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ImmunoGuide®

Instructions for Use

Ranibizumab ELISA (mAb-based) (Aqueous Humour)

Enzyme immunoassay for the specific determination
of free Ranibizumab in aqueous humour

REF: TM09034



12X8



2-8°C

For Research Use Only - Not for Use in Diagnostic Procedures

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1. INTENDED USE

Enzyme immunoassay for the specific determination of free Ranibizumab in aqueous humour. For research use only - not for use in diagnostic procedures. Not for use in serum or plasma*.

2. SUMMARY AND EXPLANATION

The drug Ranibizumab (trade name Lucentis®) is a recombinant humanized IgG1 kappa isotype monoclonal antibody fragment (Fab) designed for intraocular use. Ranibizumab binds to and inhibits the biologic activity of human vascular endothelial growth factor A (VEGF-A). Ranibizumab, which lacks an Fc region, has a molecular weight of approximately 48 kilodaltons.

The *ImmunoGuide* Ranibizumab ELISA (mAb-based) kit can be efficiently used for measuring free Ustekinumab levels in aqueous humour.

3. PRINCIPLE OF THE TEST

This ELISA is based on a Ranibizumab-specific monoclonal antibody (catcher Ab, *ImmunoGuide* clone 2A1). Diluted standards and samples are incubated in the microtiter plate coated with IG-2A1 mAb. After incubation, the wells are washed. A biotinylated anti-human kappa light chain monoclonal antibody (clone *ImmunoGuide* 7G8(Z)) is added and binds to the kappa light chain part of Ranibizumab. Following incubation, wells are washed, and the horseradish peroxidase (HRP)-conjugated streptavidin is added and binds to the biotinylated 7G8(Z) mAb. Following incubation, wells are washed and the bound enzymatic activity is detected by addition of chromogen-substrate. The colour developed is proportional to the amount of Ranibizumab in the sample or standard. Results of samples can be determined by using the standard curve. Preincubation of Ranibizumab with recombinant human VEGF inhibited the reaction. Therefore, the *ImmunoGuide* Ranibizumab ELISA (mAb-based) measures the free form of Ranibizumab.

4. WARNINGS AND PRECAUTIONS

1. Before starting the assay, read the instructions completely and carefully. Use the valid version of the package insert provided with the kit. Be sure that everything is understood. For further information (clinical background, test performance, automation protocols, alternative applications, literature, etc.) please refer to the local distributor.
2. In case of severe damage of the kit package, please contact **IBL-America** or your supplier in writing, latest one week after receiving the kit. Do not use damaged components in test runs, but keep safe for complaint related issues.
3. Obey lot number and expiry date. Do not mix reagents of different lots. Do not use expired reagents.
4. Follow good laboratory practice and safety guidelines. Wear lab coats, disposable latex gloves and protective glasses where necessary.
5. Reagents of this kit containing hazardous material may cause eye and skin irritations. See MATERIALS SUPPLIED and labels for details.
6. Chemicals and prepared or used reagents have to be treated as hazardous waste according the national biohazard safety guidelines or regulations.
7. Avoid contact with Stop solution. It may cause skin irritations and burns.
8. If any component of this kit contains human serum or plasma it is indicated and if so, it have been tested and were found to be negative for HIV I/II, HBsAg and HCV. However, the presence of these or other infectious agents cannot be excluded absolutely and therefore reagents should be treated as potential biohazards in use and for disposal.
9. Some reagents contain preservatives. In case of contact with eyes or skin, flush immediately with water.

5. STORAGE AND STABILITY OF THE KIT

The kit is shipped at ambient temperature and should be stored at 2-8°C. Keep away from heat or direct sun light. The storage and stability of specimen and prepared reagents is stated in the corresponding chapters. The microtiter strips are stable up to the expiry date of the kit in the broken, but tightly closed bag when stored at 2-8°C.

