ELISA Kit

Catalog # KRC5221 (96 tests)

Rat

VEGF

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Contents and Storage

Storage

Store at 2 to 8°C.

Contents

Reagents Provided	***96 Test Kit***
Rt VEGF Standard, lyophilized, recombinant Rt VEGF. Contains 0.1% sodium azide. Refer to vial label for quantity and reconstitution volume.	2 vials
Standard Diluent Buffer. Contains 0.1% sodium azide; 25 mL per bottle.	1 bottle
Incubation Buffer. Contains 0.05% sodium azide; 12 mL per bottle.	1 bottle
Rt VEGF High and Low Controls, lyophilized, recombinant Rt VEGF. Contains 0.1% sodium azide. Refer to vial label for reconstitution volume and range.	2 vials
Rt VEGF Antibody Coated Wells, 96 Well Plate.	1 plate
Rt VEGF Biotin Conjugate, (Biotin-labeled anti-VEGF). Contains 0.1% sodium azide; 11 mL per bottle.	1 bottle
Streptavidin-HRP (100X). Contains 3.3 mM thymol; 0.125 mL per vial.	1 vial
Streptavidin-HRP Diluent. Contains 3.3 mM thymol; 25 mL per bottle.	1 bottle
Wash Buffer Concentrate (25X); 100 mL per bottle.	1 bottle
Stabilized Chromogen, *Tetramethylbenzidine (TMB);* 25 mL per bottle.	1 bottle
Stop Solution; 25 mL per bottle.	1 bottle
Plate Covers, adhesive strips.	4

Disposal Note

This kit contains materials with small quantities of sodium azide. Sodium azide reacts with lead and copper plumbing to form explosive metal azides. Upon disposal, flush drains with a large volume of water to prevent azide accumulation. Avoid ingestion and contact with eyes, skin and mucous membranes. In case of contact, rinse affected area with plenty of water. Observe all federal, state and local regulations for disposal.

Safety

All blood components and biological materials should be handled as potentially hazardous. Follow universal precautions as established by the Centers for Disease Control and Prevention and by the Occupational Safety and Health Administration when handling and disposing of infectious agents.

Introduction

Purpose

The Invitrogen Rat VEGF (Rt VEGF) ELISA is to be used for the quantitative determination of Rt VEGF in serum, plasma, buffered solution and tissue culture medium. The assay will recognize both natural and recombinant Rt VEGF.

For Research Use Only. CAUTION: Not for human or animal therapeutic or diagnostic use.

Principle of the Method

The Invitrogen Rt VEGF kit is a solid phase sandwich Enzyme Linked-Immuno-Sorbent Assay (ELISA). A highly purified antibody has been coated onto the wells of the microtiter strips provided.

During the first incubation, standards of known Rt VEGF content, controls, and unknown samples are pipetted into the coated wells. After washing, biotinylated second antibody, is added.

After washing, Streptavidin-Peroxidase (enzyme) is added. This binds to the biotinylated antibody to complete the four-member sandwich. After a third incubation and washing to remove all the unbound enzyme, a substrate solution is added, which is acted upon by the bound enzyme to produce color. The intensity of this colored product is directly proportional to the concentration of Rt VEGF present in the original specimen.

Background Information

Vascular endothelial growth factor (VEGF, known alternatively as VEGF-A, vascular permeability factor and vasculotropin) is a member of a family of proteins that also includes VEGF-B, VEGF-C, VEGF-D, and placenta growth factor (PlGF). VEGF is the subject of intense investigation because of its role in promoting angiogenesis (new blood vessel formation), a process necessary in embryonic development and female reproductive function, but which also enhances tumor growth and metastasis.

Rat VEGF is encoded by a single gene that maps to chromosome 9q12. Alternative splicing yields three predominant VEGF variants, VEGF 120, 164, and 188 (corresponding to the human homologs 121, 165, and 189, respectively) and two rare splice variants, 205 (corresponding to 206 in human) and 144 (corresponding to 145 in human). These splice variants differ in their affinities for heparin and the various VEGF receptors. The shorter splice variants are soluble, while longer splice variants associate with extracellular matrix components, possibly through interaction with proteoglycans. The various VEGF forms are synthesized as precursor proteins containing hydrophobic signal sequences. The mature proteins are glycosylated, disulfide-linked homodimers with M_r=34-42 kDa.

