PRODUCT INFORMATION & MANUAL

Human VEGF R2/KDR Valukine™ ELISA

Catalog Number: VAL119

For the quantitative determination of natural and recombinant human Vascular Endothelial Growth Factor Receptor 2 (VEGF R2/KDR) concentrations

For research use only.
Not for diagnostic or therapeutic procedures.

Bio-Techne China Co. Ltd
P: +86 (21) 52380373 P: 8009881270 F: +86 (21) 52381001
info.cn@bio-techne.com

Please refer to the kit label for expiry date.
Novus kits are guaranteed for 3 months from date of receipt

Version 202409.4

TABLE OF CONTENTS

I. BACKGROUND

Vascular Endothelial Growth Factor Receptor 2 (VEGF R2), also known as kinase insert domain receptor (KDR) in humans or fetal liver kinase-1 (Flk-1) in mice, is a member of the class III subfamily of receptor tyrosine kinases (RTKs) that also includes VEGF R1 (Flt-1) and VEGF R3 (Flt-4). All three receptors contain seven Ig-like repeats within their extracellular domains and kinase insert domains in their intracellular regions. They are preferentially expressed in the proliferating endothelium of vessels lining and/or penetrating solid tumors (1). VEGF R2, however, is more widely distributed and expressed in all vessel-derived endothelial cells in comparison to VEGF R1 (2).

VEGF R2 binds VEGF with high affinity (3). In vitro studies further demonstrate that PlGF/VEGF heterodimers can bind with high affinity to soluble VEGF R2, but PlGF homodimers fail to bind this receptor (4). In contrast to soluble VEGF R1, soluble VEGF R2 cannot compete with VEGF for binding to human endothelial cells expressing both VEGF R1 and VEGF R2. Soluble VEGF R2 can only partially inhibit cell migration, whereas soluble VEGF R1 can almost completely block VEGF-induced cell proliferation and migration (5). The binding of VEGF to soluble VEGF R2, but not VEGF R1, is also dependent on heparin (5).

The VEGF/VEGF R2 signaling pathway plays an important role in tumor angiogenesis and other diseases where "pathological angiogenesis" is involved. Inactivation of functional VEGF R2 by a blocking antibody can disrupt angiogenesis and prevent tumor cell invasion (6, 7). Angiogenesis induced by the HIV-1 Tat protein is mediated by VEGF R2 on vascular endothelial cells (8). Tat specifically binds and activates VEGF R2. Tat-induced angiogenesis is blocked by agents that can block VEGF R2 (8, 9).

II. OVERVIEW

A. PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. An antibody specific for human VEGF R2 has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any human VEGF R2 present is bound by the immobilized antibody. After washing away any unbound substances, a biotinylated detect antibody specific for human VEGF R2 is pipetted into the wells. After washing away any unbound substances, Streptavidin-HRP is pipetted into the wells. Following a wash to remove any unbound reagent, TMB substrate (Chromogenic agent) is added to the wells and color develops in proportion to the amount of human VEGF R2 bound in the initial step. The color development is stopped, and the intensity of the color is measured.

B. LIMITATIONS OF THE PROCEDURE

- **FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.**
- This kit is suitable for cell culture supernate and human serum.
- The kit should not be used beyond the expiration date on the kit label.
- Do not mix or substitute reagents with those from other lots or sources.
- If samples generate values higher than the highest standard, dilute the samples with Calibrator Diluent (1×) and repeat the assay.
- Any variation in operator, pipetting technique, washing technique, incubation time or temperature, and kit age can cause variation in binding.

III. ADVANTAGES

A. PRECISION

Intra-assay Precision (Precision within an assay)

Three samples were tested twenty times on one plate to assess intra-assay precision.

Inter-assay Precision (Precision between assays)

Three samples were tested in twenty separate assays to assess inter-assay precision.

	Intra-assay Precision			Inter-assay Precision		
Sample	1	2	3	1	2	3
Mean (pg/mL)	113.5	218.9	789.8	116.0	223.4	799.5
Standard Deviation	3.0	11.8	18.3	5.4	11.1	29.0
CV%	2.7	5.4	2.3	4.6	5.0	3.6

B. RECOVERY

The recovery of human VEGF R2 spiked to three levels throughout the range of the assay in cell culture media was evaluated. The recovery ranged from 114.2 to 118.3% with an average of 116.1%.

C. SENSITIVITY

The minimum detectable dose (MDD) of human VEGF R2 is typically less than 6.8 pg/mL.

The MDD was determined by adding two standard deviations to the mean optical density value of twenty zero standard replicates and calculating the corresponding concentration.

D. CALIBRATION

This immunoassay is calibrated against a highly purified NS0-expressed recombinant human VEGF R2 produced at R&D Systems®.

E. LINEARITY

To assess the linearity of the assay, different samples containing or spiked with high concentrations of human VEGF R2 and diluted with Calibrator Diluent (1×) to produce samples with values within the dynamic range of the assay.

Dilution	Average % of Expected	Range (%)
1:2	99.7	95.0-104.0
1:4	102.4	95.4-106.0
1:8	102.2	92.7-108.3
1:16	95.3	81.1-104.8

F. SAMPLE VALUES

Human serum - Ten human serum samples were evaluated for the presence of human VEGF R2 in this assay. All samples measured ranged from 7196 to 12321 pg/mL with an average of 9279 pg/mL.

