PRODUCT INFORMATION & MANUAL

Human VEGF Valukine™ ELISA

Catalog Number: VAL106

For the quantitative determination of natural and recombinant human VEGF concentrations

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

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

Version 202606.6

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I. BACKGROUND

Vascular endothelial growth factor (VEGF or VEGF-A), also known as vascular permeability factor (VPF), is a potent mediator of both angiogenesis and vasculogenesis in the fetus and adult (1-3). It is a member of the PDGF family that is characterized by the presence of eight conserved cysteine residues in a cystine knot structure and the formation of antiparallel disulfide-linked dimers (4). Humans express alternately spliced isoforms of 121, 145, 165, 183, 189, and 206 amino acids (aa) in length (4). VEGF165 appears to be the most abundant and potent isoform, followed by VEGF121 and VEGF189 (3, 4). Isoforms other than VEGF121 contain basic heparin-binding regions and are not freely diffusible (4). Human VEGF165 shares 88% aa sequence identity with corresponding regions of mouse and rat VEGF. VEGF is expressed in multiple cells and tissues including skeletal and cardiac muscle (5, 6), hepatocytes (7), osteoblasts (8), neutrophils (9), macrophages (10), keratinocytes (11), brown adipose tissue (12), $CD34^+$ stem cells (13), endothelial cells (14), fibroblasts, and vascular smooth muscle cells (15). VEGF expression is induced by hypoxia and cytokines such as IL-1, IL-6, IL-8, oncostatin M and TNF-α (3, 4, 9, 16). VEGF isoforms are differentially expressed during development and in the adult (3).

VEGF dimers bind to two related receptor tyrosine kinases, VEGF R1 (also called Flt-1) and VEGF R2 (Flk-1/KDR), and induce their homodimerization and autophosphorylation (3, 4, 7, 17, 18). These receptors have seven extracellular immunoglobulin-like domains and an intracellular split tyrosine kinase domain. They are expressed on vascular endothelial cells and a range of non-endothelial cells. Although VEGF affinity is highest for binding to VEGF R1, VEGF R2 appears to be the primary mediator of VEGF angiogenic activity (3, 4). VEGF165 also binds the semaphorin receptor, neuropilin-1, which promotes complex formation with VEGF R2 (19).

VEGF is best known for its role in vasculogenesis. During embryogenesis, VEGF regulates the proliferation, migration, and survival of endothelial cells (3, 4), thus regulating blood vessel density and size, but playing no role in determining vascular patterns. VEGF promotes bone formation through osteoblast and chondroblast recruitment and is also a monocyte chemoattractant (20-22). After birth, VEGF maintains endothelial cell integrity and is a potent mitogen for micro- and macro-vascular endothelial cells. In adults, VEGF functions mainly in wound healing and the female reproductive cycle (3). In diseased tissues, VEGF promotes vascular permeability. It is thus thought to contribute to tumor metastasis by promoting both extravasation and tumor angiogenesis (23, 24). Various strategies have been employed therapeutically to antagonize VEGF-mediated tumor angiogenesis (25). Circulating VEGF levels correlate with disease activity in autoimmune diseases such as rheumatoid arthritis, multiple sclerosis and systemic lupus erythematosus (26).

II. OVERVIEW

A. PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. An antibody specific for human VEGF has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any human VEGF present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked antibody specific for human VEGF is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, TMB substrate (Chromogenic agent) is added to the wells and color develops in proportion to the amount of human VEGF 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, human serum and human plasma.
- 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 RD5K/RD6U 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 forty separate assays to assess inter-assay precision.

CELL CULTURE SUPERNATE ASSAY

	Intra-assay Precision			**Inter-assay Precision**		
Sample	1	2	3	1	2	3
Mean (pg/mL)	29.1	123	531	32.8	128	495
Standard Deviation	1.9	5.0	18.4	2.8	6.4	33.0
CV%	6.5	4.1	3.5	8.5	5.0	6.7

SERUM/PLASMA ASSAY

	Intra-assay Precision			**Inter-assay Precision**		
Sample	1	2	3	1	2	3
Mean (pg/mL)	53.7	235	910	64.5	250	1003
Standard Deviation	3.6	10.6	46.2	5.7	17.4	61.7
CV%	6.7	4.5	5.1	8.8	7.0	6.2

B. RECOVERY

The recovery of human VEGF spiked to levels throughout the range of the assay in various matrices was evaluated.

Sample Type	Average % Recovery	Range (%)
Cell culture media (n=5)	102	95-111
Human serum (n=5)	102	92-115
Human EDTA plasma (n=5)	97	82-113
Human heparin plasma (n=5)	93	82-102
Human citrate plasma (n=5)	100	88-113

C. SENSITIVITY

Using Calibrator Diluent RD5K the minimum detectable dose (MDD) of human VEGF is typically less than 5.0 pg/mL. Using Calibrator Diluent RD6U the MDD is typically less than 9.0 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 *Sf 21*-expressed recombinant human $VEGF_{165}$ produced at R&D Systems.

The NIBSC/WHO $VEGF_{165}$ 1st WHO Reference Reagent 02/286 (recombinant human DNA) was evaluated in this kit. The dose response curve of the reference reagent 02/286 parallels the Valukine standard curve. To convert sample values obtained with the Valukine Human VEGF kit to approximate NIBSC/WHO 02/286 Units, use the equation below.

NIBSC/WHO (02/286) approximate value (U/mL) = 0.002 × Valukine VEGF value (pg/mL)

Note: *Based on data generated in April 2011.*

E. LINEARITY

To assess the linearity of the assay, samples were containing or spiked with high concentrations of human VEGF in various matrices and diluted with calibrator diluent RD5K/RD6U to produce samples with values within the dynamic range of the assay.

