Murine VEGF-A ELISA Kit

Instructions for use

Catalogue numbers: 1x96 tests: 660.140.096
2x96 tests: 660.140.192

For research use only

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1. Intended use

The mouse VEGF-A ELISA is an enzyme-linked immunosorbent assay for the quantitative detection of mouse VEGF-A. **The mouse VEGF-A ELISA is for research use only. Not for diagnostic or therapeutic procedures.**

2. Introduction

2.1. Summary

Normal tissue function depends on a regular supply of oxygen through the blood vessels. Understanding the formation of blood vessels has become the focus of a major research effort throughout the last decade. Vasculogenesis in the embryo is the process by which new blood vessels are generated *de novo* from primitive precursor cells. Angiogenesis is the process of new blood vessel formation from pre-existing vasculatures. It plays an essential role in development, normal tissue growth, wound healing, the female reproductive cycle (placental development, ovulation, corpus luteum) and also plays a major role in various diseases. Special interest is focused on tumor growth, since tumors cannot grow more than a few millimeters in size without developing a new blood supply. This process is described as tumor angiogenesis which is also essential for the spread and growth of tumor cell metastasis.

One of the key molecules for angiogenesis and for the survival of the endothelium is vascular endothelial growth factor (VEGF-A). It is a specific endothelial cell mitogen and a strong vascular permeability factor (VPF). VEGF-A is a heparin-binding glycoprotein, secreted as a homodimer of 45 kDa by many different cell types. VEGF-A also causes vasodilation through the nitiric oxide synthase pathway in endothelial cells and can activate migration in monocytes. Many different splice variants of VEGF-A have been described, but $VEGF_{165}$ is the most predominant protein and anchors with its heparin binding domain to extracellular matrix and to heparin sulfate. During the past few years, several other members of the VEGF family have been cloned, including VEGF-B, -C- and -D. In terms of vascular angiogenesis, which mainly is regulated by VEGF-A, lymphangiogenesis is mainly regulated by VEGF-C and -D.

VEGF-A transcription is highly activated by hypoxia and by oncogenes like H-ras and several transmembrane tyrosine kinases, such as epidermal growth factor receptor and ErbB2. Together these pathways account for a marked upregulation of VEGF-A in tumors compared to normal tissues and are often of prognostic importance and relevance.

VEGF-A can be detected in both plasma and serum samples of patients, with much higher levels in serum. Extremely high levels can be detected in the cystic brain fluid of brain tumor patients or in ascites fluid of patients. Platelets release VEGF-A upon aggregation and may be another major source of VEGF-A delivery to tumors. Several other studies have shown that association of high serum levels of VEGF-A with poor prognosis in cancer patients may be correlated with an elevated platelet count. Tumors can release cytokines and growth factors that stimulate the production of megakaryocytes in the marrow and elevate the platelet count. This can result in another, indirect increase of VEGF-A delivery to tumors.

Furthermore, VEGF-A is implicated in several other pathological conditions associated with enhanced angiogenesis or enhanced vascular permeability. Examples where VEGF-A plays an important role are psoriasis and rheumatoid arthritis, as well as the ovarian hyperstimulation syndrome. Diabetic retinopathy is associated with high intraocular levels of VEGF-A, and inhibition of VEGF-A function may result in infertility by blockage of corpus luteum function. Direct demonstration of the importance of VEGF-A in tumor growth has been achieved using dominant negative VEGF receptors to block in vivo proliferation, as well as blocking antibodies to VEGF or to one of the VEGF receptors. Interference with VEGF-A function has therefore become of major interest for drug development to block angiogenesis and metastasis. More than 110 pharmaceutical companies world-wide are involved in the development of such antagonists. Their

approaches include antagonists of VEGF-A or its receptors, selective tyrosine kinase inhibitors, targeting of drugs and toxins to VEGF receptors and gene therapy regulated by the same hypoxia pathway that controls VEGF-A production. Targeting the VEGF signalling may be of major therapeutic importance for many diseases and serves as a basis for the design of future (anti)-angiogenic treatments.

