

# Human VEGF-R2/KDR ELISA Kit

Enzyme-linked Immunosorbent Assay for quantitative detection of human VEGF-R2/KDR

**Catalog Numbers** BMS2019 or BMS2019TEN

**Pub. No.** MAN0016477 **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 Human VEGF-R2/KDR ELISA Kit is an enzyme-linked immunosorbent assay for the quantitative detection of human VEGF-R2/KDR.

## Summary

Angiogenesis is a fundamental physiological process, both during the development of the organism and in adult life, requiring the well-coordinated action of a variety of growth factors and adhesion molecules in endothelial cells. So far, VEGF-A (also called VEGF) and its receptors represent the best characterized signaling pathway in developmental and tumour angiogenesis. VEGF-A binds to two receptor-tyrosine kinases: VEGFR-1/Flt-1 and VEGFR-2/KDR. VEGFR-2 is the major mediator of the mitogenic, angiogenic and permeability-enhancing effects of VEGF-A.

The VEGFR's possess an approximately 750 amino acid residue extracellular domain, which is organized into seven immunoglobulin (Ig)-like folds. This extracellular domain (also called ectodomain) is followed by a single transmembrane region, a juxtamembrane domain, a split tyrosine-kinase domain interrupted by a 70-amino acid kinase insert and a C-terminal tail. Alternative splicing or proteolytic processing of VEGFR's give rise to secreted variants of VEGFR-2, also called soluble VEGFR-2 (VEGFR-2). Although the VEGFR's are primarily expressed in the vascular system, sensitive methods like sandwich ELISA's have allowed the detection of VEGFR expression in non-endothelial cells like hematopoietic stem cells.

For literature update refer to our website.

## Principles of the test

An anti-human VEGF-R2/KDR coating antibody is adsorbed onto microwells.

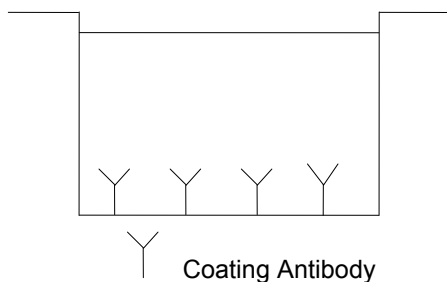


Fig. 1 Coated microwell.

Human VEGF-R2/KDR present in the sample or standard binds to antibodies adsorbed to the microwells. A biotin-conjugated anti-human VEGF-R2/KDR antibody is added and binds to human VEGF-R2/KDR captured by the first antibody.

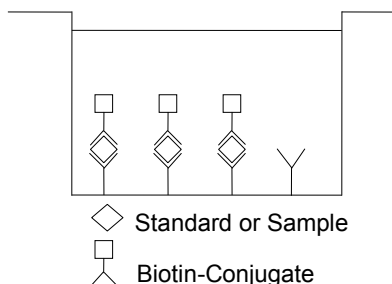


Fig. 2 First incubation.

Following incubation unbound biotin-conjugated anti-human VEGF-R2/KDR antibody is removed during a wash step. Streptavidin-HRP is added and binds to the biotin-conjugated anti-human VEGF-R2/KDR antibody.

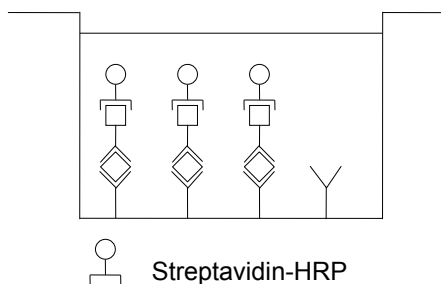


Fig. 3 Second incubation.

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

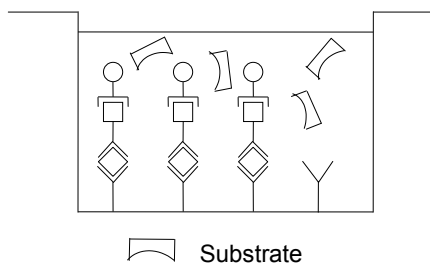


Fig. 4 Third incubation.