6. SPECIMEN COLLECTION, HANDLING AND STORAGE

Aqueous humour

The usual precautions for obtaining aqueous humour should be observed. It is important to preserve the chemical integrity of an aqueous humour sample from the moment it is collected until it is assayed. Samples appearing turbid should be centrifuged before testing to remove any particulate material.

Storage:	2-8°C	≤-20°C (Aliquots)	Keep away from heat or direct sun light Avoid repeated freeze-thaw cycles
Stability:	3 d	6 mon	

7. CONTENTS OF THE KIT

QUANTITY	COMPONENT
1 x 12 x 8	Microtiter ELISA Plate Break apart strips coated with anti-Ranibizumab mouse monoclonal antibody.
5 x 0.5 mL	Ranibizumab Standards A-E, Concentrate (10X) 300; 100; 30; 10; and 0 ng/mL Used for construction of the standard curve. Contains Ranibizumab, proteins, preservative and stabilizer.
1 x 12 mL	Assay Buffer Blue colored. Ready to use. Contains proteins and preservative.
1 x 60 mL	Dilution Buffer, Concentrate (5X) Orange colored. Contains proteins and preservative.
1 x 12 mL	Biotinylated α-hKappa mAb Green colored. Ready to use. Contains biotinylated anti-human kappa light chain monoclonal antibody, proteins, preservative and stabilizers
1 x 12 mL	Enzyme Conjugate Red colored. Ready to use. Contains horseradish peroxidase(HRP)-conjugated streptavidin (HRP-Streptavidin), Proclin [®] and stabilizers.
1 x 12 mL	TMB Substrate Solution Ready to use. Contains 3,3',5,5'-Tetramethylbenzidine (TMB).
1 x 12 mL	Stop Solution Ready to use. 1 N Hydrochloric acid (HCl)..
1 x 50 mL	Wash Buffer, Concentrate (20x) Contains buffer, Tween [®] 20 and Kathon [™] .
3 x 1	Adhesive Seal For sealing microtiter plate during incubation.

8. MATERIALS REQUIRED BUT NOT SUPPLIED

1. Micropipettes (< 3% CV) and tips to deliver 5-1000 μ L.
2. Bidistilled or deionised water and calibrated glasswares (e.g. flasks or cylinders).
3. Wash bottle, automated or semi-automated microtiter plate washing system.
4. Microtiter plate reader capable of reading absorbance at 450 nm (reference wavelength at 600-650 nm is optional).
5. Absorbent paper towels, standard laboratory glass or plastic vials, and a timer.

9. PROCEDURE NOTES

1. Any improper handling of samples or modification of the test procedure may influence the results. The indicated pipetting volumes, incubation times, temperatures and pre-treatment steps have to be performed strictly according to the instructions. Use calibrated pipettes and devices only.
2. Once the test has been started, all steps should be completed without interruption. Make sure that required reagents, materials and devices are prepared readily at the appropriate time. Allow all reagents and specimens to reach room temperature (20-25 °C) and gently swirl each vial of liquid reagent and sample before use. Mix reagents without foaming.
3. Avoid contamination of reagents, pipettes and wells/tubes. Use new disposable plastic pipette tips for each reagent, standard or specimen. Do not interchange the caps of vials. Always cap not used vials. Do not reuse wells or reagents.
4. Use a pipetting scheme to verify an appropriate plate layout.
5. Incubation time affects results. All wells should be handled in the same order and time sequences. It is recommended to use an 8-channel Micropipettor for pipetting of solutions in all wells.
6. Microplate washing is important. Improperly washed wells will give erroneous results. It is recommended to use a multichannel pipette or an automatic microplate washing system. Do not allow the wells to dry between incubations. Do not scratch coated wells during rinsing and aspiration. Rinse and fill all reagents with care. While rinsing, check that all wells are filled precisely with Wash Buffer, and that there are no residues in the wells.
7. Humidity affects the coated wells. Do not open the pouch until it reaches room temperature. Unused wells should be returned immediately to the resealed pouch including the desiccant.