Major sources of VEGF include macrophages, epithelial cells of the kidney and lung, pituitary follicular cells, corpus luteum, and smooth muscle cells. VEGF expression is regulated by several factors. Decreased pO_2 elevates the level of hypoxia inducible factor-1 (HIF-1), a transcription factor that binds to promoter regions of both VEGF and erythropoietin, enhancing the expression of both of these proteins. Studies with mutants suggest that von Hippel-Lindau tumor suppressor may negatively influence VEGF expression. Cytokines, growth factors, and lipid inflammatory mediators that enhance VEGF production include EGF, TGF-β, KGF, IL-1α, IL-6, and IGF-1, and PGE2.

At least three VEGF receptors have been identified, including VEGFR-1 (flt-1), VEGFR-2 (flk-1 or KDR), and VEGFR-3 (flt-4). These VEGF receptors are characterized by the presence of seven immunoglobulin-like domains, and a split intracellular tyrosine kinase domain. Ligand interaction cross-links two receptors, permitting autotransphosphorylation of the receptors. Neuropilins 1 and 2, two transmembrane proteins with short cytoplasmic domains, also bind VEGF. The neuropilins enhance VEGF receptor signaling, and their role as VEGF co-receptors is currently under investigation. Proteoglycans on the cell surface may also serve as VEGF co-receptors. Signaling through VEGFR-1 results in phosphorylation of Fyn and Yes, while signaling through VEGFR-2 results in the recruitment of Shc, Grb2, Nck, SHP-1, and SHP-2, and the activation of MAPK cascades. VEGF is also observed to enhance the PI3K/AKT pathway, as well as the phosphorylation and recruitment of FAK and paxillin.

VEGF is a potent mitogen for cells derived from arteries, veins, and lymphatics. VEGF acts as a survival factor for dermal endothelial cells by stimulating the production of the anti-apoptotic proteins Bcl-2 and A1, and protects motoneurons from ischemic death. VEGF is chemotactic with monocytes and lens epithelial cells. VEGF stimulates the formation of colonies by granulocyte-macrophage progenitor cells, stimulates the proliferation of IL-2-dependent lymphocytes, and inhibits dendritic cell differentiation. VEGF has also been shown to be necessary for endochondrial bone formation.

Methods

Materials Needed But Not Provided

- Microtiter plate reader (at or near 450 nm) with software
- Calibrated adjustable precision pipettes
- Distilled or deionized water
- Plate washer: automated or manual (squirt bottle, manifold dispenser, etc.)
- Glass or plastic tubes for diluting solutions
- Absorbent paper towels
- Calibrated beakers and graduated cylinders

Procedural Notes

1. When not in use, kit components should be refrigerated. All reagents should be warmed to room temperature before use.
2. **Microtiter plates should be allowed to come to room temperature before opening the foil bags.** Once the desired number of strips has been removed, immediately reseal the bag and store at 2 to 8°C to maintain plate integrity.
3. Samples should be collected in pyrogen/endotoxin-free tubes.
4. Samples should be frozen if not analyzed shortly after collection. Avoid multiple freeze-thaw cycles of frozen samples. Thaw completely and mix well prior to analysis.
5. When possible, avoid use of badly hemolyzed or lipemic sera. If large amounts of particulate matter are present, centrifuge or filter prior to analysis.
6. It is recommended that all standards, controls and samples be run in duplicate.
7. When pipetting reagents, maintain a consistent order of addition from well-to-well. This ensures equal incubation times for all wells.
8. **Do not mix or interchange different reagent lots from various kit lots**.
9. Do not use reagents after the kit expiration date.
10. Absorbances should be read immediately, but can be read up to 2 hours after assay completion. For best results, keep plate covered in the dark.
11. In-house controls or kit controls should be run with every assay. If control values fall outside pre-established ranges, the accuracy of the assay is suspect.
12. All residual wash liquid must be drained from the wells by efficient aspiration or by decantation followed by tapping the plate forcefully on absorbent paper. ***Never*** insert absorbent paper directly into the wells.
13. Because Stabilized *Chromogen* is light sensitive, avoid prolonged exposure to light. Avoid contact between chromogen and metal, or color may develop.