Dilution		Cell culture media (n=5)	Human serum (n=5)	Human EDTA plasma (n=5)	Human heparin Plasma (n=5)	Human citrate plasma (n=5)
1:2	Average % of Expected	98	97	97	94	95
	Range (%)	94-100	91-103	82-107	87-99	90-100
1:4	Average % of Expected	96	97	98	93	94
	Range (%)	93-99	93-104	91-106	85-98	88-99
1:8	Average % of Expected	93	96	96	92	92
	Range (%)	88-102	93-103	89-106	85-101	85-97
1:16	Average % of Expected	93	94	94	94	92
	Range (%)	88-105	91-101	84-106	83-103	85-98

F. SAMPLE VALUES

Human serum/plasma - Samples from apparently healthy volunteers were evaluated for the presence of human VEGF in this assay. No medical histories were available for the donors used in this study.

Sample Type	Mean of Detectable (pg/mL)	% Detectable	Range (pg/mL)
Human serum (n=37)	220	100	62-707
Human EDTA plasma (n=37)	61	24	ND-115
Human heparin plasma (n=37)	41	22	ND-55
Human citrate plasma (n=37)	—	0	ND

Cell Culture Supernates:

Human peripheral blood mononuclear cells (10×10^6 cells/mL) were cultured in RPMI supplemented with 5% fetal bovine serum, 50 µM β-mercaptoethanol, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin sulfate. The cells were cultured unstimulated or stimulated with 10 µg/mL PHA for 5 days. Aliquots of the cell culture supernates were removed, assayed for levels of human VEGF, and measured 87.1 pg/mL and 1041 pg/mL, respectively.

PC-3 human prostate cancer cells were cultured in RPMI supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin sulfate. The cells were cultured unstimulated or stimulated with 60 nM of PMA for 24 hours. Aliquots of the cell culture supernates were removed, assayed for levels of human VEGF, and measured 3373 pg/mL and 3652 pg/mL, respectively.

JEG-3 human epithelial choriocarcinoma cells were cultured in MEM + NEAA supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin sulfate. The cells were cultured unstimulated or stimulated with 100 ng/mL of LPS for 1 day. Aliquots of the cell culture supernates were removed, assayed for levels of human VEGF, and measured 1538 pg/mL and 1938 pg/mL, respectively.

G. SPECIFICITY

This assay recognizes natural and recombinant human VEGF. This assay also recognizes recombinant human $VEGF_{165b}$.

The factors listed below were prepared at 50 ng/mL in calibrator diluent RD5K/RD6U and assayed for cross-reactivity. Preparations of the following factors at 50 ng/mL in a mid-range VEGF control were assayed for interference. The following factors showed no cross-reactivity or interference.

Recombinant human:	**Recombinant mouse:**	**Recombinant zebrafish:**
PDGF-AA	PDGF-CC	VEGF
PDGF-AB	PlGF-2	**Natural proteins:**
PDGF-BB	$VEGF_{120}$	human PDGF
PDGF-CC	$VEGF_{164}$	porcine PDGF
PDGF-DD	VEGF R3	**Recombinant Bovine:**
PlGF	**Recombinant rat:**	$VEGF_{164}$
PlGF-2	PDGF-AA	
$VEGF_{165}$/PlGF	PDGF-AB	
$VEGF\text{-}B_{167}$	PDGF-BB	
VEGF-C	$VEGF_{164}$	
VEGF-D		
VEGF R3		

The factors listed below were prepared at 10 ng/mL in calibrator diluent RD5K/RD6U and assayed for cross-reactivity. Preparations of the following factors at 10 ng/mL in a mid-range VEGF control were assayed for interference. The following factors showed no cross-reactivity or interference.

Recombinant bovine:
$VEGF_{164}$

VEGF-related factors showing cross-reactivity or interference.

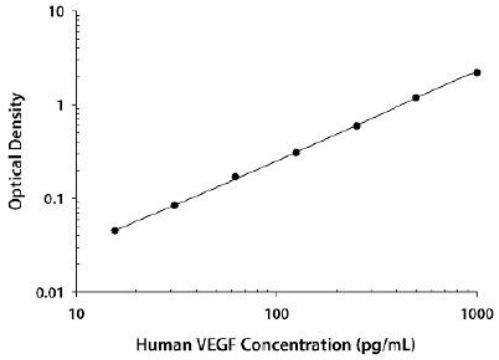
Recombinant human VEGF R1/Flt-1	Interference at levels ⩾ 500 pg/mL
Recombinant human VEGF R2/KDR	Interference at levels ⩾ 2000 pg/mL
Recombinant mouse VEGF R1/Flk-1	Interference at levels ⩾ 500 pg/mL
Recombinant mouse VEGF R2/KDR	Interference at levels ⩾ 4000 pg/mL
Recombinant canine VEGF	Cross-reacts approximately 67%
Recombinant feline VEGF	Cross-reacts approximately 82%

IV. EXPERIMENT

EXAMPLE STANDARD

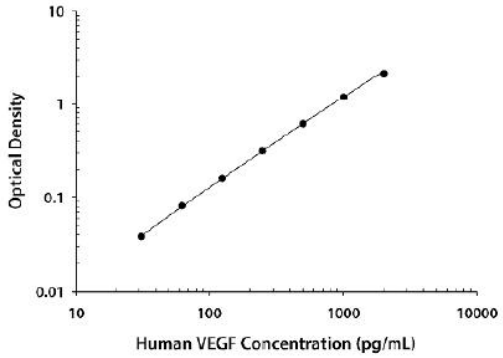
These standard curves are provided for demonstration only. A standard curve should be generated for each set of samples assayed.

