

# Mouse VEGF-A ELISA Kit

Enzyme-linked Immunosorbent Assay for quantitative detection of mouse VEGF-A

**Catalog Numbers** BMS619-2 and BMS619-2TEN

**Pub. No.** MAN0018173 **Rev.** A.0 [30]

**WARNING!** Read the Safety Data Sheets (SDSs) and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves. Safety Data Sheets (SDSs) are available from [thermofisher.com/support](http://thermofisher.com/support).

## Product description

The mouse VEGF-A ELISA is an enzyme-linked immunosorbent assay for the quantitative detection of mouse VEGF-A.

## 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 nitric oxide synthase pathway in endothelial cells and can activate migration in monocytes. Many different splice variants of VEGF-A have been described, but VEGF<sub>165</sub> 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.

For literature update refer to our website.

## Principles of the test

An anti-mouse VEGF-A coating antibody is adsorbed onto microwells.

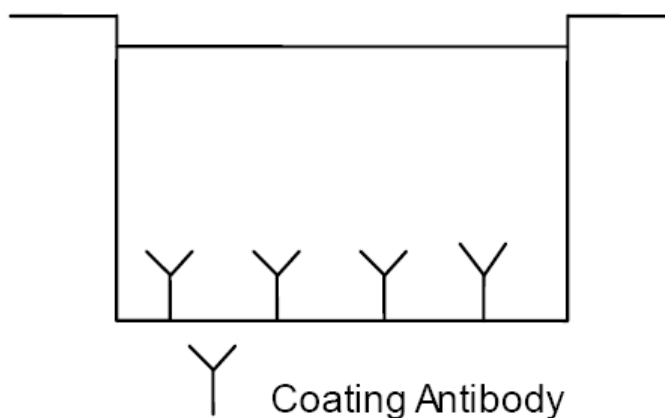


Fig. 1 Coated microwell

Mouse VEGF-A present in the sample or standard binds to antibodies adsorbed to the microwells.

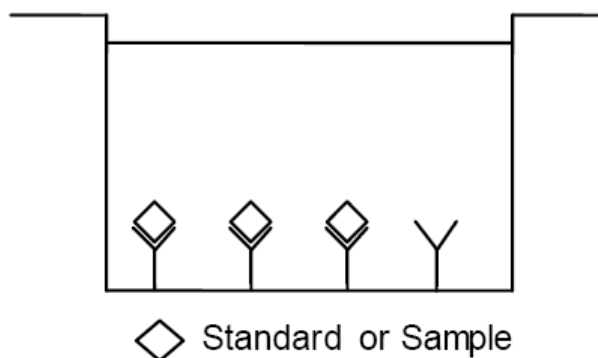


Fig. 2 First incubation

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.

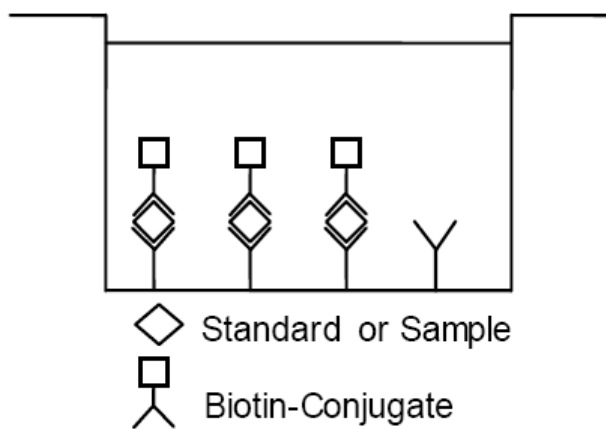


Fig. 3 Second incubation

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.

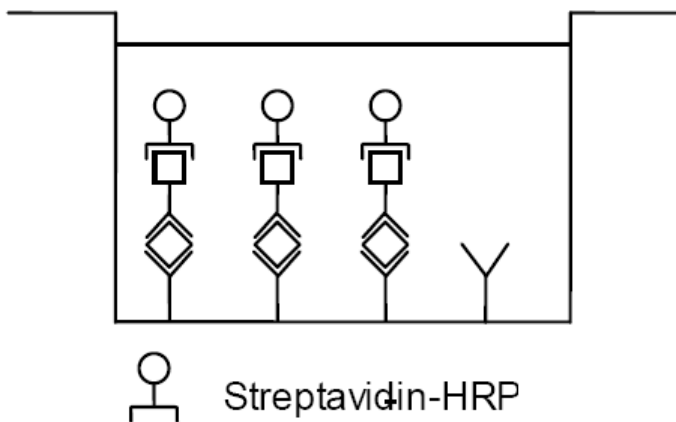


Fig. 4 Third incubation

Following incubation unbound Streptavidin-HRP is removed during a wash step, and substrate solution reactive with HRP is added to the wells.