Directions for Washing

- **Incomplete washing will adversely affect the test outcome**. All washing must be performed with the *Wash Buffer Concentrate (25X)* provided.
- Washing can be performed manually as follows: completely aspirate the liquid from all wells by gently lowering an aspiration tip into the bottom of each well. Take care not to scratch the inside of the well. After aspiration, fill the wells with at least 0.4 ml of diluted *Wash Buffer*. Let soak for 15 to 30 seconds, then aspirate the liquid. Repeat as directed under Assay Procedure. After the washing procedure, the plate is inverted and tapped dry on absorbent tissue.
- Alternatively, the diluted *Wash Buffer* may be put into a squirt bottle. If a squirt bottle is used, flood the plate with the diluted *Wash Buffer*, completely filling all wells. After the washing procedure, the plate is inverted and tapped dry on absorbent tissue.
- If using an automated washer, follow the washing instructions carefully.

Preparation of Reagents

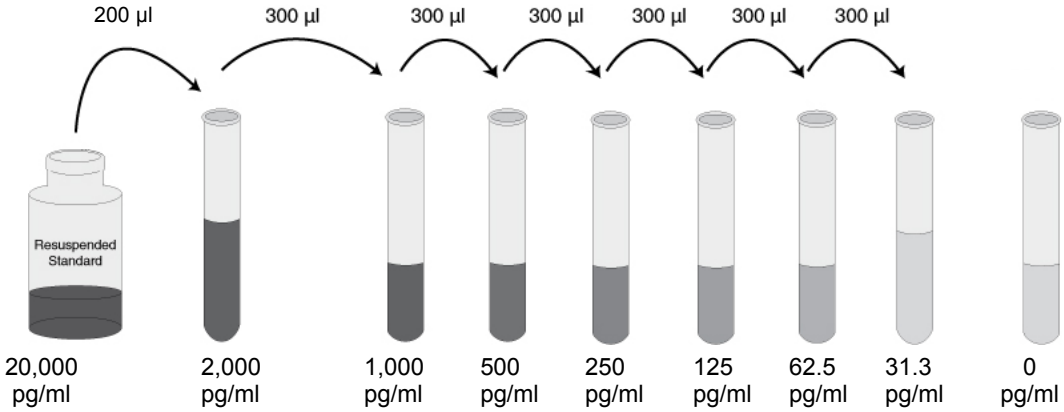
Dilution of Standard

Note: Either glass or plastic tubes may be used for standard dilutions.

1. Reconstitute standard to 20,000 pg/ml with *Standard Diluent Buffer*. Refer to the standard vial label for instructions. Swirl or mix gently and allow to sit for 10 minutes to ensure complete reconstitution. Use the standard within 15 minutes of reconstitution.
2. Add 0.2 ml of the reconstituted standard to a tube containing 1.8 ml *Standard Diluent Buffer*. Label as 2000 pg/ml Rt VEGF. Mix.
3. Add 0.300 ml of *Standard Diluent Buffer* to each of 6 tubes labeled 1,000, 500, 250, 125, 62.5, and 31.3 pg/ml Rt VEGF.
4. Make serial dilutions of the standard as described in the following dilution diagram. Mix thoroughly between steps.

Important

Remaining reconstituted standard should be discarded. Return the *Standard Diluent Buffer* to the refrigerator.



Preparing SAV-HRP

Note: Prepare within 15 minutes of usage. The *Streptavidin-HRP (100X)* is in 50% glycerol, which is viscous. To ensure accurate dilution, allow *Streptavidin-HRP (100X)* to reach room temperature. Gently mix. Pipette *Streptavidin-HRP (100X)* slowly. Remove excess concentrate solution from pipette tip by gently wiping with clean absorbent paper.

1. Dilute 10 µl of this 100X concentrated solution with 1 ml of *Streptavidin-HRP Diluent* for each 8-well strip used in the assay. Label as Streptavidin-HRP Working Solution.
2. Return the unused *Streptavidin-HRP (100X)* to the refrigerator.

# of 8-Well Strips	Volume of Streptavidin-HRP (100X)	Volume of Diluent
2	20 µl solution	2 ml
4	40 µl solution	4 ml
6	60 µl solution	6 ml
8	80 µl solution	8 ml
10	100 µl solution	10 ml
12	120 µl solution	12 ml

Dilution of Wash Buffer

1. Allow the *Wash Buffer Concentrate (25X)* to reach room temperature and mix to ensure that any precipitated salts have redissolved. Dilute 1 volume of the *Wash Buffer Concentrate (25X)* with 24 volumes of deionized water (e.g., 50 ml may be diluted up to 1.25 liters, 100 ml may be diluted up to 2.5 liters). Label as Working Wash Buffer.
2. Store both the concentrate and the Working Wash Buffer in the refrigerator. The diluted buffer should be used within 14 days.

Assay Procedure

Be sure to read the *Procedural Notes* section before carrying out the assay.