G. SPECIFICITY

This assay recognizes both natural and recombinant human VEGF R2. The following factors prepared at 50 ng/mL were assayed and exhibited no cross-reactivity or interference.

Recombinant human：	**Recombinant mouse：**
VEGF-D	$VEGF_{164}$
VEGF R3/Fc Chimera	

A sample containing 25 ng/mL of recombinant human VEGF R1/Fc Chimera reads as 40 pg/mL (0.16% cross-reactivity).

A sample containing 12.5 ng/mL of recombinant mouse VEGF R2/Fc Chimera reads as 46 pg/mL (0.36% cross-reactivity).

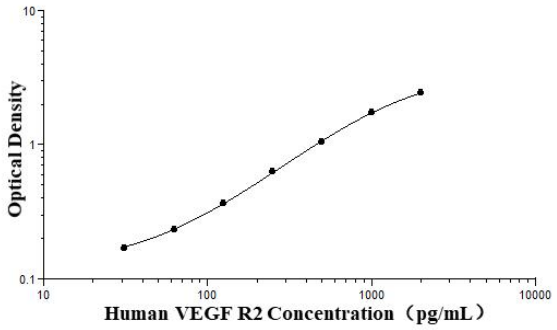
Recombinant human $VEGF_{121}$ and recombinant mouse $VEGF_{120}$ do not cross-react in this assay but do interfere at concentrations > 1.6 ng/mL.

Recombinant human $VEGF_{165}$ does not cross-react in this assay but does interfere at concentrations > 3.1 ng/mL.

IV. EXPERIMENT

EXAMPLE STANDARD

The standard curve is provided for demonstration only. A standard curve should be generated for each set of samples assayed.



pg/ml	O.D.	Average	Corrected
0	0.101 0.101	0.101	–
31.25	0.167 0.170	0.168	0.067
62.5	0.236 0.234	0.235	0.134
125	0.368 0.359	0.363	0.262
250	0.635 0.625	0.630	0.529
500	1.053 1.036	1.044	0.943
1000	1.736 1.717	1.727	1.626
2000	2.444 2.407	2.426	2.325

V. KIT COMPONENTS AND STORAGE

A. MATERIALS PROVIDED

Parts	Description	Size
Human VEGF R2 Microplate	96 well polystyrene microplate (12 strips of 8 wells) coated with an antibody against human VEGF R2.	1 plate
Human VEGF R2 Standard	Recombinant human VEGF R2 in a buffered protein base; lyophilized. Refer to the vial label for reconstitution volume.	2 vials
Human VEGF R2 Detection Antibody	Biotinylated human VEGF R2 antibody , lyophilized. Refer to the vial label for reconstitution volume.	1 vial
Calibrator Diluent (2×)	A 2× concentrated buffered diluent used to dilute standard and samples.	1 vial
Streptavidin-HRP A (200×)	200× Streptavidin conjugated to horseradish peroxidase.	1 vial
Reagent Diluent (10×)	A 10× concentrated buffered protein base used to dilute detection antibody and HRP.	1 vial
Normal Goat Serum	Diluted, heat-inactive normal goat serum	1 vial
Wash Buffer Concentrate (25×)	A 25× concentrated solution of buffered surfactant with preservatives.	1 vial
TMB Substrate	TMB ELISA Substrate Solution/TMB Substrate Solution.	1 vial
Stop Solution	2 N sulfuric acid.	1 vial
Plate Sealers	Adhesive strips.	3 strips

B. STORAGE

<table>
<tr><td>Unopened Kit</td><td colspan="2">Store at 2-8 °C. Do not use past kit expiration date.</td></tr>
<tr><td rowspan="8">Opened/ Reconstituted Reagents</td><td>Streptavidin-HRP A</td><td rowspan="5">May be stored for up to 1 month at 2-8 °C.*</td></tr>
<tr><td>Wash Buffer (1×)</td></tr>
<tr><td>TMB Substrate</td></tr>
<tr><td>Stop Solution</td></tr>
<tr><td>Normal Goat Serum</td></tr>
<tr><td>Standard</td><td>Prepare fresh for each assay.
Standards may be stored for up to 1 month at -20 °C.*</td></tr>
<tr><td>Detection Antibody</td><td>Aliquot and store for up to 1 month at -20 °C in a manual defrost freezer. *</td></tr>
<tr><td>Calibrator Diluent (2×)</td><td>May be stored for up to 1 month at 2-8 °C.*
Use and discard diluted Calibrator Diluent (1×). Prepare fresh for each assay.</td></tr>
<tr><td>Reagent Diluent (10×)</td><td>May be stored for up to 1 month at 2-8 °C.*
Use and discard diluted Reagent Diluent (1×). Prepare fresh for each assay.</td></tr>
<tr><td>Microplate Wells</td><td>Return unused wells to the foil pouch containing the desiccant pack, reseal along entire edge of zip-seal. May be stored for up to 1 month at 2-8 °C.*</td></tr>
</table>

* Provided this is within the expiration date of the kit.