2.2. Principle of the method

An anti-mouse VEGF-A coating antibody is adsorbed onto microwells.
Mouse VEGF-A present in the sample or standard binds to antibodies adsorbed to the microwells.
Following incubation unbound biological components are removed during a wash step and a biotin-conjugated anti-mouse VEGF-A antibody is added and binds to mouse VEGF-A captured by the first antibody.
Following incubation unbound biotin-conjugated anti-mouse VEGF-A antibody is removed during a wash step. Streptavidin-HRP is added and binds to the biotin-conjugated anti-mouse VEGF-A antibody.
Following incubation unbound Streptavidin-HRP is removed during a wash step, and substrate solution reactive with HRP is added to the wells.
A coloured product is formed in proportion to the amount of mouse VEGF-A present in the sample or standard. The reaction is terminated by addition of acid and absorbance is measured at 450 nm. A standard curve is prepared from 7 mouse VEGF-A standard dilutions and mouse VEGF-A sample concentration determined.

3. Reagents provided and reconstitution

REAGENTS (store at 2-8°C)	Quantity 1x96 well-kit 660.140.096	Quantity 2x96 well-kit 660.140.192	RECONSTITUTION
96-wells precoated microtiter plate	1	2	Ready-to-use
Plate covers	2	4	
Biotin-Conjugate anti-mouse VEGF-A polyclonal antibody	1 vial	2 vials	Dilute 100 times in Assay Buffer (120µl)
Streptavidin-HRP	1 vial	2 vials	Dilute 100 times in Assay Buffer (150µl)
mVEGF-A Standard: 4 ng/ml	2 vials	4 vials	Reconstitute as indicated on the vial
Conjugate Diluent Concentrate	1 vial	2 vials	(5 ml) 20X concentrate. Dilute in distilled water
Sample Diluent	1 vial	2 vials	(10 ml) Ready-to-use
Assay Diluent	1 vial	2 vials	(12 ml) Ready-to-use
Wash Buffer Concentrate	1 vial	2 vials	(50 ml) 20X concentrate. Dilute in distilled water
Substrate Solution	1 vial	2 vials	(15 ml) Ready-to-use
Stop Solution (1M Phosphoric acid)	1 vial	2 vials	(15 ml) Ready-to-use

4. Materials required but not provided

- Microtitre plate reader fitted with appropriate filters (450nm required with optional 620nm reference filter)
- Microplate washer or wash bottle
- 10, 50, 100, 200 and 1,000µl adjustable single channel micropipettes with disposable tips
- 50-300µl multi-channel micropipette with disposable tips
- Multichannel micropipette reagent reservoirs
- Distilled water
- Vortex mixer
- Miscellaneous laboratory plastic and/or glass, if possible sterile

5. Storage Instructions

Store kit reagents between 2° and 8°C. Immediately after use remaining reagents should be returned to cold storage (2° to 8°C). Expiry of the kit and reagents is stated on labels.

Expiry of the kit components can only be guaranteed if the components are stored properly, and if, in case of repeated use of one component, this reagent is not contaminated by the first handling.

6. Specimen collection, processing & storage

Cell culture supernatant, serum* and plasma (EDTA) were tested with this assay. Other body fluids might be suitable for use in the assay. Remove serum from the clot as soon as possible after clotting.

Samples containing a visible precipitate must be clarified prior to use in the assay. Do not use grossly hemolyzed or lipemic specimens.

Samples should be aliquoted and must be stored frozen at -20°C to avoid loss of bioactive mouse VEGF-A. If samples are to be run within 24 hours, they may be stored at 2° to 8°C.
Avoid repeated freeze-thaw cycles. Prior to assay, the frozen sample should be brought to room temperature slowly and mixed gently.

* Pay attention to a possibly elevated serum level of mouse VEGF-A due to VEGF-A release by platelets during platelet activation (sampling process).

Do not thaw samples in a 37°C water bath. Do not vortex or sharply agitate samples.