Allow all reagents to reach room temperature before use. Gently mix all liquid reagents prior to use.

Note: A standard curve must be run with each assay.

1. Determine the number of 8-well strips needed for the assay. Insert these in the frame(s) for current use. (Re-bag extra strips and frame. Store these in the refrigerator for future use.)
2. Add 50 µl of the *Incubation Buffer* to all wells. Well(s) reserved for chromogen blank should be left empty.
3. Add 100 µl of the *Standard Diluent Buffer* to the zero standard wells. Well(s) reserved for chromogen blank should be left empty.
4. For the standard curve, add 100 µl of standards to the appropriate microtiter wells. See **Preparation of Reagents**. For all samples (controls, serum, plasma, buffered solution and cell culture medium), add 50 µl of *Standard Diluent Buffer* to each well followed by 50 µl of sample..
5. Cover plate with *plate cover* and incubate for **2 hours at room temperature**.
6. Thoroughly aspirate or decant solution from wells and discard the liquid. Wash wells 4 times. See **Directions for Washing**.
7. Add 100 µl of biotinylated *Rt VEGF Biotin Conjugate* solution into each well except the chromogen blank(s). Tap on the side of the plate for 30 seconds to mix.
8. Cover plate with *plate cover* and incubate for **1 hour at room temperature**.
9. Thoroughly aspirate or decant solution from wells and discard the liquid. Wash wells 4 times. See **Directions for Washing**.
10. Add 100 µl Streptavidin-HRP Working Solution to each well except the chromogen blank(s). See **Preparation of Reagents**.
11. Cover plate with the *plate cover* and incubate for **30 minutes at room temperature**.
12. Thoroughly aspirate or decant solution from wells and discard the liquid. Wash wells 4 times. See **Directions for Washing**.
13. Add 100 µl of *Stabilized Chromogen* to each well. The liquid in the wells will begin to turn blue.
14. Incubate for **30 minutes at room temperature and in the dark**. ***Note*: Do not cover the plate with aluminum foil or metalized mylar**. The incubation time for chromogen substrate is often determined by the microtiter plate reader used. Many plate readers have the capacity to record a maximum optical density (O.D.) of 2.0. The O.D. values should be monitored and the substrate reaction stopped before the O.D. of the positive wells exceeds the limits of the instrument. The O.D. values at 450 nm can only be read after the *Stop Solution* has been added to each well. If using a reader that records only to 2.0 O.D., stopping the assay after 20 to 25 minutes is suggested.
15. Add 100 µl of *Stop Solution* to each well. Tap side of plate gently to mix. The solution in the wells should change from blue to yellow.

16. Read the absorbance of each well at 450 nm having blanked the plate reader against a chromogen blank composed of 100 µl each of *Stabilized Chromogen* and *Stop Solution*. Read the plate within 30 minutes after adding the *Stop Solution.*
17. Use a curve fitting software to generate the standard curve. A four parameter algorithm provides the best standard curve fit.
18. Read the concentrations for unknown samples and controls from the standard curve. **Multiply value(s) obtained for serum, plasma and tissue culture samples by 2 to correct for the overall 1:2 dilution in step 4.** Samples producing signals greater than that of the highest standard should be further diluted in the *Standard Diluent Buffer* and reanalyzed.

Typical Data (Example)

The following data were obtained for the various standards over the range of 0 to 2000 pg/ml Rt VEGF.

Standard Rt VEGF (pg/ml)	Optical Density (450 nm)
2000	3.38
1000	2.45
500	1.33
250	0.73
125	0.41
62.5	0.26
31.3	0.19
0	0.11

Performance Characteristics

Sensitivity

The minimum detectable dose of Rt VEGF is < 15 pg/ml. This was determined by adding two standard deviations to the mean O.D. obtained when the zero standard was assayed 30 times, and calculating the corresponding concentration.

Precision

1. Intra-Assay Precision

Samples of known Rt VEGF concentration were assayed in replicates of 16 to determine precision within an assay.

	Sample 1	Sample 2	Sample 3
Mean (pg/ml)	136.1	853.9	1760.2
SD	7.0	39.6	95.0
%CV	5.2	4.6	5.2

SD = Standard Deviation
CV = Coefficient of Variation

2. Inter-Assay Precision

Samples were assayed 48 times in multiple assays to determine precision between assays.

