ELISA Kit Components	Amount	Cat/Part No.
Anti-VEGF Microwell Strip Plate	8-well strips (12)	100-231
VEGF Standard, lyophilized	2 vials	100-232
Anti-VEGF Detecting Antibody (100X)	0.15 ml	100-233
Streptavidin HRP Conjugate (100X)	0.15 ml	S-HRP100
Sample Diluent Concentrate (20X)	10 ml	SD-20T
Wash Solution Concentrate (100X)	10 ml	WB-100
TMB Substrate	12 ml	80091
Stop Solution	12 ml	80101
Product Manual	1 ea	100-230-VEM

Instruction Manual No. M-100-230-VEM

Mouse VEGF

ELISA Kit Cat. No. 100-230-VEM

For Quantitative Determination of Vascular Endothelium Growth Factor in Biological Fluids

ALPHA DIAGNOSTIC INTERNATIONAL

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DRAFT MANUAL: PLEASE CONSULT THE MANUAL SUPPLIED WITH THE KIT FOR ANY LOT SPECIFIC CHNAGES.

INTENDED USE

The Mouse Vascular Endothelium Growth Factor (VEGF) ELISA Kit is an in vitro immunoassay for research use in the quantification of VEGF in cultures of mouse cells and in appropriately qualified samples from serum, saliva, or other tissue fluids.

RESEARCH USE OF THE TEST

Angiogenesis accounts for the formation of vasculature from previously avascular organs such as the brain and kidneys. Angiogenic activity in the adult is required during the normal tissue repair, and for the remodeling of the female reproductive organs (ovulation and placental development). Certain pathological conditions, such as tumor growth and diabetic retinopathy, also require angiogenesis. Study of tumor angiogenesis has led to the identification of several proteins including basic fibroblast growth factors (bFGF) and vascular endothelial growth factors.

VEGF is a potent angiogenic and growth cytokine whose role in vasculogenesis is to regulate blood vessel density and size. It also, helps maintain cell integrity and helps to promote bone formation. VEGF plays a role in initiating angiogenisis by inducing MMP-1 production in umbilical vein endothelial cells. Mouse VEGF is produced from a single gene containing 8 exons. Alternative splicing will produce 3 isoforms of VEGF that differ in heparin binding ability.

Alpha Diagnostics has developed an immunoassay for detection and quantification of VEGF in mouse samples used in research involved with angiogenesis. The kit is suitable for testing a variety of sample types, in accordance with appropriate validation of linearity and recovery.

PRINCIPLE OF THE TEST

The Mouse VEGF ELISA kit is based on the binding of mouse VEGF in samples to two antibodies, one immobilized on the microtiter wells, and the other conjugated to biotin, which then binds to a streptavidin horseradish peroxidase (HRP) conjugate. 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 VEGF 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 VEGF in samples is calculated from a standard curve of purified recombinant mouse VEGF of designated concentration.

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 label. Stabilities of the working solutions are indicated under Reagent Preparation.

ASSAY CHARACTERISTICS

Specificity & Sensitivity
The antibodies used in this kit have been affinity purified using a purified recombinant mouse VEGF immunosorbent from animals immunized with purified recombinant mouse VEGF. Human VEGF is highly cross-reactive in this assay. The assay can detect less than 5 pg/ml.

Recovery
Stored sera from 5 normal adult Swiss mice assayed as less than 20 pg/ml at 1:5 dilutions. Recovery was essentially 100% when 200 pg/ml recombinant VEGF was spiked into the sera, or into diluent with 10% neonatal bovine serum.

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 Each lab should assay internal positive control samples, which represent the lab's expected sample population and that are maintained stabilized. A Negative Diluent Control should also be run.

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-continuously increasing or low signals may indicate problems with technique, protocol directions and/or reagent preparation, use or stability. A Negative Diluent Control should be of lower signal than the lowest standard. 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.

CALCULATION OF RESULTS

The results may be calculated using any immunoassay software package. The four-parameter curve-fit is recommended. If software is not available, VEGF concentrations may be determined as follows:

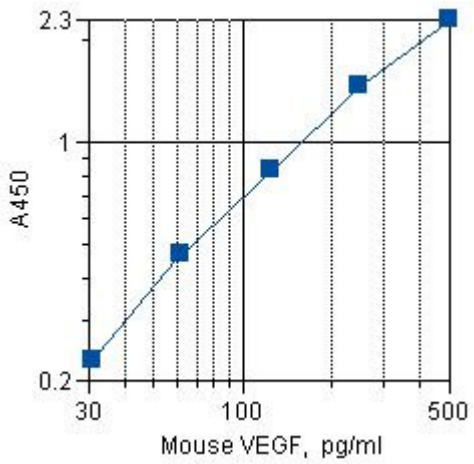
1. Calculate the mean OD of duplicate samples.
2. On graph paper plot the mean OD of the standards (y-axis) against the concentration (pg/ml) of VEGF (x-axis). Draw the best fit curve through these points to construct the standard curve. A point-to-point construction is most common and reliable.
3. The VEGF concentrations in unknown samples and controls can be determined by interpolation from the standard curve.
4. Multiply the values obtained for the samples by the dilution factor of each sample.
5. Samples producing signals higher than the 500 pg/ml standard should be further diluted and re-assayed.

TYPICAL RESULTS

The following data are for illustration purposes only. A complete standard curve should be run in every assay to determine sample values.