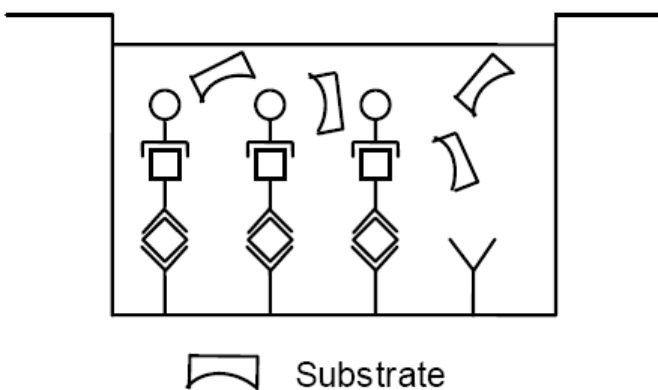


Fig. 5 Fourth incubation

A colored 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.

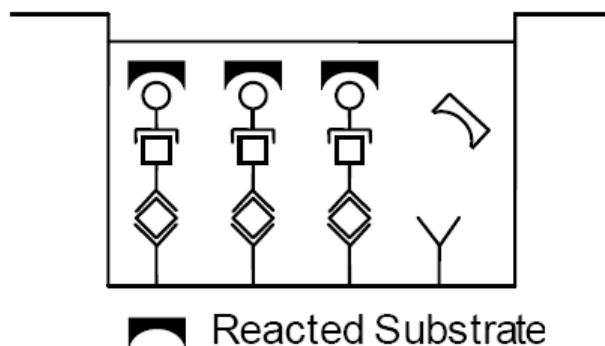


Fig. 6 Stop reaction

## Reagents provided

### Reagents for mouse VEGF-A ELISA BMS619-2 (96 tests)

- 1 aluminium pouch with a Microwell Plate (12 strips with 8 well each) coated with polyclonal antibody to mouse VEGF-A
- 1 vial (120 µL) Biotin-Conjugate anti-mouse VEGF-A polyclonal antibody
- 1 vial (150 µL) Streptavidin-HRP
- 2 vials mouse VEGF-A Standard lyophilized, 4 ng/mL upon reconstitution
- 1 vial (10 mL) Sample Diluent
- 1 vial (12 mL) Assay Diluent
- 1 vial (5 mL) Conjugate Diluent Concentrate 20x
- 1 bottle (50 mL) Wash Buffer Concentrate 20x (PBS with 1% Tween™ 20)
- 1 vial (15 mL) Substrate Solution (tetramethyl-benzidine)
- 1 vial (15 mL) Stop Solution (1M Phosphoric acid)
- 6 Adhesive Films

### Reagents for mouse VEGF-A ELISA BMS619-2TEN (10x96 tests)

- 10 aluminium pouches with a Microwell Plate (12 strips with 8 well each) coated with polyclonal antibody to mouse VEGF-A
- 10 vials (120 µL) Biotin-Conjugate anti-mouse VEGF-A polyclonal antibody
- 10 vials (150 µL) Streptavidin-HRP
- 10 vials mouse VEGF-A Standard lyophilized, 4 ng/mL upon reconstitution
- 6 vials (10 mL) Sample Diluent
- 10 vials (12 mL) Assay Diluent
- 3 vial (5 mL) Conjugate Diluent Concentrate 20x
- 8 bottles (50 mL) Wash Buffer Concentrate 20x (PBS with 1% Tween™ 20)
- 10 vials (15 mL) Substrate Solution (tetramethyl-benzidine)
- 1 vial (100 mL) Stop Solution (1M Phosphoric acid)
- 30 Adhesive Films

## Storage instructions – ELISA kit

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.

## Sample collection and storage instructions

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

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

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 (for sample stability refer to "Sample stability" on page 6).

Avoid repeated freeze-thaw cycles. Prior to assay, the frozen sample should be brought to room temperature slowly and mixed gently.