A colored product is formed in proportion to the amount of human VEGF-R2/KDR 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 human VEGF-R2/KDR standard dilutions and human VEGF-R2/KDR sample concentration determined.

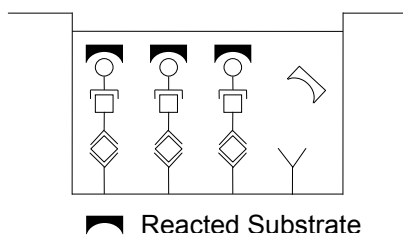


Fig. 5 Stop reaction.

## Reagents provided

### Reagents for human VEGF-R2/KDR ELISA BMS2019 (96 tests)

1 aluminum pouch with a Microwell Plate (12 strips of 8 wells each) coated with monoclonal antibody to human VEGF-R2/KDR

1 vial (70 µL) Biotin-Conjugate anti-human VEGF-R2/KDR monoclonal antibody

1 vial (150 µL) Streptavidin-HRP

2 vials human VEGF-R2/KDR Standard lyophilized, 10 ng/mL upon reconstitution

1 vial (5 mL) Assay Buffer Concentrate 20x (PBS with 1% Tween™ 20, 10% BSA)

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)

4 Adhesive Films

### Reagents for human VEGF-R2/KDR ELISA BMS2019TEN (10x96 tests)

10 aluminum pouches with a Microwell Plate (12 strips of 8 wells each) coated with monoclonal antibody to human VEGF-R2/KDR

10 vials (70 µL) Biotin-Conjugate anti-human VEGF-R2/KDR monoclonal antibody

10 vials (150 µL) Streptavidin-HRP

10 vials human VEGF-R2/KDR Standard lyophilized, 10 ng/mL upon reconstitution

7 vials (5 mL) Assay Buffer Concentrate 20x (PBS with 1% Tween™ 20, 10% BSA)

6 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)

20 Adhesive Films

## Storage instructions – ELISA kit

Store kit reagents between 2°C and 8°C. Immediately after use remaining reagents should be returned to cold storage (2°C 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, citrate, heparin), 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.

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

Samples should be aliquoted and must be stored frozen at -20°C to avoid loss of bioactive human VEGF-R2/KDR. If samples are to be run

within 24 hours, they may be stored at 2°C 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)
- 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 pipet 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 samples.
- Rubber or disposable latex gloves should be worn while handling kit reagents or samples.
- Avoid contact of substrate solution with oxidizing agents and metal.
- Avoid splashing or generation of aerosols.
- To avoid microbial contamination or cross-contamination of reagents or samples that 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 samples and all potentially contaminated materials as if 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.
2. Mix gently to avoid foaming.
3. Transfer to a clean wash bottle and store at 2°C to 25°C. The Wash Buffer (1x) is stable for 30 days.
4. 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                  |

### Assay buffer (1x)

1. Pour the entire contents (5 mL) of the Assay Buffer 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°C to 8°C. The Assay Buffer (1x) is stable for 30 days.
3. Assay Buffer (1x) may also be prepared as needed according to the following table:

| Number of Strips | Assay Buffer 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 Assay Buffer (1x) in a clean plastic tube as needed according to the following table:

| Number of Strips | Biotin-Conjugate (mL) | Assay Buffer (1x) (mL) |
|------------------|-----------------------|------------------------|
| 1 - 6            | 0.03                  | 2.97                   |
| 1 - 12           | 0.06                  | 5.94                   |

### Streptavidin-HRP

**Note:** The Streptavidin-HRP should be used within 30 minutes after dilution.

Make a 1:200 dilution of the concentrated Streptavidin-HRP solution with Assay Buffer (1x) in a clean plastic tube as needed according to the following table:

| Number of Strips | Streptavidin-HRP (mL) | Assay Buffer (1x) (mL) |
|------------------|-----------------------|------------------------|
| 1-6              | 0.03                  | 5.97                   |
| 1-12             | 0.06                  | 11.94                  |

### Human VEGF-R2/KDR standard

1. Reconstitute human VEGF-R2/KDR standard by addition of distilled water. 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 = 10 ng/mL).
2. Allow the standard to reconstitute for 10-30 minutes. Mix well prior to making dilutions.