C. OTHER SUPPLIES REQUIRED

- Microplate reader capable of measuring absorbance at 450 nm, with the correction wavelength set at 540 nm or 570 nm.
- Pipettes and pipette tips.
- Deionized or distilled water.
- Squirt bottle, manifold dispenser, or automated microplate washer.
- Test tubes for dilution of standards.
- 100 mL and 500 mL graduated cylinder.

D. PRECAUTION

- Some components in this kit contain a preservative which may cause an allergic skin reaction. Avoid breathing mist.
- The Stop Solution provided with this kit is an acid solution. Wear protective gloves, clothing, eye, and face protection. Wash hands thoroughly after handling.

VI. PREPARATION

A. SAMPLE COLLECTION AND STORAGE

Cell Culture Supernates - Remove particulates by centrifugation and assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles. Samples may require dilution with Calibrator Diluent (1×).

Serum - Use a serum separator tube (SST) and allow samples to clot for 30 minutes at room temperature before centrifugation for 15 minutes at 1000 × g. Remove serum and assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles. Samples may require dilution with Calibrator Diluent (1×).

B. SAMPLE PREPARATION

Human serum samples recommend a 10-fold dilution. A suggested 10-fold dilution is 30 µL of sample + 270 µL of Calibrator Diluent (1×). Optimal dilutions should be determined by the end user.

C. REAGENT PREPARATION

Note: *Bring all reagents to room temperature before use.*

Wash Buffer (1×) - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Dilute 20 mL of Wash Buffer Concentrate (25×) into deionized or distilled water to prepare 500 mL of Wash Buffer (1×).

Calibrator Diluent (1×) - Use deionized or distilled water to prepare Calibrator Diluent (1×).

Reagent Diluent (1×) - Use deionized or distilled water to prepare Reagent Diluent (1×).

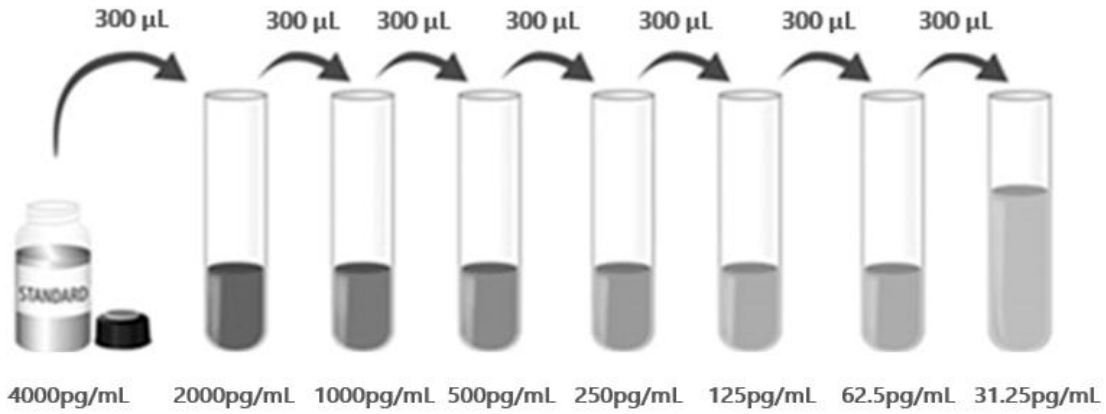
Detection Antibody (1×) - Centrifuge briefly before opening. Reconstitution volume refer to vial label to prepare Detection Antibody (100×). Allow the Detection Antibody to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions. Aliquot and store if needed. Dilute to Detection Antibody (1×) with Reagent Diluent (1×) with 2% heat inactivated Normal Goat Serum (NGS) (200 uL NGS per 10 mL solution). Prepare at least 15 minutes prior to use.

Streptavidin-HRP A (1×) - Centrifuge briefly before opening. Dilute to the working concentration specified on the vial label using Reagent Diluent (1×).

Human VEGF R2 Standard - Centrifuge briefly before opening. Refer to the vial label for the reconstitution volume*. This reconstitution produces a stock solution of 4000 pg/mL. Allow the standard to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions.

*If you have any question, please seek help from our Technical Support.

Pipette 300 µL of Calibrator Diluent (1×) into each tube. Use the stock solution to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The 2000 pg/mL standard serves as the high standard. The **Calibrator Diluent (1×)** serves as the zero standard (0 pg/mL).



D. TECHNICAL HINTS

- When mixing or reconstituting protein solutions, always avoid foaming.
- To avoid cross-contamination, change pipette tips between additions of each standard level, between sample additions, and between reagent additions. Also, use separate reservoirs for each reagent.
- It is recommended that the samples be pipetted within 15 minutes.
- To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary.
- TMB Substrate should remain colorless until added to the plate. Keep TMB Substrate protected from light. TMB Substrate should change from colorless to gradations of blue.
- Stop Solution should be added to the plate in the same order as the TMB Substrate. The color developed in the wells will turn from blue to yellow upon addition of the Stop Solution. Wells that are green in color indicate that the Stop Solution has not mixed thoroughly with the TMB Substrate.

