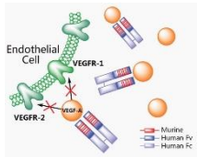
## INTENDED USE

The **Lucentis** (Ranibizumab) Anti-VEGF ELISA Kit is an immunoassay for quantifying active Lucentis in biological solutions which may include vitreous samples and ranibizumab processing solutions. The presence of endogenous animal or human IgG1 does not interfere in the assay.

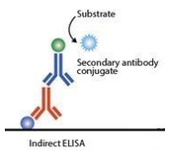
## GENERAL INFORMATION




VEGF (Vascular Epidermal Growth Factor; VEGF-A) is a dimeric (kDa 42) signal glycoprotein that stimulates production of new blood vessels. VEGF-A has been shown to have a pathological role in neovascularization and leakage in models of ocular angiogenesis and vascular occlusion, contributing to neovascular macular degeneration, macular edema following retinal vein occlusion, and diabetic macular edema.

VEGF-A is the target of Ranibizumab (Lucentis by Roche). It is produced by an E. coli expression system, and purified to generate a Fab fragment (~48 Kda, no Fc region). Lucentis is derived from the same parent molecule as bevacizumab (avastin). Many diseases of the eye, such as age-related macular degeneration (AMD) and diabetic retinopathy, damage the retina and cause blindness when blood vessels around the retina grow abnormally and leak fluid, causing the layers of the retina to separate. Lucentis binds to the receptor binding site of active forms of VEGF-A, thereby inhibiting binding to cell receptors and reducing endothelial cell proliferation, vascular leakage and new blood vessel formation.

## PRINCIPLE OF THE TEST




The Lucentis ELISA kit is based upon capture of active Lucentis to VEGF antigen coated on the plate. Bound Lucentis is then detected by anti-human IgG HRP. After a washing step, chromogenic substrate is added and color is developed by the enzymatic reaction of HRP on the TMB substrate, which is directly proportional to the amount of Lucentis present in the sample. Stopping Solution is added to terminate the reaction, and absorbance at 450nm is then measured using an ELISA microtiter well reader. The concentration of Lucentis in samples and control is calculated from a curve of standards containing known concentrations of Lucentis.



## KIT CONTENTS

The microtiter well plate and all other reagents, if unopened, are stable at 2-8$^{o}$C until the expiration date printed on the box label. Stabilities of the working solutions are indicated under Reagent Preparation.

**To Be Reconstituted:** Store as indicated.

| Component | Preparation Instructions |
|---|---|
| **Sample Diluent Concentrate (20x)** Cat.#. SD-20T, 10ml | Dilute the entire volume, 10ml + 190ml with distilled or deionized water into a clean stock bottle. Label as **Working Sample Diluent** and store at 2-8$^{o}$C until the kit lot expires or is used up. |
| **Wash Solution Concentrate (100x)** Cat. # WB-100, 10ml | Dilute the entire volume 10ml + 990ml with distilled or deionized water into a clean stock bottle. Label as **Working Wash Solution** and store at ambient temperature until kit is used entirely. |
| **Anti-Human IgG-HRP Conjugate Concentrate (100x)** Part No. H-HuF-LUG, 0.15ml | Peroxidase conjugated anti-human IgG in buffer with protein, detergents and antimicrobial as stabilizers. Dilute fresh as needed; 10 ul of concentrate to 1 ml of **Working Sample Diluent** is sufficient for 1 8-well strip. Use within the working day and discard. Return 100X to 2-8$^{o}$C storage. |

**Ready For Use:** Store as indicated on labels.

| Component | Part | Amt | Contents |
|---|---|---|---|
| **Antigen Coated Strip Plate** | 200-801 | 8-well strips (12) | Coated with recombinant VEGF antigen and post-coated with stabilizers. |
| **Lucentis Standards** | | | |
| 1.6 ng/ml<br>4 ng/ml<br>8 ng/ml<br>16 ng/ml<br>32 ng/ml | 200-883B<br>200-883C<br>200-883D<br>200-883E<br>200-883F | 0.65 ml<br>0.65 ml<br>0.65 ml<br>0.65 ml<br>0.65 ml | Five (5) vials, each containing Lucentis with designated concentrations; diluted in buffer with protein, detergents and non-azide antimicrobials as stabilizers. |
| **Positive Control** [Lucentis ] range on label | 200-882 | 0.65 ml | Lucentis of stated concentration range; diluted in buffer with protein, detergents and non-azide antimicrobials as stabilizers. |
| **TMB Substrate** | 80091 | 12 ml | Chromogenic substrate for HRP containing TMB and peroxide. |
| **Stop Solution** | 80101 | 12 ml | Dilute sulfuric acid. |