The standard has to be used immediately after reconstitution and cannot be stored.

### External standard dilution

1. Label 7 tubes, one for each standard point: S1, S2, S3, S4, S5, S6, S7.
2. Prepare 1:2 serial dilutions for the standard curve as follows: Pipet 225 µL of Assay Buffer (1x) into each tube.
3. Pipet 225 µL of reconstituted standard (concentration of standard = 10 ng/mL) into the first tube, labeled S1, and mix (concentration of standard 1 = 5 ng/mL).
4. Pipet 225 µL of this dilution into the second tube, labeled S2, and mix thoroughly before the next transfer.
5. Repeat serial dilutions 5 more times thus creating the points of the standard curve (see Figure 6).

Assay Buffer (1x) serves as blank.

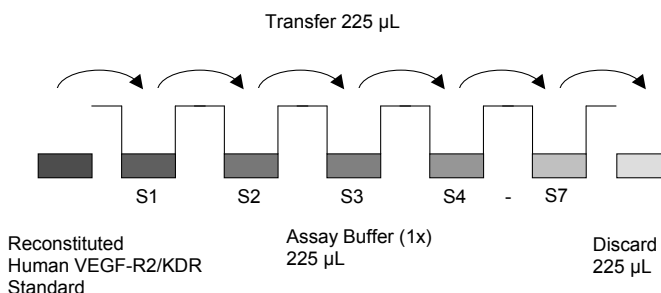


Fig. 6 Dilute standards - tubes.

## Test protocol

1. Predilute your samples before starting with the test procedure. Dilute serum, plasma and cell culture samples 1:25 with Assay Buffer (1x) according to the following scheme: 20 µL sample + 480 µL Assay Buffer (1x).
2. 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°C to 8°C sealed tightly.
3. 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.
4. Standard dilution on the microwell plate (alternatively the standard dilution can be prepared in tubes, see "External standard dilution" on page 3):

Add 100 µL of Assay Buffer (1x) in duplicate to all standard wells. Pipet 100 µL of prepared standard (see Preparation of Standard "Human VEGF-R2/KDR standard" on page 3, concentration = 10,000 pg/mL) in duplicate into well A1 and A2 (see Table 1). Mix the contents of wells A1 and A2 by repeated aspiration and ejection (concentration of standard 1, S1 = 5000 pg/mL), and transfer 100 µL to wells B1 and B2 (see Figure 7). Take care not to scratch the inner surface of the microwells. Continue this procedure 5 times, creating two rows of human VEGF-R2/KDR standard dilutions ranging from 5000-78 pg/mL. Discard 100 µL of the contents from the last microwells (G1, G2) used.

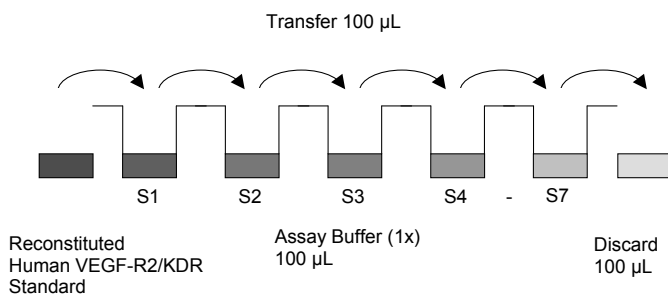


Fig. 7 Dilute standards - microwell plate.

