

AviBion Human VEGF ELISA Kit

User Manual

REF

VEGF023

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For the quantitative determination of human vascular endothelial growth factor (VEGF) concentrations in cell culture supernates, serum, and plasma.

Regulatory Status: For research use only (RUO)

Please contact Orgenium's customer service representatives for inquiries, feedback or non-conforming products.



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1. INTENDED USE

Orgenium Laboratories' vascular endothelial growth factor (VEGF) ELISA is an enzyme-linked immunosorbent assay for the quantitative detection of human VEGF levels in cell culture supernatants, plasma (heparin and citrate) and human serum.

2. INTRODUCTION

VEGF, also known as vascular permeability factor (VPF), is characterized by its highly specific mitogenic activity for endothelial cells and its angiogenic effect observed *in vitro* and *in vivo* (1). The VEGF family consists of four isoforms; VEGF-A (also called VEGF), VEGF-B, VEGF-C, and VEGF-D (2, 3). VEGF-A is considered the most important of these with respect to tumor angiogenesis, especially the splice variant isoforms VEGF121 and VEGF165 were found to be secreted by a wide spectrum of cell types, including smooth muscle cells (4), fibroblasts and epithelial cells (5) keratinocytes (6) macrophages (7), cardiac myocytes (8) and various tumor cells (1). Both environmental and epigenetic factors can lead up-regulation of VEGF in tumor cells (5, 6). It has been shown that inhibition of VEGF with a specific monoclonal antibody can suppress tumor growth *in vivo*.

Orgenium Laboratories' human VEGF ELISA kit is an *in vitro* enzyme-linked immunosorbent assay for the quantitative measurement of human VEGF in serum, plasma, cell culture supernatants, and urine. This assay employs an antibody specific for human VEGF coated on a 96-well plate. Standards, samples and biotinylated anti-human VEGF are pipetted into the wells and VEGF present in a sample is captured by antibody immobilized to the wells and by the biotinylated VEGF specific detection antibody. After washing away unbound biotinylated antibody, HRP-conjugated streptavidin is pipetted to the wells. The wells are again washed. Following this second wash step, TMB substrate solution is added to the wells, resulting in color development proportional to the amount of VEGF bound. The Stop Solution changes the color from blue to yellow, and the intensity of the color is measured at 450 nm.

3. CONTENTS OF THE KIT

Test components	Amount/Volume
<p>96 Well Plate with 12 Strips Break-apart microtiter test strips each with 8 VEGF antibody coated single wells Ready for use</p>	1 frame
<p>VEGF₁₆₅ Standard 6000 pg/ml Diluted & Stabilized Recombinant Human VEGF₁₆₅ (see label for stock concentration) Ready for use.</p>	1 ml
<p>Biotinylated VEGF antibody. Stabilized Ready for use.</p>	15 ml
<p>HRP-Conjugated Avidin. Stabilized Ready for use.</p>	15 ml
<p>20x Washing solution concentrate (sufficient for 1000ml) Dilute 1:20</p>	50 ml
<p>Sample Diluent Ready for use</p>	100 ml
<p>Stop solution 2 N H₂SO₄ Ready for use</p>	8 ml
<p>TMB-Substrate Ready for use</p>	8 ml
<p>Quality control certificate</p>	1

4. STORAGE AND STABILITY

Reagent	Storage	Stability
<p>Antibody coated 96 well plates with 12 strips.</p> <p>Break-apart microtiter test strips each with 8 antibody coated single wells</p>	<p>Store at 2-8°C in closed aluminum-bag with desiccant</p> <p>Strips which are not used must be stored in the re-sealable aluminum bag in humidity free and airtight conditions</p>	3 months after opening
<p>VEGF₁₆₅ Standard Ready for use</p>	Store at 2-8°C	3 months after opening
<p>Biotinylated antibody. Ready for use.</p>	<p>Store at 2-8°C</p> <p><i>Avoid contamination (Use clean sterile tips)</i></p>	3 months after opening
<p>HRP-Conjugated Avidin. Ready for use.</p>	<p>Store at 2-8°C</p> <p><i>Avoid contamination (Use clean sterile tips)</i></p>	3 months after opening
<p>Sample Diluent</p>	<p>Store at 2-8°C</p> <p><i>Avoid contamination (Use clean sterile tips or pipettes)</i></p>	3 months after opening
<p>20x Concentrated Wash Buffer</p> <p>Diluted Wash Buffer</p>	<p>Store at Room Temperature.</p> <p>1x working dilution</p> <p><i>Bottles used for the working dilution should be cleaned regularly, discard cloudy solutions</i></p>	<p>Until expiry date at room temperature</p> <p>3 working days at room temperature or 2 weeks at +4°C.</p>
<p>TMB-Substrate Solution</p>	<p>Ready for use solution at 2-8°C, protected from light!</p> <p><i>Avoid contamination (Use clean sterile tips)</i></p>	Until expiry date (written on the bottle).
<p>Stop Solution</p>	Store at Room Temperature	Until expiry date at room temperature