7. Safety & precautions for use

- Handling of reagents, serum or plasma specimens should be in accordance with local safety procedures, e.g. CDC/NIH Health manual : "Biosafety in Microbiological and Biomedical Laboratories" 1984.
- Laboratory gloves should be worn at all times.
- Avoid any skin contact with H_2SO_4 and TMB. In case of contact, wash thoroughly with water.
- Do not eat, drink, smoke or apply cosmetics where kit reagents are used.
- Do not pipette by mouth.
- When not in use, kit components should be stored refrigerated as indicated on vials or bottles labels.
- All reagents should be warmed to room temperature before use. Lyophilized standards should be discarded after use.
- Once the desired number of strips has been removed, immediately reseal the bag to protect the remaining strips from deterioration.
- Cover or cap all reagents when not in use.
- Do not mix or interchange reagents between different lots.
- Do not use reagents beyond the expiration date of the kit.
- Use a clean disposable plastic pipette tip for each reagent, standard, or specimen addition in order to avoid cross contamination, for the dispensing of H_2SO_4 and substrate solution, avoid pipettes with metal parts.
- Use a clean plastic container to prepare the washing solution.
- Thoroughly mix the reagents and samples before use by agitation or swirling.
- All residual washing liquid must be drained from the wells by efficient aspiration or by decantation followed by tapping the plate forcefully on absorbent paper. Never insert absorbent paper directly into the wells.
- The TMB solution is light sensitive. Avoid prolonged exposure to light. Also, avoid contact of the TMB solution with metal to prevent colour development. Warning TMB is toxic avoid direct contact with hands. Dispose off properly.
- If a dark blue colour develops within a few minutes after preparation, this indicates that the TMB solution has been contaminated and must be discarded. Read absorbance’s within 1 hour after completion of the assay.
- When pipetting reagents, maintain a consistent order of addition from well-to-well. This will ensure equal incubation times for all wells.
- Follow incubation times described in the assay procedure.
- Dispense the TMB solution within 15 min of the washing of the microtiter plate.

8. Assay Preparation

Bring all reagents to room temperature before use.
If crystals have formed in the **Buffer Concentrates**, warm them gently until they have completely dissolved.

8.1. Assay Design

Determine the number of microwell strips required to test the desired number of samples plus appropriate number of wells needed for running zeros and standards. Each sample, standard and zero should be tested **in duplicate**. Remove sufficient Microwell Strips for testing from the aluminium pouch immediately prior to use. Return any wells not required for this assay with desiccant to the pouch. Seal tightly and return to 2-8°C storage.

Example plate layout (example shown for a 7 point standard curve)

	Standards		Sample Wells									
	1	2	3	4	5	6	7	8	9	10	11	12
A	4000	4000										
B	2000	2000										
C	1000	1000										
D	500	500										
E	250	250										
F	125	125										
G	62.5	62.5										
H	Zero	Zero										

All remaining empty wells can be used to test samples in duplicate

8.2. Preparation of Wash Buffer

Pour entire contents (50 ml) of the Wash Buffer Concentrate (20x) into a clean 1000 ml graduated cylinder. Bring to final volume of 1000 ml with glass-distilled or deionized water.
Mix gently to avoid foaming.
Transfer to a clean wash bottle and store at 2° to 25°C. Please note that Wash Buffer (1x) is stable for 30 days.
Wash Buffer (1x) may also be prepared as needed according to the following table:

Number of Strips	Wash Buffer Concentrate (20x) (ml)	Distilled Water (ml)
1 - 6	25	475
1 - 12	50	950

8.3. Preparation of Conjugate Diluent Buffer

Pour the entire contents (5 ml) of the **Conjugate Diluent Concentrate** (20x) into a clean 100 ml graduated cylinder. Bring to final volume of 100 ml with distilled water. Mix gently to avoid foaming.
Store at 2° to 8°C. Please note that the Conjugate Diluent (1x) is stable for 30 days.