	Sample 1	Sample 2	Sample 3
Mean (pg/ml)	145.8	902.0	1799.2
SD	13.5	70.3	109.9
%CV	9.3	7.8	6.1

SD = Standard Deviation
CV = Coefficient of Variation

Linearity of Dilution

Rat serum, EDTA plasma, and tissue culture medium spiked with recombinant Rt VEGF were serially diluted in *Standard Diluent Buffer* over the range of the assay. Linear regression analysis of samples versus the expected concentration yielded average correlation coefficients of 0.986 for serum, 0.976 for EDTA plasma, and 0.999 for tissue culture medium.

Recovery

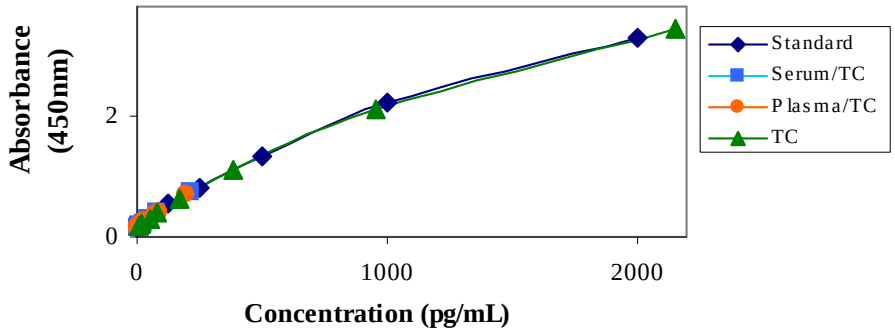
The recoveries of recombinant Rt VEGF added to rat serum, EDTA plasma, citrate plasma, and tissue culture medium containing 10% fetal bovine or calf serum were measured on the Invitrogen Rt VEGF ELISA.

Sample Type	Average % Recovery
Serum	94
EDTA plasma	91
Citrate plasma	100
RPMI+10% fetal bovine serum	113
DMEM+10% calf serum	108

Serum, plasma, and tissue culture medium were pre-diluted 2-fold as described in the assay procedure.

Parallelism

Rat serum, plasma, and tissue culture samples were serially diluted in the *Standard Diluent Buffer*. The optical density of each dilution was plotted against the Rt VEGF standard curve. Parallelism was demonstrated by the figure below and indicated that the standard accurately reflects the Rt VEGF content in natural samples.



Specificity

Buffered solutions of a panel of substances ranging in concentrations from 4,000 to 50,000 pg/mL were assayed with the Invitrogen Rat VEGF kit and found to have no cross-reactivity: Human Eotaxin, GM-CSF, IFN-α, IFN-γ, IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12p40/p70, IL-13, IL-15, IL-17, IP-10, MCP-1, MIG, MIP-1α, MIP-1β, RANTES, and VEGF; Rat GM-CSF, IFN-γ, IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-10, IL-12, IL-13, MIP-2, RANTES, and TNF-α; Mouse Eotaxin, GM-CSF, IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-12+p40, KC, MCP-1, and TNF-α. The cross-reactivity with recombinant mouse VEGF was determined to be 45%.

Expected Values

Thirty normal serum and plasma samples, and sixteen cell culture supernatants were evaluated for the presence of Rt VEGF in this assay.

Sample	Range (pg/ml)
Serum (n=20)	0-64
EDTA plasma (n=5)	0
Citrate plasma (n=5)	0
Rat Splenocytes, un-stimulated	0-63
Rat Splenocytes, stimulated	84-1999

High Hook Dose Effect

No hook effect was observed with concentrations up to 1 mg/ml.

Limitations of the Procedure

Do not extrapolate the standard curve beyond the top standard point; the dose-response is non-linear in this region and accuracy is difficult to obtain. Dilute all samples above the top standard point with *Standard Diluent Buffer*; reanalyze these and multiply results by the appropriate dilution factor.

The influence of various drugs, aberrant sera (hemolyzed, hyperlipidemic, jaundiced, etc.) and the use of biological fluids in place of serum samples have not been thoroughly investigated. The rate of degradation of native Rt VEGF in various matrices has not been investigated. The immunoassay literature contains frequent references to aberrant signals seen with some sera, attributed to heterophilic antibodies. Though such samples have not been seen to date, the possibility of this occurrence cannot be excluded.