In case of an external standard dilution (see “External standard dilution” on page 3), pipet 100 µL of these standard dilutions (S1–S7) in the standard wells according to 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>5000 pg/mL | Standard 1<br>5000 pg/mL | Sample 1 | Sample 1 |
| B | Standard 2<br>2500 pg/mL | Standard 2<br>2500 pg/mL | Sample 2 | Sample 2 |
| C | Standard 3<br>1250 pg/mL | Standard 3<br>1250 pg/mL | Sample 3 | Sample 3 |
| D | Standard 4<br>625 pg/mL  | Standard 4<br>625 pg/mL  | Sample 4 | Sample 4 |
| E | Standard 5<br>313 pg/mL  | Standard 5<br>313 pg/mL  | Sample 5 | Sample 5 |
| F | Standard 6<br>156 pg/mL  | Standard 6<br>156 pg/mL  | Sample 6 | Sample 6 |
| G | Standard 7<br>78 pg/mL   | Standard 7<br>78 pg/mL   | Sample 7 | Sample 7 |
| H | Blank                    | Blank                    | Sample 8 | Sample 8 |

- Add 100 µL of Assay Buffer (1x) in duplicate to the blank wells.
- Add 50 µL of Assay Buffer (1x) to the sample wells.
- Add 50 µL of each prediluted sample in duplicate to the sample wells.
- Prepare Biotin-Conjugate (see “Biotin-Conjugate” on page 3).
- Add 50 µL of Biotin-Conjugate to all wells.
- 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.)
- Prepare Streptavidin-HRP (see “Streptavidin-HRP” on page 3).
- Remove adhesive film and empty wells. Wash microwell strips 6 times according to step 3. Proceed immediately to the next step.
- Add 100 µL of diluted Streptavidin-HRP to all wells, including the blank wells.
- Cover with an adhesive film and incubate at room temperature (18°C to 25°C) for 1 hour on a microplate shaker. (Shaking is absolutely necessary for an optimal test performance.)
- Remove adhesive film and empty wells. Wash microwell strips 6 times according to step 3. Proceed immediately to the next step.
- Pipet 100 µL of TMB Substrate Solution to all wells.
- Incubate the microwell strips at room temperature (18°C to 25°C) for about 30 minutes. Avoid direct exposure to intense light.

The color development on the plate should be monitored and the substrate reaction stopped (see next step) 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.

- Stop the enzyme reaction by quickly pipetting 100 µL 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°C to 8°C in the dark.
- Read absorbance of each microwell on a spectrophotometer 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% of the mean value.
- Create a standard curve by plotting the mean absorbance for each standard concentration on the ordinate against the human VEGF-R2/KDR 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 human VEGF-R2/KDR 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 human VEGF-R2/KDR concentration.
- If instructions in this protocol have been followed, samples have been diluted 1:50 (1:25 external predilution, 1:2 dilution on the plate: 50 µL sample + 50 µL Assay Buffer (1x)) and the concentration read from the standard curve must be multiplied by the dilution factor (x 50).
- Calculation of 1:25 prediluted samples with a concentration exceeding standard 1 may result in incorrect, low human VEGF-R2/KDR levels (Hook Effect). Such samples require further external predilution according to expected human VEGF-R2/KDR values with Assay Buffer (1x) in order to precisely quantitate the actual human VEGF-R2/KDR level.
- It is suggested that each testing facility establishes a control sample of known human VEGF-R2/KDR 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.  
**Note:** Do not use this standard curve to derive test results. Each laboratory must prepare a standard curve for each group of microwell strips assayed.