10. PRE-TEST SETUP INSTRUCTIONS

10.1. Preparation of Components*

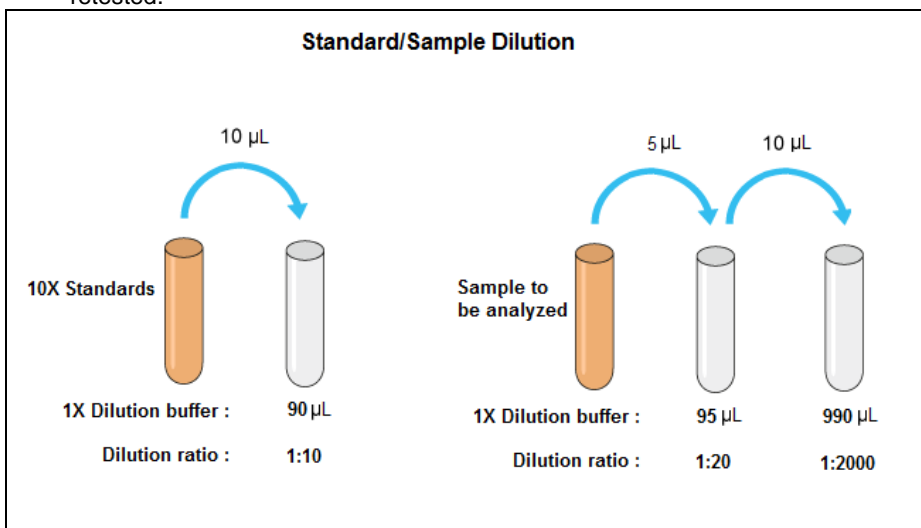
Dilute/ dissolve	Component		Diluent	Relation	Remarks	Storage	Stability
10 mL	Wash Buffer	up to 200 mL	Distilled Water	1:20	Warm up at 37°C to dissolve crystals. Mix vigorously.	2-8 °C	4 w
10 mL	Dilution Buffer	up to 50 mL	Distilled Water	1:5		2-8 °C	4 w

* Prepare Wash and Dilution Buffers before starting the assay procedure.

10.2. Dilution of Standards and Samples

The dilutions depicted below are examples of how to obtain final dilutions for standards and samples. Standards and samples should be properly diluted as homogenous mixture before starting the assay procedure. It is recommended mixing the standards and samples well to homogenous solution at each dilution step.

1. 10 μL of standard is added to 90 μL of 1X dilution buffer, giving the total volume of 100 μL and a dilution of 1:10.
2. 5 μL of sample is added to 95 μL of 1X dilution buffer, giving the total volume of 100 μL and a dilution of 1:20. Further then, 10 μL of 1:20 diluted sample is added to 990 μL of 1X dilution buffer, giving the total volume of 1000 μL and a final dilution of 1:2000.
3. Samples with a drug concentration above the measuring range should be rated as ">highest standard". The result should not be extrapolated. The sample in question should be further diluted with 1X Dilution Buffer and then retested.



11. TEST PROCEDURE

11.1. GENERAL REMARKS

11.1.1. Before performing the assay, samples and assay kit should be brought to room temperature (about 30 minutes beforehand) and ensure the homogeneity of the solution.

11.1.2. All Standards should be run with each series of unknown samples.

11.1.3. Standards should be subject to the same manipulations and incubation times as the samples being tested.

11.1.4. All steps of the test should be completed without interruption.

11.1.5. Use new disposable plastic pipette tips for each reagent, standard or specimen in order to avoid cross contamination.

11.1.6. The total pipetting time needed for dispensing all samples into the wells should not exceed 5 minutes. If this is difficult to achieve the samples should be pre-dispensed in a separate neutral polypropylene microplate and then transferred into the reaction ELISA plate by a multi channel pipette. Such neutral plates are available from the kit manufacturer upon request.