Appendix

Troubleshooting Guide

Elevated background	*Cause:* Insufficient washing and/or draining of wells after washing. Solution containing either biotin or SAV-HRP can elevate the background if residual is left in the well. *Solution:* Wash according to the protocol. Verify the function of automated plate washer. At the end of each washing step, invert plate on absorbent tissue on countertop and allow to completely drain, tapping forcefully if necessary to remove residual fluid. *Cause:* Contamination of substrate solution with metal ions or oxidizing reagents. *Solution:* Use distilled/deionized water for dilution of wash buffer and use plastic equipment. DO NOT COVER plate with foil. *Cause:* Contamination of pipette, dispensing reservoir or substrate solution with SAV-HRP conjugate. *Solution:* Do not use chromogen that appears blue prior to dispensing onto the plate. Obtain new vial of chromogen. *Cause:* Incubation time is too long or incubation temperature is too high. *Solution:* Reduce incubation time and/or temperature.
Elevated sample/ standard ODs	*Cause:* Incorrect dilution of standard stock solution; intermediary dilutions not followed correctly. *Solution*: Follow the protocol instructions regarding the dilution of the standard. *Cause:* Incorrect dilution of the SAV-HRP conjugate. *Solution:* Warm solution of SAV-HRP concentrate to room temperature, draw up slowly and wipe tip with kim-wipe to remove excess. Dilute ONLY in SAV diluent provided. *Cause*: Incubation times extended. *Solution:* Follow incubation times outlined in protocol. *Cause:* Incubations carried out at 37°C when RT is dictated. *Solution:* Perform incubations at RT (= 25 ± 2°C) when instructed in the protocol.
Poor standard curve	*Cause:* Improper preparation of standard stock solution. *Solution:* Dilute lyophilized standard as directed by the vial label only with the standard diluent buffer or in a diluent that most closely matches the matrix of your sample. *Cause:* Reagents (lyophilized standard, standard diluent buffer, etc.) from different kits, either different cytokine or different lot number, were substituted. *Solution:* NEVER substitute any components from another kit. *Cause*: Errors in pipetting the standard or subsequent steps. *Solution*: Always dispense into wells quickly and in the same order. Do not touch the pipette tip on the individual microwells when dispensing. Use calibrated pipettes and the appropriate tips for that device.

Weak/no color develops	*Cause:* Reagents not at RT (25 ± 2°C) at start of assay. *Solution:* Allow ALL reagents to warm to RT prior to commencing assay. *Cause:* Incorrect storage of components, e.g., not stored at 2 to 8°C. *Solution:* Store all components exactly as directed in protocol and on labels. *Cause:* Working SAV-HRP solution made up longer than 15 minutes before use in assay. *Solution:* Use the diluted SAV-HRP within 15 minutes of dilution. *Cause:* TMB solution lost activity. *Solution 1:* The TMB solution should be clear before it is dispensed into the wells of the microtiter plate. An intense aqua blue color indicates that the product is contaminated. Please contact Technical Support if this problem is noted. To avoid contamination, we recommend that the quantity required for an assay be dispensed into a disposable trough for pipetting. Any TMB solution left in the trough should be discarded. *Solution 2:* Avoid contact of the TMB solution with items containing metal ions. *Cause:* Attempt to measure analyte in a matrix for which the ELISA assay has not been optimized. *Solution:* Please contact Technical Support for advice when using nonvalidated sample types. *Cause:* Wells have been scratched with pipette tip or washing tips. *Solution:* Use caution when dispensing and aspirating into and out of microwells.
Poor Precision	*Cause:* Errors in pipetting the standards, samples or subsequent steps. *Solution:* Always dispense into wells quickly and in the same order. Do not touch the pipette tip on the individual microwells when dispensing. Use calibrated pipettes and the appropriate tips for that device. Check for any leaks in the pipette tip. *Cause:* Repetitive use of tips for several samples or different reagents. *Solution:* Use fresh tips for each sample or reagent transfer. *Cause:* Wells have been scratched with pipette tip or washing tips. *Solution:* Use caution when dispensing and aspirating into and out of microwells.

Technical Support

Contact Us

For more troubleshooting tips, information, or assistance, please call, email, or go online to www.invitrogen.com/ELISA.

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Explanation of symbols

Symbol	Description	Symbol	Description
REF	Catalogue Number	LOT	Batch code
RUO	Research Use Only	IVD	*In vitro* diagnostic medical device
	Use by		Temperature limitation
	Manufacturer	EC REP	European Community authorised representative
[-]	Without, does not contain	[+]	With, contains
	Protect from light		Consult accompanying documents
	Directs the user to consult instructions for use (IFU), accompanying the product.		

Notes

Rat VEGF Assay Summary