Conjugate Diluent (1x) may also be prepared as needed according to the following table:

Number of Strips	Conjugate Diluent Concentrate (20x) (ml)	Distilled Water (ml)
1 - 6	2.5	47.5
1 - 12	5.0	95.0

8.4. Preparation of Standard

Reconstitute **mouse VEGF-A standard** by addition of Sample Diluent (for subsequent measurement of **serum** or **plasma samples**) or distilled water (for subsequent measurement of **cell culture supernatant samples**).

Reconstitution volume is stated on the label of the standard vial. Allow the reconstituted standard to sit for 10-30 minutes. Swirl or mix gently to insure complete and homogeneous solubilization (concentration of reconstituted standard = 4000 pg/ml).

Label 6 tubes, one for each standard point. S2, S3, S4, S5, S6, S7.
Then prepare 1:2 serial dilutions for the standard curve as follows:
Pipette 150 µl of Sample Diluent (for subsequent measurement of serum or plasma samples) or Assay Diluent (for subsequent measurement of cell culture supernatant samples) into each tube.
Pipette 150 µl of reconstituted standard (serves as highest standard S1 = 4 ng/ml) into the first tube, labelled S2 and mix (concentration of standard 2 = 2 ng/ml).
Pipette 150 µl of this dilution into the second tube and mix thoroughly before the next transfer.
Repeat serial dilutions 4 more times thus creating the points of the standard curve ranging from 4000 to 62.5 pg/ml.

Sample Diluent (serum or plasma samples) or Assay Diluent (cell culture supernatant samples) serve as blank.

8.5. Preparation of Biotin-Conjugate

Please note that the Biotin-Conjugate should be used within 30 minutes after dilution.

Make a 1:100 dilution of the concentrated **Biotin-Conjugate** solution with Conjugate Diluent (1x) in a clean plastic tube as needed according to the following table:

Number of Strips	Biotin-Conjugate (ml)	Conjugate Diluent (1x) (ml)
1 - 6	0.06	5.94
1 - 12	0.12	11.88

8.6. Preparation of Streptavidin-HRP

Please note that the Streptavidin-HRP should be used within 30 minutes after dilution.

Make a 1:100 dilution of the concentrated **Streptavidin-HRP** solution with Conjugate Diluent (1x) in a clean plastic tube as needed according to the following table:

Number of Strips	Streptavidin-HRP (ml)	Conjugate Diluent (1x) (ml)
1 - 6	0.06	5.94
1 - 12	0.12	11.88

9. Method

We strongly recommend that every vial is mixed thoroughly without foaming prior to use.

Prepare all reagents as shown in section 8.
Note: Final preparation of Biotin conjugate (section 8.5) and Streptavidin-HRP (section 8.6) should occur immediately before use.

Assay Step		Details
1.	Wash	a) Dispense 0.4 ml of **1x washing solution** into each well b) Aspirate the contents of each well c) Repeat steps a and b Do not allow wells to dry before use
2.	Addition	Add 50µl of **Assay diluent** to all wells
3.	Preparation	**Prepare Standard curve** as shown in section 8.4
4.	Addition	Add 50µl of each **Standard, Sample and appropriate Blank (Sample or Assay Diluent)** in duplicate to appropriate number of wells
5.	Incubation	Cover with a plastic plate cover and incubate at room temperature (18 to 25°C) for **2 hour(s) on a microplate shaker** set at 400 rpm
6.	Wash	Remove the cover and wash the plate as follows: a) Aspirate the liquid from each well b) Dispense 0.4 ml of **1x washing solution** into each well c) Aspirate the contents of each well d) Repeat step b and c another five times
7.	Addition	Add 100µl of diluted **biotin-conjugate** to all wells
8.	Incubation	Cover with a plastic plate cover and incubate at room temperature (18 to 25°C) for **1 hour(s) on a microplate shaker** set at 400 rpm
9.	Wash	Repeat wash step 6.
10.	Addition	Add 100µl of **Streptavidin-HRP** solution into all wells
11.	Incubation	Cover with a plastic plate cover and incubate at room temperature (18 to 25°C) for **1 hour on a microplate shaker** set at 400 rpm
12.	Wash	Repeat wash step 6.
13.	Addition	Add 100µl of ready-to-use **TMB Substrate Solution** into all wells
14.	Incubation	Incubate in the dark for **30 minutes*** at room temperature. Avoid direct exposure to light by wrapping the plate in aluminium foil.
15.	Addition	Add 100µl of **Stop Reagent** into all wells
Read the absorbance value of each well (immediately after step 15.) on a spectrophotometer using 450 nm as the primary wavelength and optionally 620 nm as the reference wave length (610 nm to 650 nm is acceptable).		