## Materials required but not provided

- 5 mL and 10 mL graduated pipettes
- 5 µL to 1000 µL adjustable single channel micropipettes with disposable tips
- 50 µL to 300 µL adjustable multichannel micropipette with disposable tips
- Multichannel micropipette reservoir
- Beakers, flasks, cylinders necessary for preparation of reagents
- Device for delivery of wash solution (multichannel wash bottle or automatic wash system)
- Microplate shaker
- Microwell strip reader capable of reading at 450 nm (620 nm as optional reference wave length)
- Glass-distilled or deionized water
- Statistical calculator with program to perform regression analysis

## Precautions for use

- All reagents should be considered as potentially hazardous. We therefore recommend that this product is handled only by those persons who have been trained in laboratory techniques and that it is used in accordance with the principles of good laboratory practice. Wear suitable protective clothing such as laboratory overalls, safety glasses and gloves. Care should be taken to avoid contact with skin or eyes. In the case of contact with skin or eyes wash immediately with water. See material safety data sheet(s) and/or safety statement(s) for specific advice.
- Reagents are intended for research use only and are not for use in diagnostic or therapeutic procedures.
- Do not mix or substitute reagents with those from other lots or other sources.
- Do not use kit reagents beyond expiration date on label.
- Do not expose kit reagents to strong light during storage or incubation.
- Do not pipette by mouth.
- Do not eat or smoke in areas where kit reagents or samples are handled.
- Avoid contact of skin or mucous membranes with kit reagents or specimens.
- Rubber or disposable latex gloves should be worn while handling kit reagents or specimens.
- Avoid contact of substrate solution with oxidizing agents and metal.
- Avoid splashing or generation of aerosols.
- In order to avoid microbial contamination or cross-contamination of reagents or specimens which may invalidate the test use disposable pipette tips and/or pipettes.
- Use clean, dedicated reagent trays for dispensing the conjugate and substrate reagent.
- Exposure to acid inactivates the conjugate.

- Glass-distilled water or deionized water must be used for reagent preparation.
- Substrate solution must be at room temperature prior to use.
- Decontaminate and dispose specimens and all potentially contaminated materials as they could contain infectious agents. The preferred method of decontamination is autoclaving for a minimum of 1 hour at 121.5°C.
- Liquid wastes not containing acid and neutralized waste may be mixed with sodium hypochlorite in volumes such that the final mixture contains 1.0% sodium hypochlorite. Allow 30 minutes for effective decontamination. Liquid waste containing acid must be neutralized prior to the addition of sodium hypochlorite.

## Preparation of reagents

1. Buffer Concentrates should be brought to room temperature and should be diluted before starting the test procedure.
2. If crystals have formed in the Buffer Concentrates, warm them gently until they have completely dissolved.

### Wash buffer (1x)

1. 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.
2. Transfer to a clean wash bottle and store at 2° to 25°C. Please note that Wash Buffer (1x) is stable for 30 days.
3. 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                  |

### Conjugate diluent (1x)

1. 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.
2. Store at 2° to 8°C. Please note that the Conjugate Diluent (1x) is stable for 30 days.
3. 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                 |

### Biotin-Conjugate

**Note:** 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 in a clean plastic tube as needed according to the following table:

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

## Streptavidin-HRP

**Note:** 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 in a clean plastic tube as needed according to the following table:

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

## Mouse VEGF-A standard

1. 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).
2. Reconstitution volume is stated on the label of the standard vial. Swirl or mix gently to insure complete and homogeneous solubilization (concentration of reconstituted standard = 4 ng/mL).
3. Allow the standard to reconstitute for 10-30 minutes. Mix well prior to making dilutions.
4. After usage remaining standard cannot be stored and has to be discarded.

## Standard dilution

1. Label 6 tubes, one for each standard point: S2, S3, S4, S5, S6, S7.
2. 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.
3. 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).
4. Pipette 150 µL of this dilution into the second tube and mix thoroughly before the next transfer.
5. Repeat serial dilutions 4 more times thus creating the points of the standard curve (see Figure 7).