5. ADDITIONAL MATERIALS REQUIRED

- Microplate reader capable of measuring absorbance at 450 nm.
- Precision pipettes (2 µl to 1 ml volumes).
- Multi-channel pipette (25 µl to 350 µl).
- Adjustable 1-25 ml pipettes for reagent preparation.
- 100 ml and 1 liter graduated cylinders.
- Absorbent paper.
- Distilled or de-ionized water.
- Log-log graph paper or computer and software for ELISA data analysis.
- Tubes to prepare standard or sample dilutions.
- Timer

6. AMOUNTS OF THE REAGENTS NEEDED TO PERFORM THE TEST

No of strips used (with 8 well each)	Reagents				
	Biotinylated antibody	Avidin-HRP	TMB Substrate	Stop Solution	Wash Buffer
	100 µl/well	100µl/well	50 µl/well	25 µl/well	300 µl/well
1 (8 wells)	900 µl	900 µl	500 µL	300 µl	30 ml
2 (16 wells)	1.8 ml	1.8 ml	1 ml	600 µL	55 ml
4 (32 wells)	3.6 ml	3.6 ml	2 ml	1.2 ml	110 ml
6 (48 wells)	5.4 ml	5.4 ml	3 ml	1.8 ml	165 ml
8 (64 wells)	7.2 ml	7.2 ml	4 ml	2.4 ml	220 ml
12 (96 wells)	11 ml	11 ml	6 ml	4 ml	350 ml

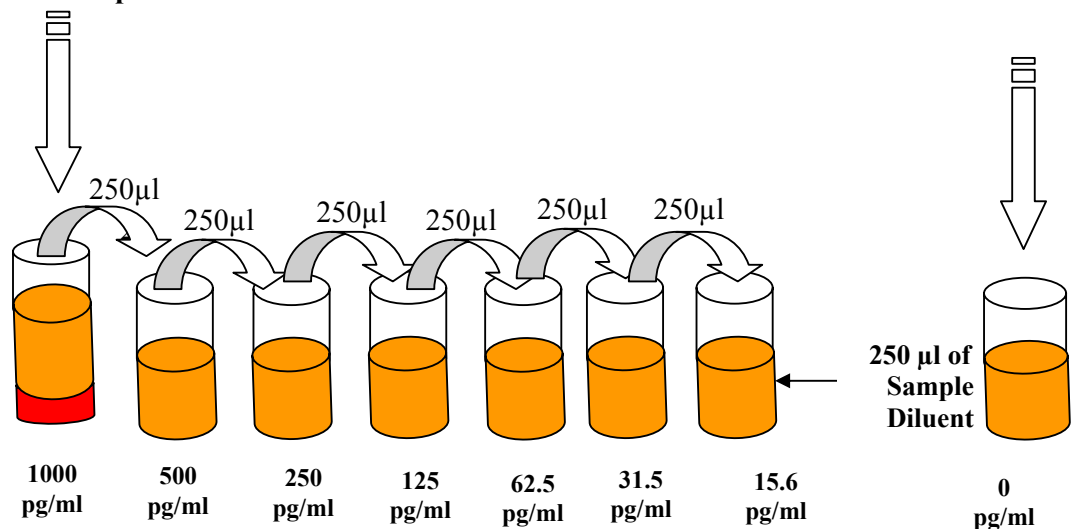
7. REAGENT AND SAMPLE PREPARATION

1. Bring all reagents and samples to room temperature (18-25°C) before use.
2. **Antibody coated plate:** Before opening the foil pouch, determine the number of strips required to test the desired number of samples, plus 16 wells needed for running standards and blanks in duplicate. Remove non-used strips from the plate-frame and return them to the foil pouch containing the desiccant for up to 1 month at 2-8°C.

3. Dilution of test standard:

- a) Take 100 µl of VEGF₁₆₅ from kit standard tube containing 6000 pg/ml of VEGF and pipet into Standard tube 1. Add 500 µl of sample diluent to obtain VEGF concentration of 1000 pg/ml in the first dilution tube (total volume 600 µl).
- b) Add 250 µl of Sample Diluent to all other 6 dilution tubes. Take 250 µl from the first tube (1000 pg/ml) and start 2 fold serial dilutions in dilution tubes as described in the figure by mixing several times with the pipet in each tube (Total of 7 dilution tubes).
- c) 300 µl of sample Diluent serves as zero standard (0 ng/ml) in tube 8.