11.2. ASSAY PROCEDURE

1.	Pipette 100 µL of Assay Buffer into each of the wells to be used.
2.	Pipette 75 µL of each 1:10 Diluted Standard, and 1:2000 Diluted Samples into the respective wells of the microtiter plate. Bubble formation during the pipetting of standards and samples must be avoided. <u>Wells</u> A1: Standard A B1: Standard B C1: Standard C D1: Standard D E1: Standard E F1 and so on: Samples (Aqueous Humour)
3.	Cover the plate with adhesive seal. Shake plate carefully by tapping several times. Incubate the plate on bench top for 60 min at room temperature (RT, 20-25°C).
4.	Remove adhesive seal. Aspirate or decant the incubation solution. Wash the plate 4 X 350 µL of Diluted Wash Buffer per well. Remove excess solution by tapping the inverted plate on a paper towel.
5.	Pipette 100 µL of Biotinylated α-h Kappa mAb into each well.
6.	Cover plate with adhesive seal. Shake plate carefully by tapping several times. Incubate the plate on a bench top for 30 min at RT.
7.	Remove adhesive seal. Aspirate or decant the incubation solution. Wash the plate 4 X 350 µL of Diluted Wash Buffer per well. Remove excess solution by tapping the inverted plate on a paper towel.
8.	Pipette 100 µL of Enzyme Conjugate (HRP-Streptavidin) into each well.
9.	Cover plate with adhesive seal. Shake plate carefully by tapping several times. Incubate the plate on a bench top for 30 min at RT.
10.	Remove adhesive seal. Aspirate or decant the incubation solution. Wash the plate 4 X 350 µL of Diluted Wash Buffer per well. Remove excess solution by tapping the inverted plate on a paper towel.
11.	Pipette 100 µL of Ready-to-Use TMB Substrate Solution into each well.
12.	Incubate 15 min at RT. Avoid exposure to direct sunlight.
13.	Stop the substrate reaction by adding 100 µL of Stop Solution into each well. Color changes from blue to yellow. Briefly mix contents by gently shaking the plate.
14.	Measure optical density (OD) with a photometer at 450 nm (Reference at OD620 nm is optional) within 15 min after pipetting the Stop Solution.

11. 3. QUALITY CONTROL

The test results are only valid if the test has been performed following the instructions. Moreover the user must strictly adhere to the rules of GLP (Good Laboratory Practice) or other applicable standards/laws. All standards/controls must be found within the acceptable ranges as stated above and/or label. If the criteria are not met, the run is not valid and should be repeated. In case of any deviation, the following technical issues should be reviewed: Expiration dates of (prepared) reagents, storage conditions, pipettes, devices, incubation conditions and washing methods.

11. 4. CALCULATION OF RESULTS

A standard curve should be constructed using the standard concentration (X-axis) versus the OD450 (or OD450/620) values (Y-axis). This can be done manually using graph paper or with a computer program. Concerning the data regression by computer, it is recommended to primarily use the "4 Parameter Logistic (4PL)" or alternatively the "point-to-point calculation". In case of manual plot there are 2 options: Semilog graph (see Fig. A) or linear graph (see Fig. B). Semilog graph paper is available at <http://www.papersnake.com/logarithmic/semilogarithmic/>.