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

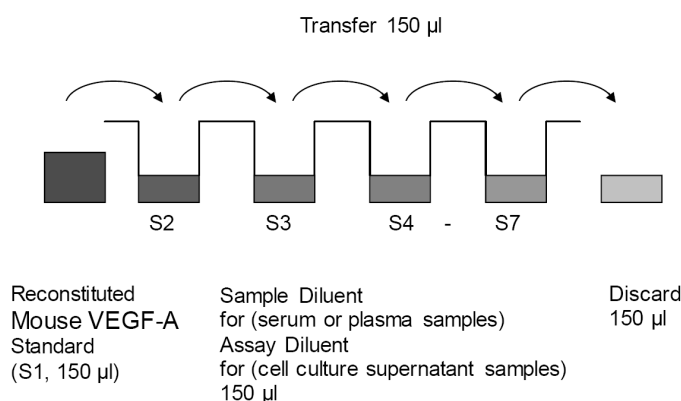


Fig. 7 Dilute standards - tubes

## Test protocol

1. Determine the number of microwell strips required to test the desired number of samples plus appropriate number of wells needed for running blanks and standards. Each sample, standard, blank and optional control sample should be assayed in duplicate. Remove extra microwell strips from holder and store in foil bag with the desiccant provided at 2°-8°C sealed tightly.
2. Wash the microwell strips twice with approximately 400 µL Wash Buffer per well with thorough aspiration of microwell contents between washes. Allow the Wash Buffer to sit in the wells for about 10 – 15 seconds before aspiration. Take care not to scratch the surface of the microwells.

After the last wash step, empty wells and tap microwell strips on absorbent pad or paper towel to remove excess Wash Buffer. Use the microwell strips immediately after washing. Alternatively microwell strips can be placed upside down on a wet absorbent paper for not longer than 15 minutes. Do not allow wells to dry.

3. Add 50 µL of Assay Diluent in duplicate to all wells.
4. Add 50 µL of each prepared Standard dilution in duplicate to the corresponding standard well (see Table 1).

**Table 1** Example of the arrangement of blanks, standards and samples in the microwell strips

|   | 1                          | 2                          | 3        | 4        |
|---|----------------------------|----------------------------|----------|----------|
| A | Standard 1<br>4000.0 pg/mL | Standard 1<br>4000.0 pg/mL | Sample 1 | Sample 1 |
| B | Standard 2<br>2000.0 pg/mL | Standard 2<br>2000.0 pg/mL | Sample 2 | Sample 2 |
| C | Standard 3<br>1000.0 pg/mL | Standard 3<br>1000.0 pg/mL | Sample 3 | Sample 3 |
| D | Standard 4<br>500.0 pg/mL  | Standard 4<br>500.0 pg/mL  | Sample 4 | Sample 4 |
| E | Standard 5<br>250.0 pg/mL  | Standard 5<br>250.0 pg/mL  | Sample 5 | Sample 5 |
| F | Standard 6<br>125.0 pg/mL  | Standard 6<br>125.0 pg/mL  | Sample 6 | Sample 6 |
| G | Standard 7<br>62.5 pg/mL   | Standard 7<br>62.5 pg/mL   | Sample 7 | Sample 7 |
| H | Blank                      | Blank                      | Sample 8 | Sample 8 |

5. Add 50 µL of each sample in duplicate to the sample wells (see Table 1).
6. Add 50 µL of Sample Diluent (for serum or plasma samples) or Assay Diluent (for cell culture supernatant samples) in duplicate to the blank wells.
7. Cover with an adhesive film and incubate at room temperature (18° to 25°C) for 2 hours on a microplate shaker. (Shaking is absolutely necessary for an optimal test performance.)
8. Prepare Biotin-Conjugate (see Preparation of Biotin-Conjugate "Biotin-Conjugate" on page 3).
9. Remove adhesive film and empty wells. Wash microwell strips 6 times according to 2. of the test protocol. Proceed immediately to the next step.
10. Add 100 µL of Biotin-Conjugate to all wells.
11. Cover with an adhesive film and incubate at room temperature (18° to 25°C) for 1 hour on a microplate shaker. (Shaking is absolutely necessary for an optimal test performance.)
12. Prepare Streptavidin-HRP (refer to Preparation of Streptavidin-HRP "Streptavidin-HRP" on page 4).
13. Remove adhesive film and empty wells. Wash microwell strips 6 times according to 2. of the test protocol. Proceed immediately to the next step.
14. Add 100 µL of diluted Streptavidin-HRP to all wells, including the blank wells.
15. Cover with an adhesive film and incubate at room temperature (18° to 25°C) for 1 hour on a microplate shaker. (Shaking is absolutely necessary for an optimal test performance.)