CELL CULTURE SUPERNATE ASSAY



(pg/mL)	O.D.	Average	Corrected
0	0.074 0.076	0.075	—
15.6	0.118 0.121	0.120	0.045
31.3	0.159 0.159	0.159	0.084
62.5	0.242 0.246	0.244	0.169
125	0.378 0.384	0.381	0.306
250	0.666 0.669	0.668	0.593
500	1.258 1.263	1.260	1.185
1000	2.233 2.302	2.268	2.193

SERUM/PLASMA ASSAY



(pg/mL)	O.D.	Average	Corrected
0	0.068 0.071	0.070	—
31.3	0.107 0.110	0.108	0.038
62.5	0.149 0.153	0.151	0.081
125	0.230 0.230	0.230	0.160
250	0.377 0.387	0.382	0.312
500	0.657 0.699	0.678	0.608
1000	1.261 1.281	1.271	1.201
2000	2.159 2.246	2.202	2.132

V. KIT COMPONENTS AND STORAGE

A. MATERIALS PROVIDED

Parts	Description	Size
Human VEGF Microplate	96 well polystyrene microplate (12 strips of 8 wells) coated with an antibody against human VEGF	1 plate
Human VEGF Conjugate	Solution of antibody against human VEGF conjugated to horseradish peroxidase	1 vial
Human VEGF Standard	Recombinant human VEGF in a buffered protein base; lyophilized. Refer to the vial label for reconstitution volume.	2 vials
Assay Diluent RD1W	A buffered protein base with preservatives.	1 vial
Calibrator Diluent RD5K	A buffered protein base with preservatives. *For cell culture supernate samples.*	1 vial
Calibrator Diluent RD6U	Animal serum with preservatives. *For serum/plasma samples. May contain a precipitate.*	1 vial
Wash Buffer Concentrate (25×)	A 25× concentrated solution of buffered surfactant	1 vial
TMB Substrate	TMB ELISA Substrate Solution/TMB Substrate Solution	2 vials
Stop Solution	2 N sulfuric acid	1 vial
Plate Sealers	Adhesive strip	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>Wash Buffer (1×)</td><td rowspan="7">May be stored for up to 1 month at 2-8°C.*</td></tr>
<tr><td>Stop Solution</td></tr>
<tr><td>Conjugate</td></tr>
<tr><td>TMB Substrate</td></tr>
<tr><td>Assay Diluent RD1W</td></tr>
<tr><td>Calibrator Diluent RD5K</td></tr>
<tr><td>Calibrator Diluent RD6U</td></tr>
<tr><td>Standard</td><td>Discard the VEGF stock solution and dilutions after 4 hours. Use a fresh standard 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.
- 500 mL graduated cylinder.

D. PRECAUTION

- Calibrator Diluent RD6U contains sodium azide which may react with lead and copper plumbing to form explosive metallic azides. Flush with large volumes of water during disposal.
- VEGF is detectable in saliva. Take precautionary measures to prevent contamination of the kit reagents while running the assay.
- The Stop Solution provided with this kit is an acid solution.
- Some components in this kit contain a preservative which may cause an allergic skin reaction. Avoid breathing mist.
- Wear protective gloves, clothing, eye, and face protection. Wash hands thoroughly after handling.

VI. PREPARATION

A. SAMPLE COLLECTION AND STORAGE

The sample collection and storage conditions listed below are intended as general guidelines. Sample stability has not been evaluated.

Cell Culture Supernates - Cell culture supernates should contain at least 1% fetal bovine serum for stability of the VEGF. 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 RD5K.

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 RD6U.

Plasma - Collect plasma using EDTA, heparin, or citrate as an anticoagulant. Centrifuge for 15 minutes at 1000 × g within 30 minutes of collection. Assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles. Samples may require dilution with Calibrator Diluent RD6U.

B. REAGENT PREPARATION

Note: Bring all reagents to room temperature before use.

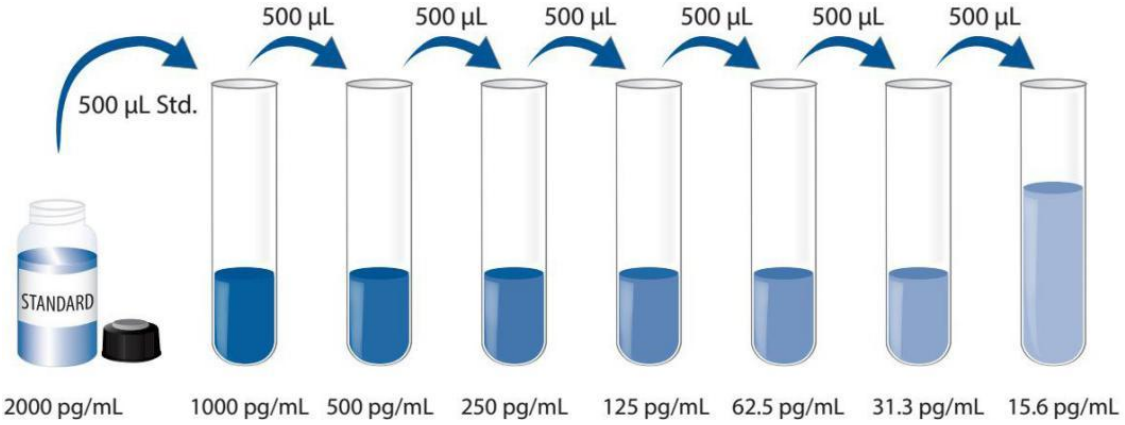
Wash Buffer (1×) - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Dilute 20 mL of Wash Buffer Concentrate (25×) into deionized or distilled water to prepare 500 mL of Wash Buffer (1×).