VII.ASSAY PROCEDURE

Note: *Bring all reagents and samples to room temperature before use. It is recommended that all samples and standards be assayed in duplicate.*

1. Prepare all reagents and working standards as directed in the previous sections.
2. Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, and reseal.
3. Add 100 µL of standard and prepared sample per well. Cover with the adhesive strip provided. **Incubate for 2 hours at room temperature.** A plate layout is provided for a record of standards and samples assayed. (Serum samples may require dilution. See Sample Preparation section.)
4. Aspirate each well and wash, repeating the process three times for a total of four washes. Wash by filling each well with Wash Buffer (400 µL) using a squirt bottle, manifold dispenser, or autowasher. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.
5. Add 100 µL of the Detection Antibody (1×) diluted in Reagent Diluent (1×) with 2% NGS, to each well. Cover with a new adhesive strip and **incubate 2 hours at room temperature.**
6. Repeat the aspiration/wash as in step4.
7. Add 100 µL of the working dilution of Streptavidin-HRP A to each well. Cover the plate and **incubate for 20 minutes at room temperature. Avoid placing the plate in direct light.**
8. Repeat the aspiration/wash as in step 4.
9. Add 100 µL of TMB Substrate to each well. **Incubate for 30 minutes at room temperature. Avoid placing the plate in direct light.**
10. Add 50 µL of Stop Solution to each well. Gently tap the plate to ensure thorough mixing.
11. Determine the optical density of each well within 10 minutes, using a microplate reader set to 450 nm. If wavelength correction is available, set to 540 nm or 570 nm. If wavelength correction is not available, subtract readings at 540 nm or 570 nm from the readings at 450 nm. This subtraction will correct for optical

imperfections in the plate. Readings made directly at 450 nm without correction may be higher and less accurate.

12. **CALCULATION OF RESULTS**

Average the duplicate readings for each standard and sample and subtract the average zero standard optical density (O.D.).Create a standard curve by reducing the data using computer software capable of generating a four parameter logistic (4-PL) curve-fit. As an alternative, construct a standard curve by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis and draw a best fit curve through the points on the graph. The data may be linearized by plotting the log of the human VEGF R2 concentrations versus the log of the O.D. and the best fit line can be determined by regression analysis. This procedure will produce an adequate but less precise fit of the data.

If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

VIII. REFERENCES

1. Barleon, B. et al. (1994) J. Cell. Biochem. 54:56.

2. Barleon, B. et al. (1997) Cancer Res. 57:5421.

3. Terman, B.I. et al. (1991) Oncogene 6:1677.

4. Barleon, B. et al. (1994) J. Cell. Biochem. 54:56.

5. Barleon, B. et al. (1997) Cancer Res. 57:5421.

6. Skobe, M. et al. (1997) Nature Med. 3:1222.

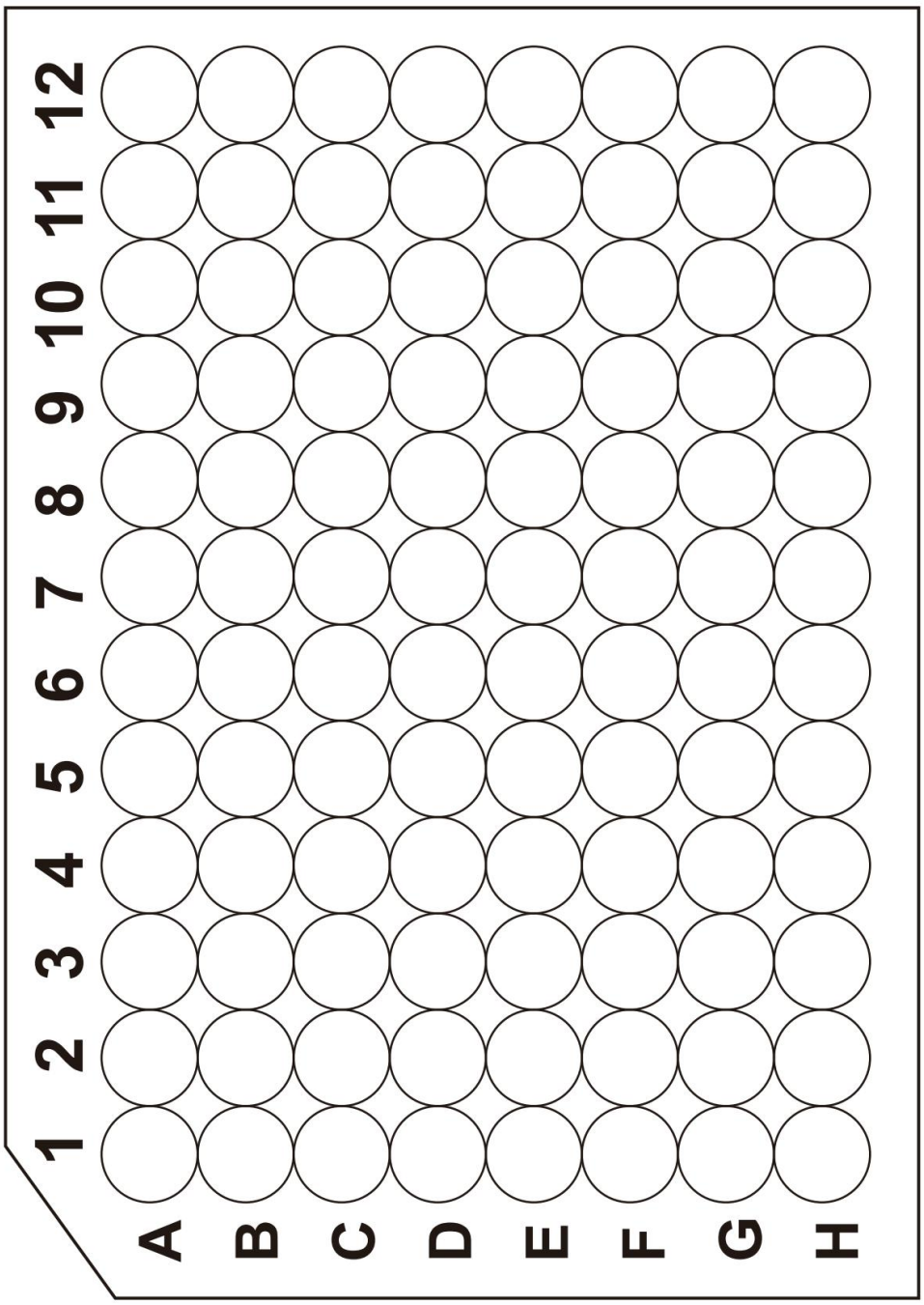
7. Brekken, R.A. et al. (2000) Cancer Res. 60:5117.