16. Remove adhesive film and empty wells. Wash microwell strips 6 times according to 2. of the test protocol. Proceed immediately to the next step.
17. Pipette 100  $\mu$ L of TMB Substrate Solution to all wells.
18. Incubate the microwell strips at room temperature (18° to 25°C) for about 30 min. Avoid direct exposure to intense light.

The color development on the plate should be monitored and the substrate reaction stopped (see next point of this protocol) before positive wells are no longer properly recordable. Determination of the ideal time period for color development has to be done individually for each assay.

It is recommended to add the stop solution when the highest standard has developed a dark blue color. Alternatively the color development can be monitored by the ELISA reader at 620 nm. The substrate reaction should be stopped as soon as Standard 1 has reached an OD of 0.9 – 0.95.

19. Stop the enzyme reaction by quickly pipetting 100  $\mu$ L of Stop Solution into each well. It is important that the Stop Solution is spread quickly and uniformly throughout the microwells to completely inactivate the enzyme. Results must be read immediately after the Stop Solution is added or within one hour if the microwell strips are stored at 2 - 8°C in the dark.
20. Read absorbance of each microwell on a spectro-photometer using 450 nm as the primary wave length (optionally 620 nm as the reference wave length; 610 nm to 650 nm is acceptable). Blank the plate reader according to the manufacturer's instructions by using the blank wells. Determine the absorbance of both the samples and the standards.

## Calculation of results

- Calculate the average absorbance values for each set of duplicate standards and samples. Duplicates should be within 20 percent of the mean value.
- Create a standard curve by plotting the mean absorbance for each standard concentration on the ordinate against the mouse VEGF-A concentration on the abscissa. Draw a best fit curve through the points of the graph (a 5-parameter curve fit is recommended).
- To determine the concentration of circulating mouse VEGF-A for each sample, first find the mean absorbance value on the ordinate and extend a horizontal line to the standard curve. At the point of intersection, extend a vertical line to the abscissa and read the corresponding mouse VEGF-A concentration.
- If instructions in this protocol have been followed, samples have not been diluted and the concentration read from the standard curve must not be multiplied by a dilution factor.
- Calculation of samples with a concentration exceeding standard 1 may result in incorrect, low mouse VEGF-A levels. Such samples require further external predilution according to expected mouse VEGF-A values with Sample Diluent (serum or plasma samples) or Assay Diluent (cell culture supernatant samples) in order to precisely quantitate the actual mouse VEGF-A level.
- It is suggested that each testing facility establishes a control sample of known mouse VEGF-A concentration and runs this additional control with each assay. If the values obtained are not within the expected range of the control, the assay results may be invalid.

- A representative standard curve is shown in Figure 8. This curve cannot be used to derive test results. Each laboratory must prepare a standard curve for each group of microwell strips assayed.

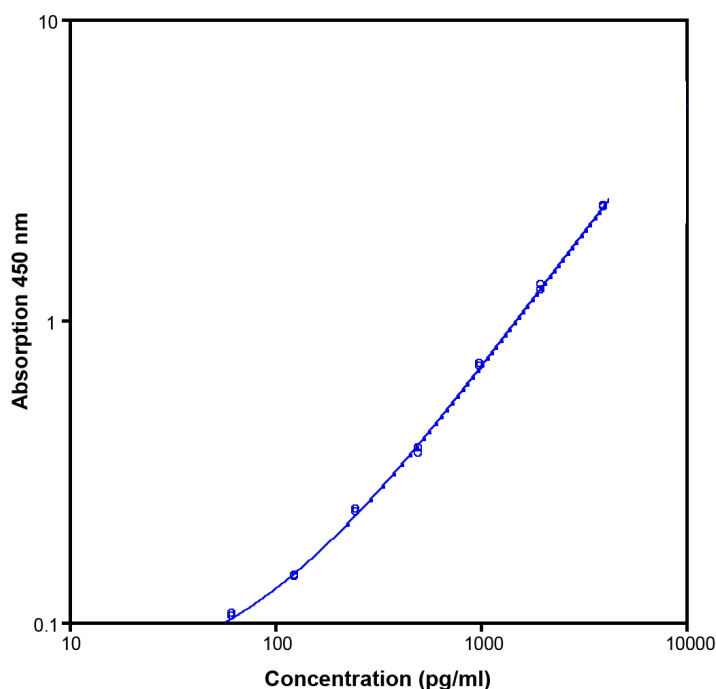


Fig. 8 Representative standard curve for mouse VEGF-A ELISA. Mouse VEGF-A was diluted in serial 2-fold steps in Sample Diluent (for serum or plasma samples) or Assay Diluent (for cell culture supernatant samples). Do not use this standard curve to derive test results. A standard curve must be run for each group of microwell strips assayed.