Human VEGF Standard - **Refer to the vial label for the reconstitution volume.**

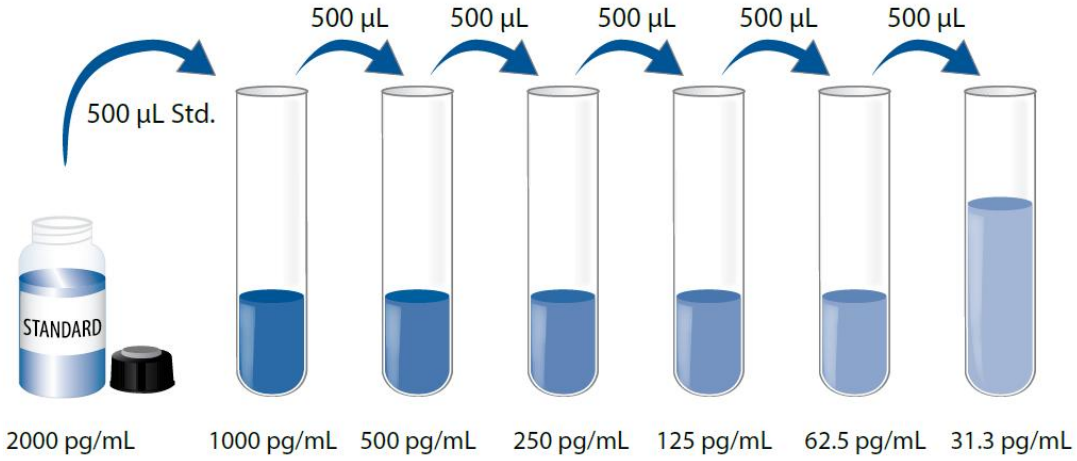
Reconstitute the Human VEGF Standard with Calibrator Diluent RD5K (*for cell culture supernate samples*) or Calibrator Diluent RD6U (*for human serum/plasma samples*). Calibrator Diluent RD6U may contain a precipitate. Mix well before and during use. This reconstitution produces a stock solution of 2000 pg/mL. Allow the standard to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions.

For Cell Culture Supernate Samples: Use polypropylene tubes. Pipette 500 µL of Calibrator Diluent RD5K into each tube. Use the stock solution to produce a dilution

series (below). Mix each tube thoroughly before the next transfer. The 1000 pg/mL dilution serves as the high standard. Calibrator Diluent RD5K serves as the zero standard (0 pg/mL).



For Serum/Plasma Samples: Use polypropylene tubes. Pipette 500 µL of Calibrator Diluent RD6U 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. Calibrator Diluent RD6U serves as the zero standard (0 pg/mL)



C. 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. **For Cell Culture Supernate Samples:** Add 50 µL of Assay Diluent RD1W to each well. **For Human Serum/Plasma Samples:** Add 100 µL of Assay Diluent RD1W to each well.
4. **For Cell Culture Supernate Samples:** Add 200 µL of standard or sample per well. **For Human Serum/Plasma Samples:** Add 100 µL of standard or sample per well. Cover with the adhesive strip provided and **incubate for 2 hours at room temperature.** A plate layout is provided to record the standards and samples assayed.
5. Aspirate each well and wash, repeating the process twice times for a total of three 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.
6. Add 200 µL of human VEGF Conjugate to each well. Cover with a new adhesive strip. **Incubate for 2 hours at room temperature.**
7. Repeat the aspiration/wash as in step 5.
8. Add 200 µL of TMB Substrate to each well. **Protect from light**.

   For Cell Culture Supernate Samples: Incubate for 20 minutes at room temperature.

   For Human Serum/Plasma Samples: Incubate for 25 minutes at room temperature.
9. Add 50 µL of Stop Solution to each well. Gently tap the plate to ensure thorough mixing.
10. 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.

11. **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 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.

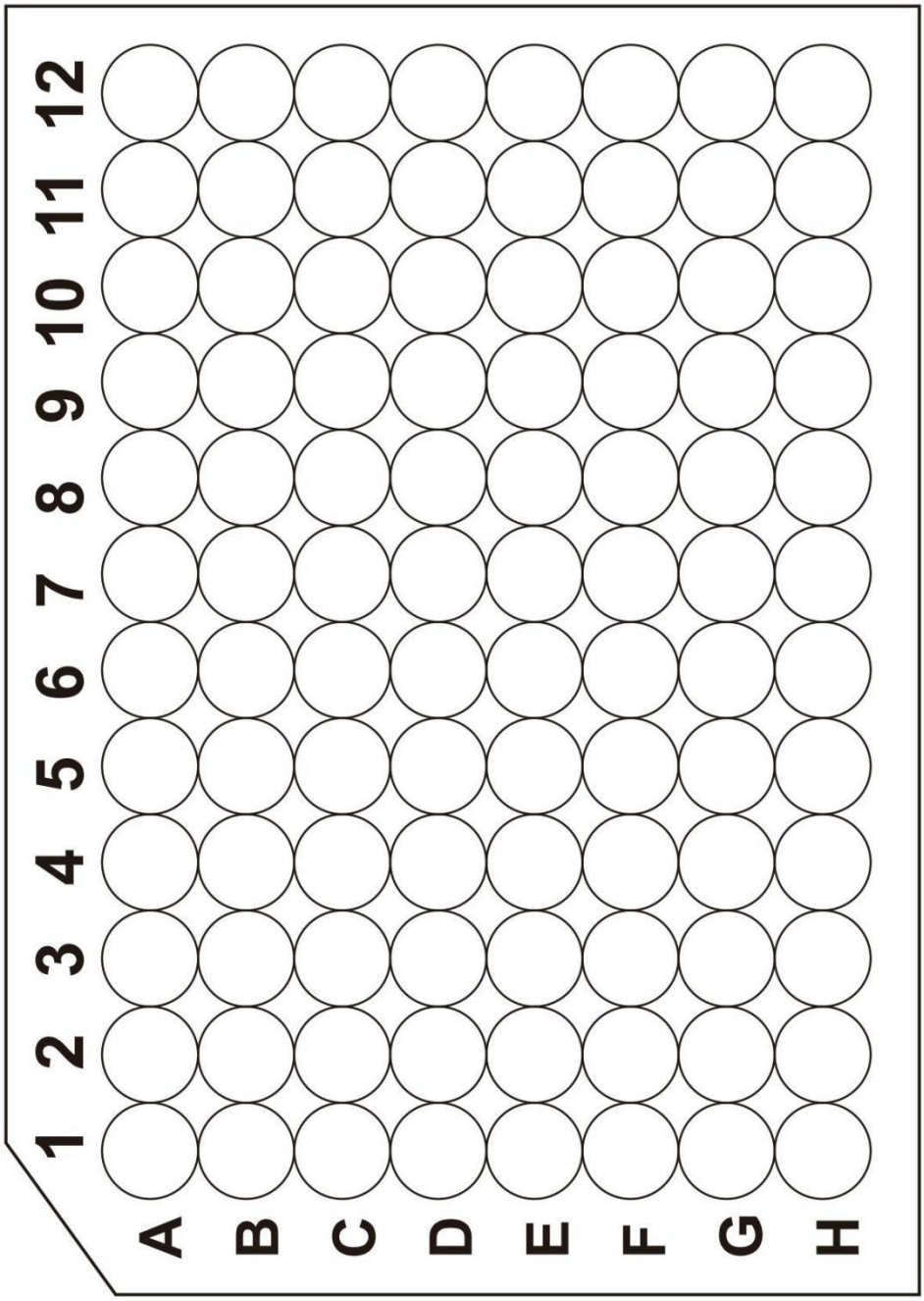
    If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