8. Albini, A. et al. (1996) Nature Med. 2:1371.

9. Morini, M. et al. (2000) Biochem. Biophys. Res. Commun. 273:267.

PLATE LAYOUT

Use this plate layout to record standards and samples assayed.



产品信息及操作手册

人 VEGF R2/KDR Valukine™ ELISA 试剂盒

目录号：VAL119

适用于定量检测天然和重组人 VEGF R2/KDR 的浓度

科研专用，不可用于临床诊断

Bio-Techne China Co. Ltd

P: +86 (21) 52380373 P: 8009881270 F: +86 (21) 52381001

info.cn@bio-techne.com

有效期详见试剂盒包装标签

Novus 试剂盒确保在你收货日期 3 个月内有效

版本号 202409.4

目录

I. 背景

血管内皮生长因子受体 2（VEGF R2）在人体中被称为激酶插入区受体（KDR），在小鼠中被称为胎儿肝脏激酶-1 受体（Flk-1），是受体酪氨酸激酶 (RTKs) III 类亚家族成员，此家族还包括 VEGF R1 (Flt-1)和 VEGF R3 (Flt-4)。这三种受体的胞外结构域均含有 7 个免疫球蛋白样的重复序列，胞内结构域均含有激酶插入区。它们优先表达于血管内膜或穿透实体肿瘤的增生的内皮细胞 (1)。与 VEGF R1 相比，VEGF R2 更广泛的分布和表达在所有血管源性内皮细胞中(2)。

VEGF R2 与 VEGF 的结合具有高亲和力 (3)。体外研究进一步证实了 PlGF/VEGF 异二聚体与可溶性 VEGF R2 具有较高的亲和力，但 PlGF 同型二聚体却不能结合此受体(4)。与可溶性 VEGF R1 相比，可溶性 VEGF R2 在与表达 VEGF R1 和 VEGF R2 的人内皮细胞结合方面无法与 VEGF 竞争。可溶性 VEGF R2 只能部分抑制细胞迁移，而可溶性 VEGF R1 几乎可以完全阻断 VEGF 诱导的细胞增殖和迁移(5)。VEGF 与可溶性 VEGF R2 而非 VEGFR1 结合也依赖于肝素(5)。

VEGF/VEGF R2 信号通路在肿瘤血管生成及其它涉及“病理性血管生成”的疾病中发挥重要作用。通过阻断抗体致使 VEGF R2 失活从而破坏血管生成，也能阻止肿瘤细胞侵袭(6,7)。HIV-1 Tat 蛋白是通过 VEGF R2 作用于血管内皮细胞来诱导血管生成的(8)。Tat 蛋白能特异性结合并激活 VEGF R2。Tat 蛋白诱导的血管生成能够被阻断 VEGF R2 的药物所阻断(8, 9)。

II. 概述

A. 检测原理

本实验采用双抗体夹心 ELISA 法。抗人 VEGF R2 捕获抗体包被于微孔板上，经过孵育，样品和标准品中的人 VEGF R2 会与固定在板上的抗体结合，游离的成分被洗去；接着加入生物素化的抗人 VEGF R2 检测抗体进行孵育，洗涤去除未结合的物质后，加入链霉亲和素标记的辣根过氧化物酶(Streptavidin-HRP)孵育。洗涤后，加入 TMB 底物溶液（显色剂），避光显色。溶液颜色与结合的目标蛋白成正比；加入终止液；用酶标仪测定吸光度。

B. 检测局限

- **仅供科研使用，不可用于体外诊断；**
- 该试剂盒适用于细胞培养上清样本和人血清样本；
- 请在试剂盒有效期内使用；
- 不同试剂盒及不同批号试剂盒的组分不能混用；
- 样本值若大于标准曲线的最高值，应将样本用标准品稀释液（1×）稀释后重新检测；
- 检测结果的不同可由多种因素引起，包括实验人员的操作、移液器的使用方式、洗板技术、反应时间或温度、试剂盒的效期等。