***Incubation time of the substrate solution is usually determined by the ELISA reader performance. Many ELISA readers only record absorbance up to 2.0 O.D. Therefore the colour development within individual microwells must be observed by the analyst, and the substrate reaction stopped before positive wells are no longer within recordable range*
Note: In case of incubation without shaking the obtained O.D. values may be lower than indicated below. Nevertheless the results are still valid.

10. Data Analysis

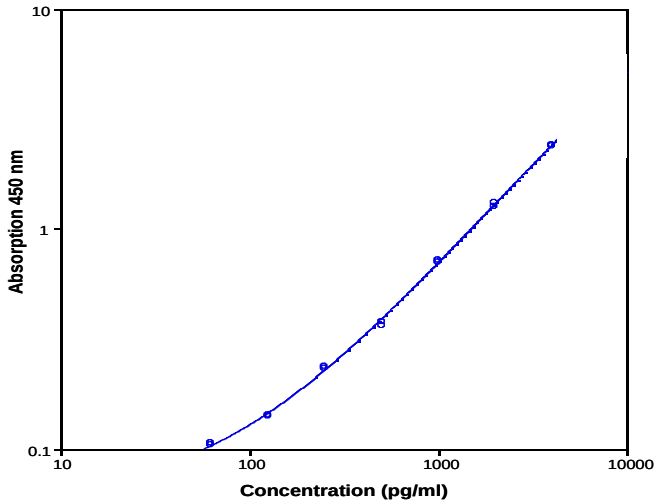
Calculate the average absorbance values for each set of duplicate standards and samples**.** Ideally duplicates should be within 20% of the mean.

Generate a linear standard curve by plotting the average absorbance of each standard on the vertical axis versus the corresponding mVEGF-A standard concentration on the horizontal axis.

The amount of mVEGF-A in each sample is determined by extrapolating OD values against mVEGF-A standard concentrations using the standard curve**.**

Example mVEGF-A Standard Curve:

Standard	mVEGF-A Conc	OD (450nm) mean	CV (%)
1	4000.0	2.394	0.3
2	2000.0	1.290	1.9
3	1000.0	0.714	1.1
4	500.0	0.371	1.9
5	250.0	0.235	0.6
6	125.0	0.142	0.9
7	62.5	0.105	0.5
Blank	0	0.065	0.7



Note: curve shown above should not be used to determine results. Every laboratory must produce a standard curve for each set of microwell strips assayed.

11. Assay limitations

Do not extrapolate the standard curve beyond the maximum standard curve point. The dose-response is non-linear in this region and good accuracy is difficult to obtain. Concentrated samples above the maximum standard concentration must be diluted with Standard diluent or with your own sample buffer to produce an OD value within the range of the standard curve. Following analysis of such samples always multiply results by the appropriate dilution factor to produce actual final concentration.

The influence of various drugs on end results has not been investigated. Bacterial or fungal contamination and laboratory cross-contamination may also cause irregular results.

Improper or insufficient washing at any stage of the procedure will result in either false positive or false negative results. Completely empty wells before dispensing fresh Washing Buffer, fill with Washing Buffer as indicated for each wash cycle and do not allow wells to sit uncovered or dry for extended periods.

Disposable pipette tips, flasks or glassware are preferred, reusable glassware must be washed and thoroughly rinsed of all detergents before use.