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PLATE LAYOUT

Use this plate layout to record standards and samples assayed.



产品信息及操作手册

人 VEGF Valukine™ ELISA 试剂盒

目录号：VAL106

适用于定量检测天然和重组人血管内皮生长因子（VEGF）的浓度

科研专用，不可用于临床诊断

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版本号 202606.6

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VIII. 参考文献 35

I. 背景

血管内皮生长因子（VEGF或VEGF-A）又叫血管通透因子，是一种针对胎儿和成人的血管新生和血管生成的有效调节因子（1-3）。血管内皮生长因子属于PDGF家族，该家族蛋白的特点是在胱氨酸结和通过反向平行二硫键结合的二聚体的结构处，存在8个保守的胱氨酸残基。经过选择性剪切和氨基酸的序列长度，人类表达不同的亚型，包括：VEGF121、VEGF145、VEGF165、VEGF183、VEGF189和VEGF206等等（4）。其中VEGF165是表达量最高的一种亚型，其次是VEGF121和VEGF189（3, 4）。除了VEGF121，其它的亚型都包含了基本的肝素结合区域且不能自由扩散（4）。人类的VEGF165与小鼠和大鼠的相应的蛋白氨基酸序列同源性为88%。VEGF在各种细胞和组织中表达，包括骨骼肌细胞和心肌细胞（5, 6）、肝细胞（7）、成骨细胞（8）、中性粒细胞（9）、巨噬细胞（10）、角质形成细胞（11）、棕色脂肪细胞（12）、$CD34^+$干细胞（13）、内皮细胞（14）、成纤维细胞、血管平滑肌细胞等等（15）。VEGF的表达收到缺氧和细胞因子的诱导，包括IL-1、IL-6、IL-8、抑瘤素M和肿瘤坏死因子α等（3, 4, 9, 16）。在发育过程和成体中，VEGF的表达量也是不同的（3）。

VEGF的二聚体与2个相关的酪氨酸激酶受体相结合，即VEGF R1（也叫Flt-1）和VEGF R2（Flk-1/KDR）。VEGF同时可以诱导后者的同型二聚体和自磷酸化过程（3, 4, 7, 17, 18）。这些受体拥有7个胞外的免疫球蛋白的域和一个胞内分开的酪氨酸受体域。血管内皮细胞和其它一些非内皮细胞都表达VEGF受体。尽管VEGF与VEGF R1的亲和力最高，但VEGF R2 却是调控VEGF的血管生成的主要因子（3, 4）。VEGF165也结合臂板蛋白（semaporin）受体，神经纤毛蛋白1（neuropilin-1），由此促进与VEGFR2的复合体形成（19）。

VEGF因其参与血管生成而著称。在胚胎发育过程中，VEGF调控内皮细胞的增殖、迁移和生存（3, 4），并由此调控血管的密度、体积；但对于血管形成的格局并不起作用。VEGF通过成骨细胞和软骨细胞的招募促进骨骼的形成，它同时也是一个单核细胞趋化因子（20-22）。在产后，VEGF维持血管内皮细胞的完整性，并且是大/小血管内皮细胞的有效丝裂原。在成体中，VEGF主要在伤口修复和女性的生殖周期发挥作用（3）。在疾病组织当中，VEGF促进血管的通透性。因此，VEGF通过外渗和肿瘤血管生成参与了肿瘤的转移过程（23, 24）。针对阻断VEGF活性的各种治疗策略正在被用于控制由VEGF诱导的肿瘤血管生成（25）。体内循环的VEGF水平与自身免疫性疾病（如类风湿关节炎、多发性硬化症、系统性红斑狼疮等）的病症程度相关（26）。