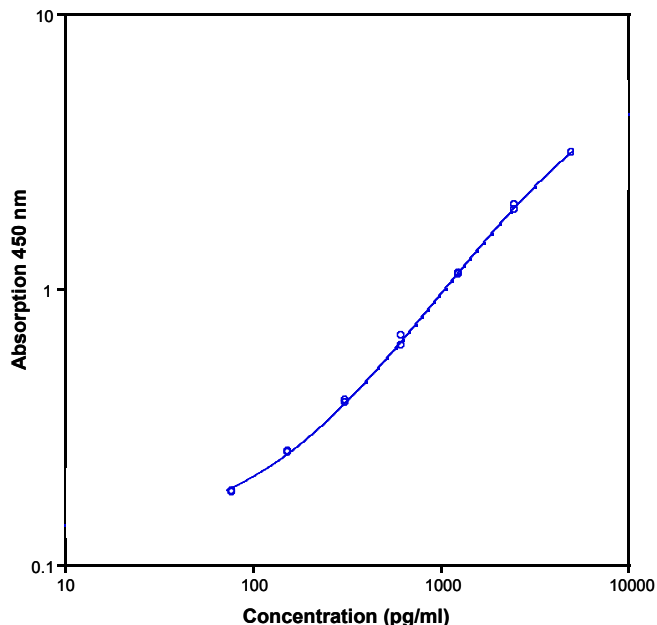


Fig. 8 Representative standard curve for Human VEGF-R2/KDR ELISA Kit. Human VEGF-R2/KDR was diluted in serial 2-fold steps in assay buffer (1x).

**Table 2** Typical data using the Human VEGF-R2/KDR ELISA Kit.  
Measuring wavelength: 450 nm  
Reference wavelength: 620 nm

| Standard | Human VEGF-R2/KDR concentration (pg/mL) | O.D. at 450 nm | Mean O.D. at 450 nm | C.V. (%) |
|----------|---|----------------|---------------------|----------|
| 1        | 5000                                    | 3.122<br>3.118 | 3.120               | 0.1      |
| 2        | 2500                                    | 2.020<br>1.936 | 1.978               | 2.1      |
| 3        | 1250                                    | 1.142<br>1.127 | 1.135               | 0.6      |
| 4        | 625                                     | 0.675<br>0.623 | 0.649               | 4.0      |
| 5        | 313                                     | 0.386<br>0.393 | 0.389               | 1.0      |
| 6        | 156                                     | 0.257<br>0.255 | 0.256               | 0.5      |
| 7        | 78                                      | 0.185<br>0.182 | 0.183               | 0.6      |
| Blank    | 0                                       | 0.093<br>0.092 | 0.093               | 0.2      |

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

- Because 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.
- The use of radioimmunotherapy has significantly increased the number of patients with human anti-mouse IgG antibodies (HAMA). HAMA may interfere with assays utilizing murine monoclonal antibodies leading to both false positive and false negative results. Serum samples containing antibodies to murine immunoglobulins can still be analyzed in such assays when murine immunoglobulins (serum, ascitic fluid, or monoclonal antibodies of irrelevant specificity) are added to the sample.

## Performance characteristics

### Sensitivity

The limit of detection of human VEGF-R2/KDR 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 7.0 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 8 serum samples containing different concentrations of human VEGF-R2/KDR. Two standard curves were run on each plate. Data below show the mean human VEGF-R2/KDR concentration and the coefficient of variation for each sample. The calculated overall intra-assay coefficient of variation was 5.3%.

**Table 3** The mean human VEGF-R2/KDR concentration and the coefficient of variation for each sample.

| Sample | Experiment | Mean concentration (pg/mL) | Coefficient of variation (%) |
|--------|------------|----------------------------|------------------------------|
| 1      | 1          | 152,357                    | 6.0                          |
|        | 2          | 119,914                    | 1.8                          |
|        | 3          | 117,181                    | 2.2                          |
| 2      | 1          | 92,056                     | 5.3                          |
|        | 2          | 81,321                     | 3.3                          |
|        | 3          | 77,605                     | 4.0                          |
| 3      | 1          | 47,453                     | 5.8                          |
|        | 2          | 40,261                     | 4.0                          |
|        | 3          | 36,194                     | 6.1                          |
| 4      | 1          | 19,234                     | 5.3                          |
|        | 2          | 16,679                     | 3.7                          |
|        | 3          | 14,978                     | 4.8                          |
| 5      | 1          | 15,509                     | 8.5                          |
|        | 2          | 13,796                     | 3.9                          |
|        | 3          | 13,131                     | 5.5                          |
| 6      | 1          | 12,818                     | 5.4                          |
|        | 2          | 11,928                     | 6.1                          |
|        | 3          | 10,974                     | 6.0                          |
| 7      | 1          | 6225                       | 10.1                         |
|        | 2          | 6068                       | 3.9                          |
|        | 3          | 5294                       | 6.7                          |
| 8      | 1          | 6805                       | 7.6                          |
|        | 2          | 7132                       | 5.4                          |
|        | 3          | 6795                       | 6.8                          |