III. 优势

A. 精确度

板内精确度（同一板内不同孔间的精确度）

已知浓度的三个样本，在同一板内分别检测 20 次，以确定板内精确度。

板间精确度（不同板之间的精确度）

已知浓度的三个样本，在不同板中分别检测 20 次，以确定板间精确度。

	板内精确度			板间精确度		
样本	1	2	3	1	2	3
平均值（pg/mL）	113.5	218.9	789.8	116.0	223.4	799.5
标准差	3.0	11.8	18.3	5.4	11.1	29.0
CV%	2.7	5.4	2.3	4.6	5.0	3.6

B. 回收率

在细胞培养基样本中掺入检测范围内不同水平的人 VEGF R2，测定其回收率。回收率范围在 114.2-118.3%，平均回收率在 116.1%。

C. 灵敏度

人 VEGF R2 的最低可测剂量（MDD）一般小于 6.8 pg/mL。

MDD 是根据 20 个重复的零标准品孔的吸光度值的平均值加两倍标准差计算得到的相对应浓度。

D. 校正

此 ELISA 试剂盒经由 R&D Systems®生产的 NS0 表达的高纯度重组人 VEGF R2 蛋白所校正。

E. 线性

不同的样本中含有或掺入高浓度的人 VEGF R2，然后用标准品稀释液（1×）将样本稀释到检测范围内，测定其线性。

稀释倍数	平均期待值（%）	范围（%）
1:2	99.7	95.0 -104.0
1:4	102.4	95.4 -106.0
1:8	102.2	92.7 -108.3
1:16	95.3	81.1 -104.8

F. 样本值

人血清样本 - 使用本试剂盒检测了 10 份人血清样本中人 VEGF R2 的水平。所有样本的检测值在 7196-12321 pg/mL 之间，平均值为 9279 pg/mL。

G. 特异性

此 ELISA 法可检测天然及重组人 VEGF R2 蛋白。对制备的 50 ng/mL 的下列因素进行了测定，无交叉反应或干扰。

Recombinant human：	**Recombinant mouse：**
VEGF-D	$VEGF_{164}$
VEGF R3/Fc Chimera	

含 25 ng/mL 重组人 VEGF R1/Fc Chimera 的样本检测值为 40 pg/mL（0.16%的交叉反应）

含 12.5 ng/mL 重组小鼠 VEGF R2/Fc Chimera 的样本检测值为 46 pg/mL（0.36%的交叉反应）

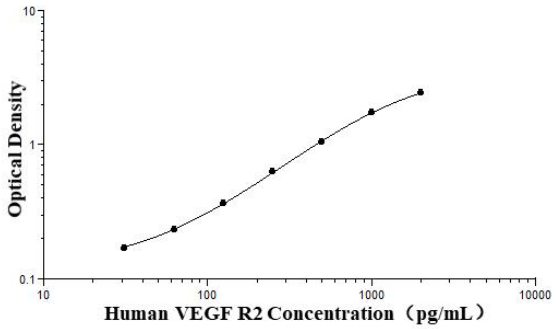
重组人 $VEGF_{121}$ 和重组小鼠 $VEGF_{120}$ 均无交叉反应，但浓度大于 1.6 ng/mL 时，对实验有干扰。

重组人 $VEGF_{165}$ 无交叉反应，但浓度大于 3.1 ng/mL 时，对实验有干扰。

IV. 实验

标准曲线实例

该标准曲线数据仅供参考，每次实验应绘制其对应的标准曲线。



pg/ml	O.D.	Average	Corrected
0	0.101 0.101	0.101	–
31.25	0.167 0.170	0.168	0.067
62.5	0.236 0.234	0.235	0.134
125	0.368 0.359	0.363	0.262
250	0.635 0.625	0.630	0.529
500	1.053 1.036	1.044	0.943
1000	1.736 1.717	1.727	1.626
2000	2.444 2.407	2.426	2.325

V. 试剂盒组成及储存

A. 试剂盒组成

组成	描述	规格
Human VEGF R2 Microplate	包被抗人 VEGF R2 抗体的 96 孔聚苯乙烯板，8 孔×12 条	1 块板
Human VEGF R2 Standard	人 VEGF R2 标准品（冻干粉），参考瓶标签进行重溶	2 瓶
Human VEGF R2 Detection Antibody	生物素化的抗人 VEGF R2 检测抗体，冻干粉，参考瓶标签进行重溶	1 瓶
Calibrator Diluent (2×)	浓缩的标准品稀释液（2×）用于稀释样品和标准品	1 瓶
Streptavidin-HRP A (200×)	200×浓缩的链霉亲和素标记的 HRP	1 瓶
Reagent Diluent (10×)	浓缩的试剂稀释液（10 ×）用于稀释检测抗体和 HRP	1 瓶
Normal Goat Serum	稀释的热灭活正常山羊血清	1 瓶
Wash Buffer Concentrate（25×）	浓缩洗涤缓冲液（25×）	1 瓶
TMB Substrate	TMB ELISA 底物溶液/TMB 底物溶液	1 瓶
Stop Solution	终止液	1 瓶
Plate Sealers	封板膜	3 张