II. 概述

A. 检测原理

本实验采用双抗体夹心ELISA法。抗人VEGF抗体包被于微孔板上，样品和标准品中的人VEGF会与固定在板上的抗体结合，游离的成分被洗去；加入辣根过氧化酶标记的抗人VEGF抗体，与结合在微孔板上的人VEGF结合而形成免疫复合物，游离的成分被洗去；加入TMB底物溶液（显色剂）。溶液颜色与结合目标蛋白成正比；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，人血清样本和人血浆样本；
- 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，应将样本用稀释液RD5K/RD6U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，在不同板间分别检测40次，以确定板间精确度。

细胞上清

	板内精确度			板间精确度		
样本	1	2	3	1	2	3
平均值 (pg/mL)	29.1	123	531	32.8	128	495
标准差	1.9	5.0	18.4	2.8	6.4	33.0
CV%	6.5	4.1	3.5	8.5	5.0	6.7

血清/血浆

	板内精确度			板间精确度		
样本	1	2	3	1	2	3
平均值 (pg/mL)	53.7	235	910	64.5	250	1003
标准差	3.6	10.6	46.2	5.7	17.4	61.7
CV%	6.7	4.5	5.1	8.8	7.0	6.2

B. 回收率

在不同类型样本中掺入检测范围内不同水平的人VEGF，测定其回收率。

样本类型	平均回收率（%）	范围（%）
细胞培养基 (n=5)	102	95-111
人血清 (n=5)	102	92-115
人EDTA血浆 (n=5)	97	82-113
人肝素血浆 (n=5)	93	82-102
人枸橼酸钠血浆 (n=5)	100	88-113

C. 灵敏度

使用标准品稀释液RD5K时，人VEGF的最低可检测剂量（MDD）通常低于5.0 pg/mL。使用标准品稀释液RD6U时，MDD通常低于9.0 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

本免疫检测方法以R&D Systems生产的高度纯化的、由*Sf21*细胞表达的重组人$VEGF_{165}$为标准品进行校准。

NIBSC/WHO $VEGF_{165}$第1代WHO参考试剂02/286（重组人DNA来源）已在本试剂盒中经过评估。该参考试剂02/286的剂量反应曲线与Valukine标准曲线相平行。如需将使用Valukine人VEGF试剂盒获得的样本数值换算为近似的NIBSC/WHO 02/286单位，请使用以下公式：

NIBSC/WHO (02/286) 近似值 (U/mL) = 0.002 × Valukine VEGF检测值 (pg/mL)

注： 基于2011年4月产生的数据。

E. 线性

在不同类型样本中含有或者掺入高浓度的人VEGF，然后用标准品稀释液RD5K/RD6U将样本稀释到检测范围内，测定其线性。

稀释倍数		细胞培养基 (n=5)	人血清 (n=5)	人EDTA血浆 (n=5)	人肝素血浆 (n=5)	人枸橼酸钠血浆 (n=5)
1:2	平均值/期待值（%）	98	97	97	94	95
	范围 (%)	94-100	91-103	82-107	87-99	90-100
1:4	平均值/期待值（%）	96	97	98	93	94
	范围 (%)	93-99	93-104	91-106	85-98	88-99
1:8	平均值/期待值（%）	93	96	96	92	92
	范围 (%)	88-102	93-103	89-106	85-101	85-97
1:16	平均值/期待值（%）	93	94	94	94	92
	范围 (%)	88-105	91-101	84-106	83-103	85-98

F. 样本预值

人血清/血浆 - 采用本检测方法对来自表观健康志愿者的样本进行了人VEGF检测。本研究未获得所用样本捐献者的既往病史信息

样本类型	平均检测值 (pg/mL)	可测率 (%)	范围 (pg/mL)
人血清 (n=37)	220	100	62-707
人 EDTA 血浆 (n=37)	61	24	ND-115
人肝素血浆 (n=37)	41	22	ND-55
人枸橼酸钠血浆 (n=37)	—	0	ND

细胞培养上清液：

人外周血单核细胞（10×10⁶个细胞/mL）培养于添加了5%胎牛血清、50 µM β-巯基乙醇、2 mM L-谷氨酰胺、100 U/mL青霉素和100 µg/mL硫酸链霉素的RPMI培养基中。细胞未经刺激，或用10 µg/mL PHA刺激5天。取细胞培养上清液的等分试样，检测人VEGF水平，未刺激组和刺激组的检测值分别为87.1 pg/mL和1041 pg/mL。

PC-3人前列腺癌细胞培养于添加了10%胎牛血清、2 mM L-谷氨酰胺、100 U/mL青霉素和100 µg/mL硫酸链霉素的RPMI培养基中。细胞未经刺激，或用60 nM PMA刺激24小时。取细胞培养上清液的等分试样，检测人VEGF水平，未刺激组和刺激组的检测值分别为3373 pg/mL和3652 pg/mL。

JEG-3人上皮绒毛膜癌细胞培养于添加了10%胎牛血清、2 mM L-谷氨酰胺、100 U/mL青霉素、100 µg/mL硫酸链霉素和NEAA的MEM培养基中。细胞未经刺激，或用100 ng/mL LPS刺激1天。取细胞培养上清液的等分试样，检测人VEGF水平，未刺激组和刺激组的检测值分别为1538 pg/mL和1938 pg/mL。

G. 特异性

本检测方法可识别天然和重组人VEGF。本检测方法也可识别重组人$VEGF_{165b}$。

将以下因子以50 ng/mL的浓度配制于标准品稀释液RD5K/RD6U中，并进行交叉反应性检测。将以下因子以50 ng/mL的浓度掺入中浓度VEGF质控品中，并进行干扰性检测。以下因子未显示交叉反应性或干扰。