#### Inter-assay

Assay to assay reproducibility within one laboratory was evaluated in 3 independent experiments. Each assay was carried out with 6 replicates of 8 serum samples containing different concentrations of human VEGF-R2/KDR. Two standard curves were run on each plate.

Data below show the mean human VEGF-R2/KDR concentration and the coefficient of variation calculated on 18 determinations of each sample. The calculated overall inter-assay coefficient of variation was 9.8%.

**Table 4** The mean human VEGF-R2/KDR concentration and the coefficient of variation of each sample.

| Sample | Mean concentration (pg/mL) | Coefficient of variation (%) |
|--------|----------------------------|------------------------------|
| 1      | 129,817                    | 15.1                         |
| 2      | 83,661                     | 9.0                          |
| 3      | 41,303                     | 13.8                         |
| 4      | 16,964                     | 12.6                         |
| 5      | 14,145                     | 8.7                          |
| 6      | 11,906                     | 7.7                          |
| 7      | 5862                       | 8.5                          |
| 8      | 6911                       | 2.8                          |

### Spike recovery

The spike recovery was evaluated by spiking 3 levels of human VEGF-R2/KDR into serum, plasma, and cell culture supernatant samples. Recoveries were determined with 4 replicates each. The amount of endogenous human VEGF-R2/KDR in unspiked serum was subtracted from the spike values.

| Sample matrix            | Spike high (%) | Spike medium (%) | Spike low (%) |
|--------------------------|----------------|------------------|---------------|
| Serum                    | 90             | 90               | 89            |
| Plasma (EDTA)            | 86             | 91               | 107           |
| Plasma (citrate)         | 92             | 97               | 108           |
| Plasma (heparin)         | 96             | 93               | 106           |
| Cell culture supernatant | 86             | 93               | 98            |

### Dilution parallelism

Serum, plasma and cell culture supernatant samples with different levels of human VEGF-R2/KDR were analyzed at serial 2 fold dilutions with 4 replicates each.

| Sample matrix            | Recovery of exp. val. |          |
|--------------------------|-----------------------|----------|
|                          | Range (%)             | Mean (%) |
| Serum                    | 83-97                 | 89       |
| Plasma (EDTA)            | 84-99                 | 88       |
| Plasma (citrate)         | 80-97                 | 89       |
| Plasma (heparin)         | 83-97                 | 88       |
| Cell culture supernatant | 84-107                | 97       |

### Sample stability

#### Freeze-thaw stability

Aliquots of serum samples (spiked or unspiked) were stored at -20°C and thawed 3 times, and the human VEGF-R2/KDR levels determined. There was no significant loss of human VEGF-R2/KDR immunoreactivity detected by freezing and thawing.

#### Storage stability

Aliquots of serum samples (spiked or unspiked) were stored at -20°C, 2°C to 8°C, room temperature, and at 37°C, and the human VEGF-R2/KDR level determined after 24 hours. There was no significant loss of human VEGF-R2/KDR immunoreactivity detected during storage under above conditions.

### Specificity

The assay detects both natural and recombinant human VEGF-R2/KDR. Cross-reactivity and interference of circulating factors of the immune system was evaluated by spiking these proteins at physiologically relevant concentrations into a human VEGF-R2/KDR positive sample. No cross-reactivity was detected, notably not with VEGF-R1 at physiological concentrations and not with VEGF-

R3/FLT-4. No interference was detected, notably not for VEGF-A and VEGF-C.