**Materials Required But Not Provided:**

- Pipettors and pipettes that deliver 100ul and 1-10ml. A multi-channel pipettor is recommended.
- Disposable glass or plastic 5-15ml tubes for diluting samples and Antibody HRP Concentrate.
- Graduated cylinder to dilute Wash Concentrate; 0.2 to 1L.
- Distilled or deionized water to dilute reagent concentrates.
- Microwell plate washer and reader at 450 nm wavelength.



## PRECAUTIONS AND SAFETY INSTRUCTIONS

Standards, Sample Diluent, and Antibody HRP contain bromonitrodioxane (BND: 0.05%, w/v). Stop Solution contains 1% sulfuric acid. Follow good laboratory practices, and avoid ingestion or contact of any reagent with skin, eyes or mucous membranes. All reagents may be disposed of down a drain with copious amounts of water. MSDS for TMB, sulfuric acid and BND can be requested or obtained from the ADI website: http://4adi.com/commerce/info/showpage.jsp?page_id=1060&category_id=2430&visit=10

## ASSAY DESIGN AND SET-UP

**Sample Collection and Handling**

Culture medium, serum and other biological fluids may be used as samples with proper dilution to avoid solution matrix interference (See Limits of the Assay, page 6). For **serum**, collect blood by venipuncture, allow clotting, and separate the serum by centrifugation at room temperature. For all samples, clarify by centrifugation and/or filtration. If samples will not be assayed immediately, store frozen for long-term storage.

DILUTE samples in **Working Sample Diluent**.
Diluted samples are stable for at least a year refrigerated.

**Assay Validation**

Validate the performance of the Lucentis sample and matrix in the assay system for recovery (see Limits of the Assay, page 6), as follows:

Recovery – a measure of the interference of the sample matrix (diluent effect) in providing accurate quantitation of Lucentis in the sample relative to the Lucentis Standards.

Prepare and run a series of dilutions of the Lucentis sample (within the Standard range) in Working Sample Diluent to determine the dilutions that give consistent and accurate quantitation. Serum and plasma require greater than 1/200 dilution to obtain consistent quantitation or complete antigen recovery.

Recovery Limits – Lucentis was spiked into dilutions of human serum & plasma, 1 pool and 11 individual samples, or Sample Diluent (Control), at a final concentration of 15.3 ng/ml.

**Results:** recovered values ranged from **82** to **98%** of Control with sera diluted 1/200. Recovery was **less** when serum was diluted less than 1/100. Low recovery suggests serum factors that interfere with Lucentis binding to the antigen on the plate.

**Plate Set-up**
Bring all reagents to room temperature (18-30$^{o}$ C) equilibration (at least 30 minutes).

- Determine the number of wells for the assay run. Duplicates are recommended, including 10 Standard wells and 2 wells for each sample and control to be assayed.
- Remove the appropriate number of microwell strips from the pouch and return unused strips to the pouch. Reseal the pouch and store refrigerated.
- Add 200 ul Working Wash Solution to each well and let stand for about 5 minutes. Aspirate or dump the liquid and pat dry on a paper towel before sample addition.



### Assay Procedure

ALL STEPS ARE PERFORMED AT ROOM TEMPERATURE. After each reagent addition, gently tap the plate to mix the well contents prior to beginning incubation.

**1. 1st Incubation [100ul – 60 min; 4 washes]**

- Add 100ul of calibrators, samples and controls each to pre-determined wells.
- Tap the plate gently to mix reagents and incubate for **60 minutes.**
- Wash wells 4 times and pat dry on fresh paper towels. As an alternative, an automatic plate washer may be used. Improper washes may lead to falsely elevated signals and poor reproducibility.