Recombinant human:	**Recombinant mouse:**	**Recombinant zebrafish:**
PDGF-AA	PDGF-CC	VEGF
PDGF-AB	PlGF-2	**Natural proteins:**
PDGF-BB	$VEGF_{120}$	human PDGF
PDGF-CC	$VEGF_{164}$	porcine PDGF
PDGF-DD	VEGF R3	**Recombinant Bovine:**
PlGF	**Recombinant rat:**	$VEGF_{164}$
PlGF-2	PDGF-AA	
$VEGF_{165}$/PlGF	PDGF-AB	
$VEGF\text{-}B_{167}$	PDGF-BB	
VEGF-C	$VEGF_{164}$	
VEGF-D		
VEGF R3		

将以下因子以10 ng/mL的浓度配制于标准品稀释液RD5K/RD6U中，并进行交叉反应性检测。将以下因子以10 ng/mL的浓度掺入中浓度VEGF质控品中，并进行干扰性检测。以下因子未显示交叉反应性或干扰。

Recombinant bovine:
$VEGF_{164}$

显示出交叉反应性或干扰的 VEGF 相关因子。

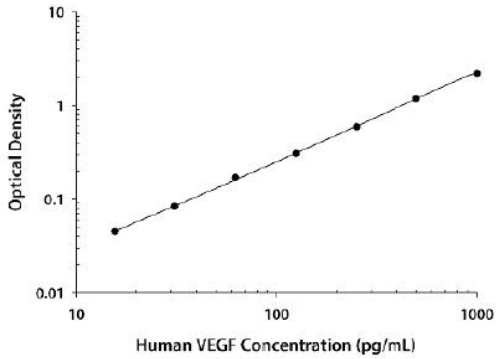
Recombinant human VEGF R1/Flt-1	Interference at levels ⩾ 500 pg/mL
Recombinant human VEGF R2/KDR	Interference at levels ⩾ 2000 pg/mL
Recombinant mouse VEGF R1/Flk-1	Interference at levels ⩾ 500 pg/mL
Recombinant mouse VEGF R2/KDR	Interference at levels ⩾ 4000 pg/mL
Recombinant canine VEGF	Cross-reacts approximately 67%
Recombinant feline VEGF	Cross-reacts approximately 82%

IV. 实验

标准曲线实例

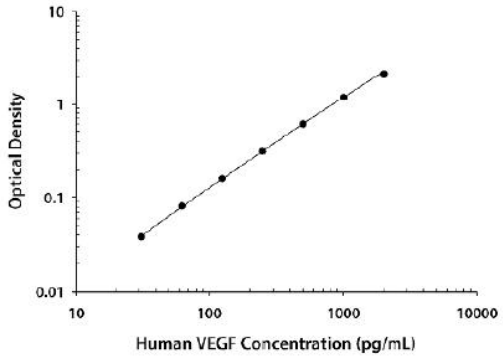
该标准曲线数据仅供参考，每次实验应绘制其对应的标准曲线。

CELL CULTURE SUPERNATE ASSAY



(pg/mL)	O.D.	Average	Corrected
0	0.074 0.076	0.075	—
15.6	0.118 0.121	0.120	0.045
31.3	0.159 0.159	0.159	0.084
62.5	0.242 0.246	0.244	0.169
125	0.378 0.384	0.381	0.306
250	0.666 0.669	0.668	0.593
500	1.258 1.263	1.260	1.185
1000	2.233 2.302	2.268	2.193

SERUM/PLASMA ASSAY



(pg/mL)	O.D.	Average	Corrected
0	0.068 0.071	0.070	—
31.3	0.107 0.110	0.108	0.038
62.5	0.149 0.153	0.151	0.081
125	0.230 0.230	0.230	0.160
250	0.377 0.387	0.382	0.312
500	0.657 0.699	0.678	0.608
1000	1.261 1.281	1.271	1.201
2000	2.159 2.246	2.202	2.132

V. 试剂盒组成及储存

A. 试剂盒组成

组成	描述	规格
Human VEGF Microplate	包被抗人VEGF抗体的96孔聚苯乙烯板，8孔× 12条	1块板
Human VEGF Conjugate	酶标检测抗人VEGF抗体	1瓶
Human VEGF Standard	人VEGF标准品（冻干粉），参考瓶标签进行重溶	2瓶
Assay Diluent RD1W	检测液RD1W	1瓶
Calibrator Diluent RD5K	标准品稀释液RD5K，*用于细胞培养上清样本*	1瓶
Calibrator Diluent RD6U	标准品稀释液RD6U，*用于人血清、血浆样本，可能有沉淀*	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	2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="8">已打开，稀释或重溶的试剂</td><td>洗涤液（1×）</td><td rowspan="6">2-8℃储存，最多30天*</td></tr>
<tr><td>终止液</td></tr>
<tr><td>酶标检测抗体</td></tr>
<tr><td>检测液RD1W</td></tr>
<tr><td>标准品稀释液RD5K</td></tr>
<tr><td>标准品稀释液RD6U</td></tr>
<tr><td>TMB底物溶液</td><td rowspan="2">已重溶和稀释的标准品仅限4小时内使用。每次实验需新鲜配制标准品。</td></tr>
<tr><td>标准品</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
- 500 mL量筒

D. 注意事项

- 标准品稀释液 RD6U 含有叠氮化钠，该物质可能与铅管或铜管反应形成易爆的金属叠氮化物。废弃处置时应用大量水冲洗。
- VEGF在唾液中可被检测到。在运行本检测时，请采取预防措施以防止试剂盒组分受到污染。
- 本试剂盒提供的终止液为酸性溶液。
- 本试剂盒中的某些组分含有防腐剂，可能引起过敏性皮肤反应。避免吸入雾状液滴。
- 穿戴防护手套、防护服、眼罩和面罩。操作后彻底清洗双手。

VI. 实验前准备

A. 样品收集及储存

以下列出的样本采集和保存条件仅供一般性参考。样本稳定性尚未经过评估。

细胞培养上清液：为确保VEGF的稳定性，细胞培养上清液中应至少含有1%的胎牛血清。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RD5K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RD6U稀释。