### Expected values

Panels of 40 serum as well as EDTA, citrate and heparin plasma samples from randomly selected apparently healthy donors (males and females) were tested for human VEGF-R2/KDR. The levels measured may vary with the sample collection used.

| Sample matrix    | Number of samples evaluated | Range (pg/mL) | Mean (pg/mL) | Standard deviation (pg/mL) |
|------------------|-----------------------------|---------------|--------------|----------------------------|
| Serum            | 40                          | 7104-30,861   | 17,553       | 5082                       |
| Plasma (EDTA)    | 40                          | 7824-22,688   | 14,017       | 3426                       |
| Plasma (citrate) | 40                          | 9255-30,288   | 18,471       | 5560                       |
| Plasma (heparin) | 40                          | 7101-21,177   | 13,207       | 3324                       |

### 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                  |

#### Assay Buffer (1x)

Add Assay Buffer Concentrate 20x (5 mL) to 95 mL distilled water.

| Number of Strips | Assay Buffer 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 Assay Buffer (1x).

| Number of Strips | Biotin-Conjugate (mL) | Assay Buffer (1x) (mL) |
|------------------|-----------------------|------------------------|
| 1-6              | 0.03                  | 2.97                   |
| 1-12             | 0.06                  | 5.94                   |

#### Streptavidin-HRP

Make a 1:200 dilution of Streptavidin-HRP in Assay Buffer (1x).

| Number of Strips | Streptavidin-HRP (mL) | Assay Buffer (1x) (mL) |
|------------------|-----------------------|------------------------|
| 1-6              | 0.03                  | 5.97                   |
| 1-12             | 0.06                  | 11.94                  |

#### Human VEGF-R2/KDR standard

Reconstitute lyophilized human VEGF-R2/KDR standard with distilled water. (Reconstitution volume is stated on the label of the standard vial.)

### Test protocol summary

**Note:** If instructions in this protocol have been followed, samples have been diluted 1:50 (50 µL 1:25 prediluted sample + 50 µL Assay Buffer (1x)) and the concentration read from the standard curve must be multiplied by the dilution factor (x 50).

1. Predilute sample with Assay Buffer (1x) 1:25.
2. Determine the number of microwell strips required.
3. Wash microwell strips twice with Wash Buffer.

- Standard dilution on the microwell plate: Add 100 µL Assay Buffer (1x) in duplicate to all standard wells. Pipette 100 µL prepared standard into the first wells and create standard dilutions by transferring 100 µL from well to well. Discard 100 µL from the last wells.

Alternatively, external standard dilution in tubes (see “External standard dilution” on page 3): Pipette 100 µL of these standard dilutions in the microwell strips.

- Add 100 µL Assay Buffer (1x) in duplicate to the blank wells.
- Add 50 µL Assay Buffer (1x) to sample wells.
- Add 50 µL prediluted sample in duplicate to designated sample wells.
- Prepare Biotin-Conjugate.
- Add 50 µL Biotin-Conjugate to all wells.
- Cover microwell strips and incubate 2 hours at room temperature (18°C to 25°C). (Shaking is absolutely necessary for an optimal test performance.)
- Prepare Streptavidin-HRP.
- Empty and wash microwell strips 6 times with Wash Buffer.
- Add 100 µL diluted Streptavidin-HRP to all wells.
- Cover microwell strips and incubate 1 hour at room temperature (18°C to 25°C). (Shaking is absolutely necessary for an optimal test performance.)
- Empty and wash microwell strips 6 times with Wash Buffer.
- Add 100 µL of TMB Substrate Solution to all wells.
- Incubate the microwell strips for about 30 minutes at room temperature (18°C to 25°C).

- Add 100 µL Stop Solution to all wells.
- Blank microwell reader and measure color intensity at 450 nm.

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