**2. 2nd Incubation [100ul – 30 min; 5 washes]**

- Add 100ul of diluted **Anti-Human IgG HRP Conjugate** to each well.
- Incubate for 30 minutes.
- Wash wells 5 times as in step 2.

**3. Substrate Incubation [100ul – 15 min]**

- Add 100ul TMB Substrate to each well. The liquid in the wells will begin to turn blue.
- Incubate for 15 minutes in the dark, e.g., place in a drawer or closet.

Note: If your microplate reader does not register optical density (OD) above 2.0, incubate for less time, or read OD at 405-410 nm (results are valid).

**4. Stop Step [Stop: 100ul]**

- Add 100ul of Stop Solution to each well.
- Tap gently to mix. The enzyme reaction will stop; liquid in the wells will turn yellow.

**5. Absorbance Reading**

- Use any commercially available microplate reader capable of reading at 450nm wavelength. Use a program suitable for obtaining OD readings, and data calculations if available.
- Read absorbance of the entire plate at 450nm using a single wavelength within 30 minutes after Stop Solution addition. If available, program to subtract OD at 630nm to normalize well background.

## STORAGE AND STABILITY

The microtiter well plate and all other reagents, if unopened, are stable at 2-8$^{o}$ C until the expiration date printed on the box label. Stabilities of the working solutions are indicated under Reagent Preparation.

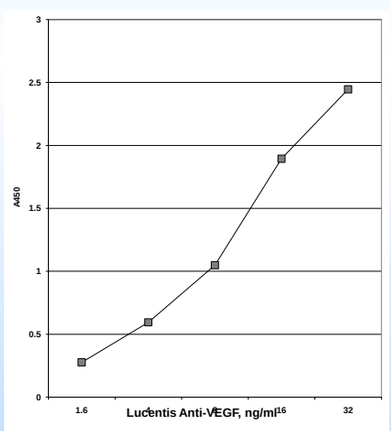
## CALCULATION OF RESULTS

1. The results may be calculated using any immunoassay software package. The four-parameter curve-fit is recommended. If software is not available, Lucentis concentrations may be determined as follows:
2. Calculate the mean OD of duplicate samples.
3. On graph paper plot the mean OD of the standards (y-axis) against the concentration (ng/ml) of Lucentis (x-axis). Draw the best fit curve through these points to construct the standard curve. A point-to-point construction is most common and reliable.
4. The Lucentis concentrations in unknown samples and controls can be determined by interpolation from the standard curve.
5. Multiply the values obtained for the samples by the dilution factor of each sample.
6. Samples producing signals higher than the 32 ng/ml standard should be further diluted and re-assayed.

**Typical Results:**

| Wells | Calibrators | | A450 nm |
|---|---|---|---|
| A1,2 | **Negative Diluent Blank** | | 0.07 |
| B1,2 | 1.6 ng/ml | **Standard** | 0.28 |
| C1,2 | 4 ng/ml | **Standard** | 0.59 |
| D1,2 | 8 ng/ml | **Standard** | 1.05 |
| E1,2 | 16 ng/ml | **Standard** | 1.89 |
| F1,2 | 32 ng/ml | **Standard** | 2.45 |
| G1,2 | **Positive** | **Control** | 1.35 |
| H1,2 | Sample | **1:500** | 1.89 |

Sample Result: **15.9** ng/ml x 500 dilution = **7.95** ug/ml






## PERFORMANCE CHARACTERISTICS

**Specificity**
The plate is coated with recombinant VEGF antigen to which Lucentis binds with high affinity. Other antibodies or binding proteins may also bind to the VEGF-antigen coated plate; however the Anti-Human IgG-HRP conjugate will not bind to mouse antibodies or non-antibody human serum proteins. Therefore, the assay is highly specific for measuring Lucentis activity only.

**Precision**
Samples containing low, medium and high concentrations of Lucentis, were assayed multiple times in the same assay (n=10) to provide within-assay precision, and as duplicates in multiple assays (n=5) to obtain between-assay reproducibility. Coefficient of variations were calculated for the concentrations using a point-to-point curve-fitting program.