血浆样本：使用EDTA，肝素或枸橼酸钠作为抗凝剂收集血浆。然后1000 × g离心15分钟，需在30分钟内收集血浆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RD6U稀释。

B. 检测前准备工作

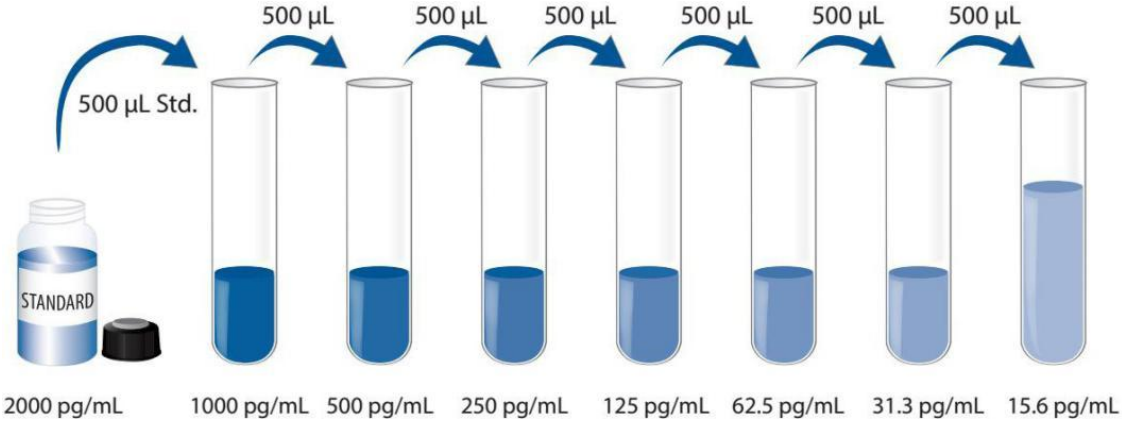
使用前请将所有试剂放置于室温

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，待结晶完成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×）。

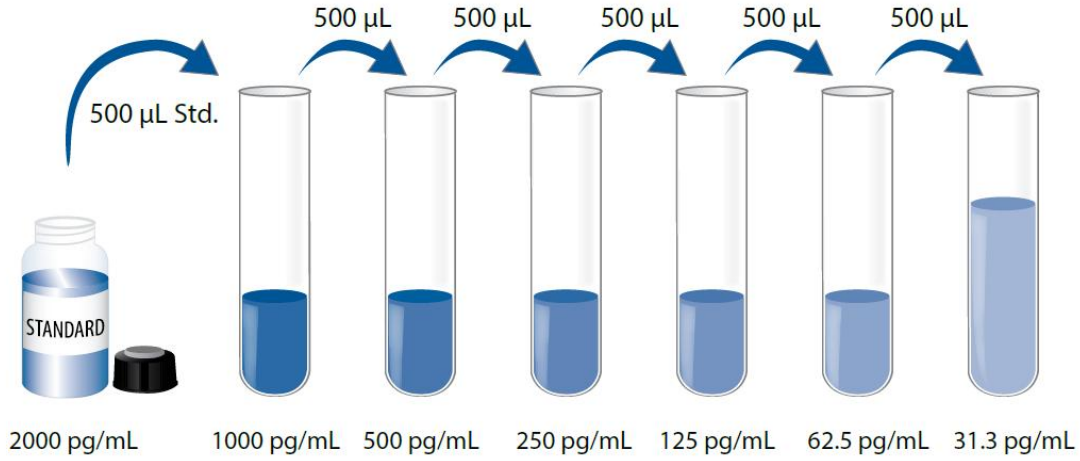
人VEGF标准品：重溶冻干标准品，**重溶体积请参考瓶身标签**。

使用标准品稀释液RD5K（*适用于细胞培养上清液样本*）或标准品稀释液 RD6U（*适用于人血清/血浆样本*）复溶人VEGF标准品。标准品稀释液RD6U中可能含有沉淀。使用前及使用过程中请充分混匀。复溶后得到浓度为2000 pg/mL的标准品储备液。在进行稀释前，让标准品静置至少 15 分钟，并伴随温和的摇动。

细胞培养上清液样本：使用聚丙烯试管。向每个试管中加入500 µL的标准品稀释液RD5K。使用储备液按下图进行系列稀释。在每次转移前，将每个试管充分混匀。1000 pg/mL的稀释液作为最高浓度标准点。标准品稀释液RD5K作为零标准点（0 pg/mL）。



血清/血浆样本： 使用聚丙烯试管。向每个试管中加入500 µL的标准品稀释液RD6U。使用储备液按下图进行系列稀释。在每次转移前，将每个试管充分混匀。2000 pg/mL的标准品作为最高浓度标准点。标准品稀释液RD6U作为零标准点（0 pg/mL）。



C. 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. **细胞上清样本：**每孔中加入50 µL检测液RD1W。

   人血清/血浆样本：每孔中加入100 µL检测液RD1W。
4. **细胞上清样本：分别将不同浓度标准品和实验样本加入相应孔中，每孔200 µL。**

   人血清/血浆样本：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；
5. 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2次，共洗3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；
6. 在每个微孔内加入200 µL酶标检测抗体。用封板膜封住反应孔，**室温孵育2小时**；
7. 重复第5步洗板操作；
8. 在每个微孔内加入200 µL TMB底物溶液。**注意避光**；

   细胞上清样本：室温孵育20分钟。

   人血清/血浆样本：室温孵育25分钟。
9. 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；
10. 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；
11. **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。

    如果样品被稀释，从标准曲线读取的浓度必须乘以稀释倍数。

VIII. 参考文献

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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

