2-8ºC) → THAW (22±4ºC) → AVOID → NO CHANGE IN RESULTS

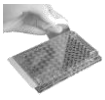


1.  Pipette **50 µl Tracer** + **50 µl Standard** + **50 µl BCR-ABLTyrosine kinase** into each well.
2. Cover plate  and incubate for 

at 37°C.
3.  Pipette **50 µl Anti- BCR-ABLTyrosine kinase Antibody** into the respective wells.
4. Cover plate  and incubate for 

at 37°C.
5.  Transfer **150 µl** of **all reaction mixtures** to coated wells.
6. Cover plate  and incubate for 

at 37°C.
7.  Aspirate and wash wells 4 times with **Wash Buffer (1X).**
8.  Pipette **100 µl Streptavidin: HRP** into each well.
9. Cover plate  and incubate for 

at 37°C.
10.  Aspirate and wash wells 4 times with **Wash Buffer (1X).**
11.  Pipette **100 µl TMB Substrate** into each well.
12. Cover plate  and incubate for 

at 37°C.
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	nM Nilotinib equivalent
1A 2A	zero std zero std			
1B 2B	6.25 nM 6.25 nM			
1C 2C	12.5 nM 12.5 nM			
1D 2D	25 nM 25 nM			
1E 2E	50 nM 50 nM			
1F 2F	100 nM 100 nM			
1G 2G	200 nM 200 nM			
1H 2H	400 nM 400 nM			
3A 4A	Sample			
3B 4B	Sample			

LIMITED WARRANTY

Krishgen Biosystems does not warrant against damages or defects arising in shipping or handling, or out of accident or improper or abnormal use of the product; against defects in products or components not manufactured by Krishgen Biosystems, or against damages resulting from such non-Krishgen Biosystems made products or components. Krishgen Biosystems passes on to customer the warranty it received (if any) from the maker thereof of such non-Krishgen made products or components. This warranty also does not apply to product to which changes or modifications have been made or attempted by persons other than pursuant to written authorization by Krishgen Biosystems.

THIS WARRANTY IS EXCLUSIVE. The sole and exclusive obligation of Krishgen Biosystems shall be to repair or replace the defective product in the manner and for the period provided above. Krishgen Biosystems shall not have any other obligation with respect to the products or any part thereof, whether based on contract, tort, strict liability or otherwise. Under no circumstances, whether based on this Limited Warranty or otherwise, shall Krishgen Biosystems be liable for incidental, special, or consequential damages.

This Limited Warranty states the entire obligation of Krishgen Biosystems with respect to the product. If any part of this Limited Warranty is determined to be void or illegal, the remainder shall remain in full force and effect.

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