Lucentis concentrations were measured with good within- and between-assay (**4.5** to **9.7** %CV) reproducibility.

| Sample | Lucentis ng/ml | Intra-assay %CV | Inter-assay %CV |
|---|---|---|---|
| Low | 4.4 | **4.5** | **9.7** |
| Medium | 10.3 | **6.8** | **5.7** |
| High | 16.4 | **8.0** | **4.7** |

**Recovery**
Lucentis was spiked into human serum or plasma diluted 1/200 in Sample Diluent (1 pooled and 11 individual samples), and assayed for anti-VEGF activity. **Recovery** was calculated comparing the observed (O) values to the expected (E) values for each diluted sample. All serum and plasma samples contained no Lucentis (E = 0).
O/E values ranged from **82%** to **98%**. See Limits of the Assay.

| Human Serum & Plasma Samples | Lucentis Concn (E) = 15.3 ng/ml | |
|---|---|---|
| | Observed (O) | O/E % |
| BC Pooled Serum | 14.3 | 93.5 |
| Serum, male A | 14.8 | 96.7 |
| Serum, female B | 13.5 | 88.2 |
| Serum, female C | 14.6 | 95.4 |
| Serum, male D | 14.2 | 92.8 |
| Plasma, female E | 12.6 | 82.4 |
| Plasma, male F | 13.4 | 87.6 |
| Plasma, female G | 14.0 | 91.5 |
| Plasma, male H | 13.5 | 88.2 |
| Plasma, male I | 14.3 | 93.5 |
| Plasma, female J | 14.5 | 94.8 |
| Plasma, female K | 15.1 | 98.7 |



## QUALITY CONTROL

**Reagents** Accurate and reproducible assay results rely on proper storage, handling and control of reagent and sample temperature. Store all reagents as indicated, and warm to room temperature only those to be used in the assay. Shelf-life of the critical reagents and samples will diminish with extended exposure to non-refrigeration, resulting in inaccurate assay results. All solutions should be clear. Cloudiness or particulates are indications of reagent contamination or instability and may interfere with proper performance of the assay. Do not use.

**Sample Controls** A Positive Serum Control is provided with the kit, assigned with an Lucentis concentration value range. Recovery in this range is an indicator of proper assay performance. Each lab should also assay internal control samples, which represent the lab's expected sample population and that are maintained stabilized. A Sample Diluent blank should also be run; OD should be <0.3 and lower than 0.5 ng/ml Standard OD.

**Standard Curve** The signal generated by the standards should be continuously increasing in OD from the lowest Standard to the highest Standard, with a difference greater than 1.2 OD. Non-uniform or low signals may indicate problems with technique, protocol directions and/or reagent preparation, use or stability. Do not rely on results generated from an assay with these issues.

**Technique** Accurate and reproducible assay results rely on good lab technique regarding pipetting, plate washing and handling of samples and reagents.

**Equipment** Precision of results relies on uniform and effective washing techniques; an automatic washer may be used. ELISA reader and pipettes should be properly calibrated.

## LIMITS OF THE ASSAY

1. The assay measures Lucentis activity, i.e., antibody that actually binds to the VEGF-antigen coated plate, relative to Lucentis standards that are presumed to be 100% active antibody. Factors in the sample that diminish Lucentis binding, e.g., VEGF antigen or other Lucentis -binding molecules, may reduce apparent Lucentis concentration in the assay (**Recovery**).

2. Assays that measure Lucentis mass concentration may not have a tight correlation with the Lucentis activity assay, e.g., full Lucentis recovery may be determined by different factors.

3. The **recovery** (accuracy of Lucentis measurement in stored serum) may be diminished if not diluted at least 1/100 in Sample Diluent (see Recovery, above and page 6). Recovery in fresh, individual human or mouse serum or plasma, or vitreous samples may differ, and has not been determined.

4. Multiple-dose intravitreal administration of Lucentis in humans has resulted in minimum-maximum concentration ranges of 0.22 – 1.5 ng Lucentis /ml of serum (product data). This is predicted to be 90,000-fold lower than vitreal concentrations and lower than the assay range of the ELISA.



Instruction Manual No. M-200-880-LUG

# Lucentis
## (Ranibizumab) Anti-VEGF

## ELISA Kit # 200-880-LUG, 96 Tests

### For Quantitation of Active Lucentis in Biological Solutions

*For research use only (RUO), not for diagnosis, cure or prevention of the disease.*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