**Table 2** Typical data using the mouse VEGF-A ELISA

Measuring wavelength: 450 nm

Reference wavelength: 620 nm

| Standard | Mouse VEGF-A Concentration (pg/mL) | O.D. at 450 nm | Mean O.D. at 450 nm | C.V. (%) |
|----------|------------------------------------|----------------|---------------------|----------|
| 1        | 4000.0                             | 2.401<br>2.388 | 2.394               | 0.3      |
| 2        | 2000.0                             | 1.266<br>1.314 | 1.290               | 1.9      |
| 3        | 1000.0                             | 0.706<br>0.722 | 0.714               | 1.1      |
| 4        | 500.0                              | 0.364<br>0.379 | 0.371               | 1.9      |
| 5        | 250.0                              | 0.236<br>0.233 | 0.235               | 0.6      |
| 6        | 125.0                              | 0.143<br>0.141 | 0.142               | 0.9      |
| 7        | 62.5                               | 0.106<br>0.104 | 0.105               | 0.5      |
| Blank    | 0                                  | 0.066<br>0.065 | 0.065               | 0.7      |

The OD values of the standard curve may vary according to the conditions of assay performance (e.g. operator, pipetting technique, washing technique or temperature effects). Furthermore shelf life of the kit may affect enzymatic activity and thus color intensity. Values measured are still valid.

## Limitations

- Since exact conditions may vary from assay to assay, a standard curve must be established for every run.

- Bacterial or fungal contamination of either screen samples or reagents or cross-contamination between reagents may cause erroneous results.
- Disposable pipette tips, flasks or glassware are preferred, reusable glassware must be washed and thoroughly rinsed of all detergents before use.
- Improper or insufficient washing at any stage of the procedure will result in either false positive or false negative results. Empty wells completely before dispensing fresh wash solution, fill with Wash Buffer as indicated for each wash cycle and do not allow wells to sit uncovered or dry for extended periods.

## Performance characteristics

### 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).

### Reproducibility

#### 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. Data below show the mean mouse VEGF-A concentration and the coefficient of variation for each sample (see Table 3). The calculated overall intra-assay coefficient of variation was 3.8%.

**Table 3** The mean mouse VEGF-A concentration and the coefficient of variation for each sample

| Sample | Experiment | Mean Mouse VEGF-A Concentration (pg/mL) | Coefficient of Variation (%) |
|--------|------------|---|------------------------------|
| 1      | 1          | 2932                                    | 3.6                          |
|        | 2          | 3158                                    | 1.7                          |
|        | 3          | 3148                                    | 1.3                          |
| 2      | 1          | 1747                                    | 1.4                          |
|        | 2          | 1920                                    | 2.1                          |
|        | 3          | 1971                                    | 4.2                          |
| 3      | 1          | 1214                                    | 3.7                          |
|        | 2          | 1306                                    | 4.2                          |
|        | 3          | 1349                                    | 3.9                          |
| 4      | 1          | 614                                     | 5.1                          |
|        | 2          | 690                                     | 6.1                          |
|        | 3          | 710                                     | 2.8                          |
| 5      | 1          | 494                                     | 3.3                          |
|        | 2          | 543                                     | 2.3                          |
|        | 3          | 547                                     | 1.0                          |
| 6      | 1          | 121                                     | 5.7                          |
|        | 2          | 143                                     | 8.9                          |
|        | 3          | 123                                     | 6.6                          |

#### 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. Data below show the mean mouse VEGF-A concentration and the coefficient of

variation calculated on 18 determinations of each sample (see Table 4). The calculated overall inter-assay coefficient of variation was 6.4%.