As with most biological assays conditions may vary from assay to assay therefore **a fresh standard curve must be prepared and run for every assay.**

12. Performance Characteristics

12.1. Sensitivity

The limit of detection of mouse VEGF-A defined as the analyte concentration resulting in an absorbance significantly higher than that of the dilution medium (mean plus 2 standard deviations) was determined to be 20.0 pg/ml (mean of 6 independent assays).

12.2. Specificity

The assay detects both natural and recombinant mouse VEGF-A. The cross reactivity and interference of circulating factors of the immune system was evaluated by spiking these proteins at physiologically relevant concentrations into a mouse VEGF-A positive serum. There was no cross reactivity or interference detected, notably not with mouse VEGF-B, VEGF-C, VEGF-D and PfGF.

Interference was detected for VEGF-R1 at concentrations > 200 pg/ml, and not for VEGF-R2.

12.3. Precision

Intra Assay

Reproducibility within the assay was evaluated in 3 independent experiments. Each assay was carried out with 6 replicates of 6 serum samples containing different concentrations of mouse VEGF-A. 2 standard curves were run on each plate. **The calculated overall intra-assay coefficient of variation was 3.8%**.

Inter-assay

Assay to assay reproducibility within one laboratory was evaluated in 3 independent experiments. Each assay was carried out with 6 replicates of 6 serum samples containing different concentrations of mouse VEGF-A. 2 standard curves were run on each plate. **The calculated overall inter-assay coefficient of variation was 6.4%.**

12.4. Dilution Parallelism

Serum, plasma and cell culture supernatant samples with different levels of mouse VEGF-A were analysed at serial 2 fold dilutions with 4 replicates each.

Sample matrix	Recovery Range (%)	Mean (%)
Serum	84 - 112	102
Plasma (EDTA)	98 - 119	106
Cell culture supernatant	78 - 105	92

12.5. Spike Recovery

The spike recovery was evaluated by spiking 4 levels of mouse VEGF-A into serum, plasma and cell culture supernatant. Recoveries were determined in 3 independent experiments with 4 replicates each.
The amount of endogenous mouse VEGF-A in unspiked serum was subtracted from the spike values.

Sample matrix *	Spike high (%)	Spike medium (%)	Spike low (%)
Serum	95	87	95
Plasma (EDTA)	88	104	nd
Cell culture supernatant	88	97	75

* Due to high endogen mouse VEGF-A levels data for low spikes are not indicated

12.6. Stability

Freeze-Thaw Stability

Aliquots of serum samples (spiked or unspiked) were stored at -20°C and thawed 5 times, and the mouse VEGF-A levels determined.
There was a significant decrease of mouse VEGF-A immunoreactivity detected. Therefore samples should be stored in aliquots at -20°C and thawed only once.

Storage Stability

Aliquots of serum samples (spiked or unspiked) were stored at -20°C, 2-8°C, room temperature (RT) and at 37°C, and the mouse VEGF-A level determined after 24 h. There was no significant loss of mouse VEGF-A immunoreactivity detected during storage at -20°C and 2-8°C.
A significant loss of mouse VEGF-A immunoreactivity was detected during storage at RT and 37°C after 24 h.

13. Assay Summary

Total procedure length : 4h30mn

Wash two times
↓
Add sample and diluted standard
↓
Incubate 2 hours at room temperature
↓
Wash six times
↓
Add 100µl of Biotin-Conjugate
↓
Incubate 1hour at room temperature
↓
Wash six times

Add 100µl of Streptavidin-HRP
↓
Incubate 1hour at room temperature
↓
Wash six times
↓
Add 100 µl of ready-to-use TMB
Protect from light. Let the color develop for 30 mn.
↓
Add 100 µl Stop Reagent
↓
Read Absorbance at 450 nm

Diaclone SAS
6 Rue Docteur Girod
25020 Besançon Cedex
France
Tel +33 (0)3 81 41 38 38
Fax +33 (0)3 81 41 36 36
Email: info@diaclone.com