Wells	Standards, Control & Samples	A450 nm	VEGF pg/ml
A1, A2	**Negative Diluent Control**	0.04	0
B1, B2	31.2 pg/ml **Standard**	0.23	31.2
C1, C2	62.5 pg/ml **Standard**	0.47	62.5
D1, D2	125 pg/ml **Standard**	0.83	125
E1, E2	250 pg/ml **Standard**	1.47	250
F1, F2	500 pg/ml **Standard**	2.30	500
G1, G2	**Sample** [Diluted 1:10]	0.92	134
	Calculated: 10-fold dilution x 134 pg/ml = **1.34** ng/ml in sample		

A typical assay Standard Curve (do not use for calculating sample values)



KIT CONTENTS

To Be Reconstituted: Store as indicated.

Component	Instructions for Use
Mouse VEGF Standard Part No. 100-232	Two (2) vials, each containing VEGF lyophilized in buffer with protein as stabilizers. Keep lyophilized vials frozen until used or kit lot expires.

Reconstitute 1 vial with 1.0 ml **Working Sample Diluent** to provide a 2000 pg/ml solution, sufficient for at least two curves. Prepare dilutions, as follows:

Standard	+ Diluent	= Final Concn
Reconstituted Standard	None	2000 pg/ml
125 ul of 2000 pg/ml	375ul	500 pg/ml
225 ul of 500 pg/ml	225ul	250 pg/ml
225 ul of 250 pg/ml	225ul	125 pg/ml
225 ul of 125 pg/ml	225ul	62.5 pg/ml
225 ul of 62.5 pg/ml	225ul	31.25 pg/ml

Use within 2 weeks of preparation. Store @ 4 C.

Sample Diluent Concentrate (20x) Cat. No. 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°C until the kit lot expires or is used up.
Wash Solution Concentrate (100x) Cat. No. WB-100, 10ml	Dilute the entire volume, 10ml, to 1L 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-Mouse VEGF Detection Antibody Concentrate (100x) Part No. 100-233, 0.15ml	Biotinylated anti-human VEGF in buffer with protein, detergents and ProClin 300 as stabilizers. Dilute fresh as needed; 10ul of concentrate to 1ml of **Working Sample Diluent** is sufficient for 1 8-well strip. Use within the working day and discard. Return concentrate to 2-8°C storage.
Streptavidin-HRP Conjugate Concentrate (100x) Part No. S-HRP100, 0.15ml	Peroxidase conjugated streptavidin in buffer with protein, detergents and ProClin 300 as stabilizers. Dilute fresh as needed; 10ul of concentrate to 1ml of **Working Sample Diluent** is sufficient for 1 8-well strip. Use within the working day and discard. Return concentrate to 2-8°C storage.

Ready For Use: Store as indicated on labels.

Component	Part No.	Amt	Contents
Anti-Mouse VEGF Microwell Strip Plate	100-231	8-well strips (12)	Coated with purified anti-mouse VEGF antibodies. Return unused strips to the pouch with desiccant; re-seal and store refrigerated.
TMB Solution	80091	12 ml	Chromogenic substrate for HRP containing TMB and peroxide.
Stop Solution	80101	12 ml	1% sulfuric acid.

Materials Required But Not Provided:

- Pipettors and pipettes that deliver 100ul and 1-10ml. A multi-channel pipetter is recommended.
- Disposable glass or plastic 5-15ml tubes for diluting samples, Detection Antibody Concentrate and Streptavidin-HRP Concentrate.
- Graduated cylinder to dilute Wash Concentrate and Sample Diluent concentrate; 200ml to 1L.
- Stock bottle to store diluted Wash Solution; 200ml to 1L.
- Distilled or deionized water to dilute reagent concentrates.
- Microwell plate reader at 450 nm wavelength.

PRECAUTIONS AND SAFETY INSTRUCTIONS

Standards, Controls, Sample Diluent, Detection Antibody and Streptravidin-HRP contain Proclin 300 (0.05%, v/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 Proclin 300, if not already on file, can be requested or obtained from the ADI website.

SPECIMEN COLLECTION AND HANDLING

Culture medium, serum and other biological fluids may be used as samples with proper dilution to avoid solution matrix interference. For serum, collect blood by venipuncture or retro-orbital puncture, allow clotting, and separate the serum by centrifugation at room temperature. For other samples, including tissue culture media, clarify the sample by centrifugation and/or filtration prior to dilution in Working Sample Diluent. If samples will not be assayed immediately, stored refrigerated for up to a few weeks, or frozen for long-term storage. Avoid freeze-thaw cycles.

ASSAY PROCEDURE

Bring all reagents to room temperature (18-30° C) equilibration (at least 30 minutes).

Use freshly diluted Standards as described on page 2. Dilute samples in Working Sample Diluent according to expected VEGF concentrations.

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. Set-up

- Determine the number of wells for the assay run, 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 200-300ul Working Wash Solution to each well and let stand for about 5 minutes before sample addition.
- Aspirate or dump the liquid and pat the plate dry on a paper towel.

2. 1st Incubation [100ul -120min; 4 washes]

- Add 100ul of standards, samples and controls each to pre-determined wells.
- Tap the plate gently to mix reagents and incubate for 120 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.

3. 2nd Incubation [100ul - 60min; 4 washes]

- Add 100ul of Working Detection Antibody to each well.
- Incubate for 60 minutes.
- Wash wells 4 times as in step 2.

4. 3rd Incubation [100ul - 30min; 5 washes]

- Add 100ul of Working Streptavidin-HRP Conjugate to each well.
- Incubate for 30 minutes.
- Wash wells 5 times as in step 2.

5. Substrate Incubation [100ul - 15min]

- 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).

6. 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.

7. 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.