B. 试剂盒储存

<table>
<tr><td>未开封试剂盒</td><td colspan="2">2-8℃储存；请在试剂盒有效期内使用</td></tr>
<tr><td rowspan="9">已打开，稀释或重溶的试剂</td><td>链霉亲和素-HRPA</td><td rowspan="5">2-8℃储存，最多 30 天*</td></tr>
<tr><td>洗涤缓冲液（1×）</td></tr>
<tr><td>TMB 底物溶液</td></tr>
<tr><td>山羊血清</td></tr>
<tr><td>终止液</td></tr>
<tr><td>标准品</td><td>使用时新鲜配制*
标准品-20℃储存，最多 30 天*</td></tr>
<tr><td>检测抗体</td><td>分装，-20℃储存，最多 30 天*</td></tr>
<tr><td>标准品稀释液(2×)</td><td>2-8℃储存，最多 30 天*
请每次使用新鲜配制的 1×标准品稀释液，多余的丢弃</td></tr>
<tr><td>试剂稀释液（10×）</td><td>2-8℃储存，最多 30 天*
请每次使用新鲜配制的 1×试剂稀释液，多余的丢弃</td></tr>
<tr><td>包被的微孔板条</td><td>将未用的板条放回带有干燥剂的铝箔袋内，密封：2-8℃储存，最多 30 天*</td></tr>
</table>

*必须在试剂盒有效期内

C. 实验所需自备试验器材

- 酶标仪（可测量 450 nm 检测波长的吸收值及 540 nm 或 570 nm 校正波长的吸收值）
- 高精度加液器及一次性吸头
- 蒸馏水或去离子水
- 洗瓶（喷瓶）、多通道洗板器或自动洗板机
- 用来稀释标准品的试管
- 100 mL 及 500 mL 量筒

D. 注意事项

- 试剂盒中的一些组分含有防腐剂，可能引起皮肤过敏反应，避免吸入。
- 试剂盒中的终止液是酸性溶液，使用时请做好眼睛、手、面部及衣服的防护。使用后请彻底洗手。

VI. 实验前准备

A. 样品收集及储存

细胞培养上清液：颗粒物应离心去除；立刻检测样本。样本收集后若不及时检测，需按一次使用量分装，冻存于≤-20℃冰箱内，避免反复冻融。样本可能需要用标准品稀释液（1×）稀释。

血清样本：用血清分离管(SST)分离血清。使血样室温凝集30分钟，然后1000 × g离心15分钟。吸取血清样本之后即刻用于检测，或者分装，≤-20℃贮存备用。避免反复冻融。样本可能需要用标准品稀释液（1×）稀释。

B. 样本准备工作

人血清样本建议用标准品稀释液（1×）10倍稀释后进行检测，即30 µL血清+270 µL标准品稀释液（1×）。最佳稀释度应由最终用户确定。

C. 检测前准备工作

使用前请将所有试剂放置于室温

洗涤液（1×）：从冰箱中取出的浓缩洗涤液可能有结晶，属于正常现象；放置室温，轻摇混匀，待结晶完全溶解后再配制洗涤液。可将 20 mL 浓缩洗涤液（25×）用蒸馏水或去离子水稀释配制成 500 mL 工作浓度的洗涤液（1×）。

标准品稀释液（1×）：使用蒸馏水或去离子水稀释配制成标准品稀释液（1×）。

试剂稀释液（1×）：使用蒸馏水或去离子水稀释配制成试剂稀释液（1×）。

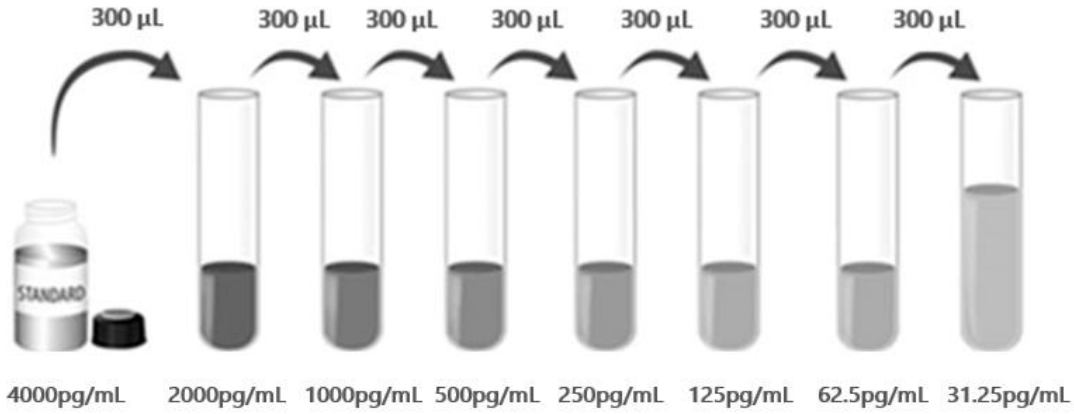
检测抗体（1×）：开盖前请瞬时离心。参考检测抗体瓶标签重溶冻干粉，制备检测抗体（100×）。轻轻震摇至少 15 分钟，其充分溶解。如有需要分装保存。再用添加了 2%热灭活的正常山羊血清（NGS）（每 10 mL 溶液加 200 µL NGS）的试剂稀释液 (1×) 稀释至检测抗体（1×），至少在使用前 15 分钟准备。