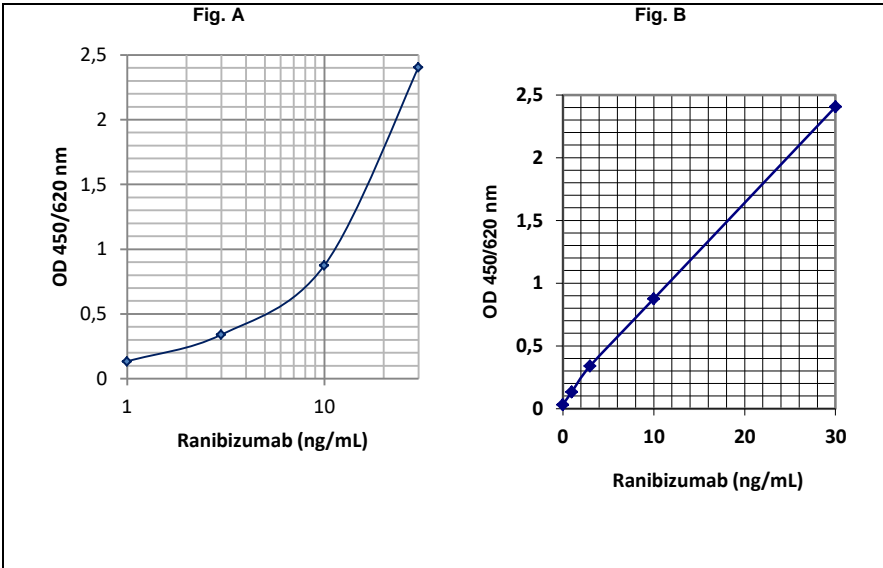
The concentration of the samples can be read from this standard curve as follows. Using the absorbance value for each sample, determine the corresponding concentration of the drug from the standard curve. This value always has to be multiplied by the individual dilution factor (usually 2000). If any diluted sample is reading higher than the highest standard, it should be further diluted appropriately with 1X Dilution Buffer and retested. Also this second dilution has to be used for calculation of the final result.

A sample with a drug concentration lower than the standard D has not a high relevance to the clinical importance. Therefore, it is proposed that a sample with an OD lower than the standard D should NOT be plotted on the standard curve, instead could be reported as "below lowest standard".

Typical Calibration Curve

(All steps were performed at 23°C. Just an example. Do not use it for calculation!)

1:10 Diluted Standard	A	B	C	D	E
Concentration (ng/mL)	30	10	3	1	0
Mean OD _{450/620 nm}	2,405	0,875	0,340	0,134	0,032



12. ASSAY CHARACTERISTICS

12.1. SPECIFICITY

There is no cross reaction with any other proteins present in native aqueous humour samples. A screening test was performed with different aqueous humour samples. All produced OD_{450/620 nm} values (ranged from 0.016 to 0.023) less than the mean OD (0.134) of standard D (1 ng/mL). In addition, binding of Ranibizumab to the solid phase is inhibited by recombinant human VEGF. Therefore, the *ImmunoGuide* Ranibizumab ELISA (mAb-Based) measures the biologically active free form of Ranibizumab, i.e. not pre-occupied by human VEGF antigen. No cross reaction was observed with the other therapeutic antibodies including Infliximab, Rituximab, Veolizumab, Trastuzumab, Nivolumab and Aflibercept at concentrations tested up to 50 µg/mL. All produced mean OD_{450/620 nm} values (ranged from 0.052 to 0.068) less than standard D.

Because the 2A1 mAb, used for coating solid phase, is also reactive against Bevacizumab, it causes full cross reaction. But a quantification of Bevacizumab in aqueous humour by this testkit is possible only by using the drug-specific standards (i.e. Bevacizumab).

12.2. SENSITIVITY

The lowest detectable level that can be clearly distinguished from the zero standard is 0,33 ng/mL (zero standard +2SD read from the curve) under the above-described conditions. If needed, the minimum measurable level of Ranibizumab in samples can be improved up to 10 times by diluting aqueous humour samples at 1:200 instead of 1:2000. Under the conditions described in section 10.2 the analytical sensitivity is 0,33 ng/mL, and corresponding to the detection limit (limit of quantification) of 0.66 µg/mL for undiluted clinical samples because the serum or plasma samples are instructed to be diluted at 1:2000 before starting the assay.

12.3. PRECISION

Intra-assay CV: <10%.

Inter-assay CV: <10%.

12.4. RECOVERY

Recovery rate was found to be >90% with aqueous humour when spiked with exogenous Ranibizumab.

13. AUTOMATION

The *ImmunoGuide* Ranibizumab ELISA (mAb-based) is suitable also for being used by an automated ELISA processor.

14. REFERENCES

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