**Table 4** The mean mouse VEGF-A concentration and the coefficient of variation of each sample

| Sample | Mean Mouse VEGF-A Concentration (pg/mL) | Coefficient of Variation (%) |
|--------|---|------------------------------|
| 1      | 3079                                    | 4.2                          |
| 2      | 1879                                    | 6.3                          |
| 3      | 1289                                    | 5.4                          |
| 4      | 672                                     | 7.5                          |
| 5      | 528                                     | 5.7                          |
| 6      | 129                                     | 9.4                          |

### 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 <sup>[1]</sup> | Spike high (%) | Spike medium (%) | Spike low (%)     |
|------------------------------|----------------|------------------|-------------------|
| Serum                        | 95             | 87               | 95                |
| Plasma (EDTA)                | 88             | 104              | nd <sup>[2]</sup> |
| Cell culture supernatant     | 88             | 97               | 75                |

<sup>[1]</sup> Due to high endogen mouse VEGF-A levels data for low spikes are not indicated.

<sup>[2]</sup> nd= nondetectable, samples measured below the lowest standard point are considered to be nondetectable.

### 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 of Exp. Val. |          |
|--------------------------|-----------------------|----------|
|                          | Range (%)             | Mean (%) |
| Serum                    | 84 - 112              | 102      |
| Plasma (EDTA)            | 98 - 119              | 106      |
| Cell culture supernatant | 78 - 105              | 92       |

### Sample 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.

### 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 PlGF.

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

## Reagent preparation summary

### Wash buffer (1x)

Add Wash Buffer Concentrate 20x (50 mL) to 950 mL distilled water.

| Number of Strips | Wash Buffer Concentrate (mL) | Distilled Water (mL) |
|------------------|------------------------------|----------------------|
| 1 - 6            | 25                           | 475                  |
| 1 - 12           | 50                           | 950                  |

### Conjugate diluent (1x)

Add Conjugate Diluent Concentrate 20x (5 mL) to 95 mL distilled water.

| Number of Strips | Conjugate Diluent Concentrate (mL) | Distilled Water (mL) |
|------------------|------------------------------------|----------------------|
| 1 - 6            | 2.5                                | 47.5                 |
| 1 - 12           | 5.0                                | 95.0                 |

### Biotin-Conjugate

Make a 1:100 dilution of Biotin-Conjugate in Conjugate Diluent (1x):

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

### Streptavidin-HRP

Make a 1:100 dilution of Streptavidin-HRP in Conjugate Diluent (1x):

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

### Mouse VEGF-A standard

Reconstitute lyophilized mouse VEGF-A standard with Sample Diluent (serum or plasma samples) or distilled water (cell culture supernatant samples). (Reconstitution volume is stated on the label of the standard vial.)

## Test protocol summary

1. Determine the number of microwell strips required.
2. Wash microwell strips twice with Wash Buffer.
3. Add 50 µL Assay Diluent in duplicate to all wells.
4. Add 50 µL of prepared Standard dilutions in duplicate to standard wells.
5. Add 50 µL of each sample in duplicate to sample wells.
6. Add 50 µL of Sample Diluent (for serum or plasma samples) or Assay Diluent (for cell culture supernatant samples) in duplicate to blank wells.
7. Cover microwell strips and incubate 2 hours at room temperature (18° to 25°C). (Shaking is absolutely necessary for an optimal test performance.)
8. Prepare Biotin-Conjugate Conjugate.
9. Empty and wash microwell strips 6 times with Wash Buffer.
10. Add 100 µL Biotin-Conjugate to all wells.
11. Cover microwell strips and incubate 1 hour at room temperature (18° to 25°C). (Shaking is absolutely necessary for an optimal test performance.)
12. Prepare Streptavidin-HRP.
13. Empty and wash microwell strips 6 times with Wash Buffer.
14. Add 100 µL diluted Streptavidin-HRP to all wells.
15. Cover microwell strips and incubate 1 hour at room temperature (18° to 25°C). (Shaking is absolutely necessary for an optimal test performance.)
16. Empty and wash microwell strips 6 times with Wash Buffer.
17. Add 100 µL of TMB Substrate Solution to all wells.
18. Incubate the microwell strips for about 30 minutes at room temperature (18° to 25°C).
19. Add 100 µL Stop Solution to all wells.
20. Blank microwell reader and measure color intensity at 450 nm.

**Note:** If instructions in this protocol have been followed, samples have not been diluted and the concentration read from the standard curve must not be multiplied by a dilution factor.

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Bender MedSystems GmbH | Campus Vienna Biocenter 2 | 1030 Vienna, Austria

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