链霉亲和素- HRP A（1×）：开盖前请瞬时离心。用试剂稀释液（1×）将链霉亲和素-HRP A（200×）稀释至工作浓度。

人 VEGF R2 标准品：开盖前请瞬时离心。参照冻干标准品瓶身注明的方式重溶冻干标准品*，得到浓度为 4000 pg/mL 标准品母液。轻微震摇至少 15 分钟，使其充分溶解。

***如有疑问，请咨询我们的技术支持。**

每个稀释管中加入 300 μL 标准品稀释液（1×）。将标准品母液参照下图做系列稀释，每管须充分混匀后再移液到下一管。2000 pg/mL 管作标准曲线最高点，标准品稀释液（1×）可用作标准品零点（0 pg/mL）。



D. 技术小提示

- 当混合或重溶蛋白液时，尽量避免起沫；
- 为了避免交叉污染，配制不同浓度标准品、上样、加不同试剂都需要更换枪头。另外不同试剂请分别使用不同的移液槽；
- 建议 15 分钟内完成一块板的上样；
- 每次孵育时，正确使用封板膜可保证结果的准确性；
- TMB 底物溶液在上板前应为无色，请避光保存；加入微孔板后，将由无色变成不同深度的蓝色；
- 终止液上板顺序应同 TMB 底物溶液上板顺序一致；加入终止液后，孔内颜色由蓝变黄；若孔内有绿色，则表明孔内液体未混匀，请充分混合。

VII.操作步骤

使用前请将所有试剂和样本放置于室温，建议所有的实验样本和标准品做复孔检测。

1. 按照上一节的说明，准备好所有需要的试剂和标准品；
2. 从已平衡至室温的密封袋中取出微孔板，未用的板条请放回铝箔袋内，重新封口；
3. 分别将不同浓度标准品和实验样本加入相应孔中，每孔 100 µL。用封板膜封住反应孔，**室温孵育 2 小时**。说明书提供了一张 96 孔模板图，可用于记录标准品和试验样本的板内位置；（血清样本需要稀释，详情参见样本制备部分。）
4. 将板内液体吸去，使用洗瓶、多通道洗板器或自动洗板机洗板。每孔加洗涤液 400 µL，然后将板内洗涤液吸去。重复操作 3 次，共 4 次。每次洗板尽量吸去残留液体会有助于得到好的实验结果。最后一次洗板结束，请将板内所有液体吸干或将板倒置，在吸水纸拍干所有残留液体；
5. 在每个微孔内加入 100 µL 用含 2%NGS 的试剂稀释液（1×）配制的检测抗体（1×）。用封板膜封住反应孔，**室温孵育 2 小时**；
6. 重复第 4 步洗板操作；
7. 在每个微孔内加入 100 µL 稀释好的链霉亲和素- HRP A 工作液。用封板膜封住反应孔，**室温孵育 20 分钟，注意避光；**
8. 重复第 4 步洗板操作；
9. 在每个微孔内加入 100 µL TMB 底物溶液，**室温孵育 30 分钟，注意避光；**
10. 在每个微孔内加入 50 µL 终止液，请轻拍微孔板，使溶液混合均匀。
11. 加入终止液后 10 分钟内，使用酶标仪测量 450 nm 的吸光度值，设定 540 nm 或 570 nm 作为校正波长。如果波长校正不可用，以 450 nm 的读数减去 540 nm 或 570 nm 的读数。这种减法将校正酶标板上的光学缺陷。没有校正而直接在 450 nm 处进行的读数可能会更高且更不准确；
12. **计算结果：**将每个标准品和样品的复孔吸光值取平均值，然后减去零标准品平均 OD 值（O.D.），使用计算机软件作四参数逻辑（4-PL）曲线拟合创建标准曲线。另一替代方法是，通过绘制 y 轴上每个标准品的平均吸光值与 x 轴上的浓度来构建标准曲线，并通过图上的点绘制最佳拟合曲线。数据可以通过绘制人 VEGF R2 浓度的对数与 O.D.的对数来线性化，并且最佳拟合线可以通过回归分析来确定。该程序将产生足够但不太精确的数据拟合。

    从标准曲线读取的浓度必须乘以稀释倍数。

VIII. 参考文献

1. Barleon, B. et al. (1994) J. Cell. Biochem. 54:56.

2. Barleon, B. et al. (1997) Cancer Res. 57:5421.

3. Terman, B.I. et al. (1991) Oncogene 6:1677.

4. Barleon, B. et al. (1994) J. Cell. Biochem. 54:56.

5. Barleon, B. et al. (1997) Cancer Res. 57:5421.

6. Skobe, M. et al. (1997) Nature Med. 3:1222.

7. Brekken, R.A. et al. (2000) Cancer Res. 60:5117.

8. Albini, A. et al. (1996) Nature Med. 2:1371.

9. Morini, M. et al. (2000) Biochem. Biophys. Res. Commun. 273:267.

96 孔模板图

请使用 96 孔模板图来记录标准品及样本在板内的位置