100 µl of Standard (6000 pg/ml)
+
500 µl of Sample Diluent



Sample preparation and dilution: Dilution of samples not required for initial screening. Samples that exceed the measuring range should be diluted serially and measured again. Samples with absorbance values >1.900 can be serially diluted 1:2, 1:4, 1:8 or further if necessary in sample diluent. The dilution factor must be taken in account when calculating the results.

Store and dilute all samples in tubes or plates made of material with low binding surface, such as polypropylene.

Sample collection and storage: Serum, EDTA-anti-coagulated plasmas, cerebrospinal fluid and culture fluids are suitable for use in the assay (caution: separate plasma/serum and blood cells within 4 hours after collection, non-separated samples must be kept in temperatures from 2 to 8°C). Do not use grossly haemolyzed or lipemic specimens. If samples are to be run within 24 hours, they may be stored at 2-8°C; otherwise samples should be stored frozen (between -18 to -32°C, preferably < -70°C). Up to 3 freeze-thaw cycles have no effect on the VEGF levels of serum or plasma samples. Nonetheless, excessive freeze-thaw cycles should be avoided. Prior to the assay, frozen samples should be thawed as quickly as possible in tap water (18-25°C). Do not use 37°C or 56°C water bath for this purpose.

4. **Wash Buffer:** If the 20x concentrated Wash Buffer contains visible crystals, warm it at 37°C and mix gently until dissolved. Dilute 25 ml of Wash Buffer Concentrate with de-ionized or distilled water to yield 500 ml of 1x Wash Buffer.
5. Vortex mix **green colored Biotinylated antibody** solution gently before use.
6. Vortex mix **blue colored peroxidase (HRP) labeled avidin** gently before use.

Caution: TMB substrate (Tetramethylbenzidine) and the Stop solution (H₂SO₄) are toxic or corrosive and should be handled with care. Use gloves during handling.

8. TEST PROCEDURE SUMMARY

1. Prepare all reagents, samples and standards.

Dilution of samples not required at initial screening.



2. Add 100 µl standard (starting from 1000 pg/mL), test samples and sample diluent as a blank into the appropriate wells of the strips.

Incubate 3 hours at room temperature. Wash 5x.



3. Add 100 µl ready for use biotin antibody promptly to each well.

Incubate 1 hour at room temperature. Wash 5x.



4. Add 100 µl ready for use HRP-Streptavidin solution.

Incubate 30 minutes at room temperature. Wash 5x.



5. Add 50 µl TMB One-Step Substrate Reagent to each well.

Incubate 20 minutes at room temperature.



6. Add 25 µl Stop Solution to each well.

Read at 450 nm against *630 nm immediately.

**Correcting for optical imperfections in the microplates by subtracting $A_{630\text{ nm}}$ is recommended, but not an essential procedure.*



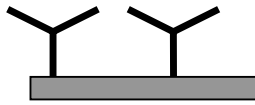
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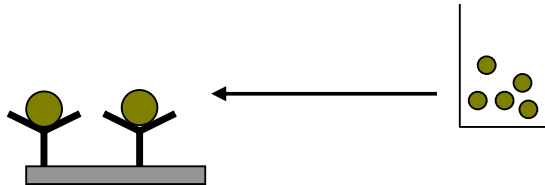
TEST PRINCIPLE



VEGF Antibody coated test well

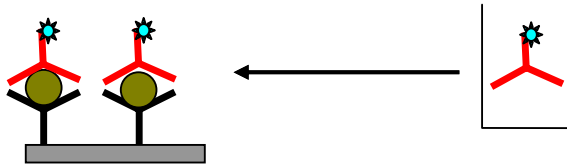
STEP 1

Add 100 μ L of standards and test samples to test well and incubate 3hrs at Room Temperature



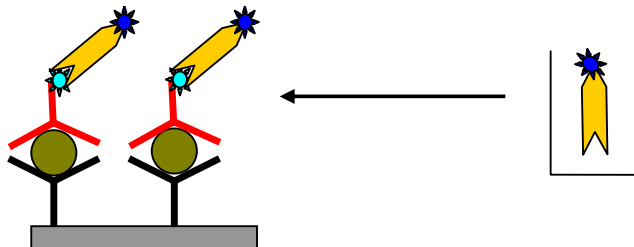
STEP 2

Add 100 μ L of Biotinylated VEGF antibody to test well and incubate 1 hr at Room Temperature



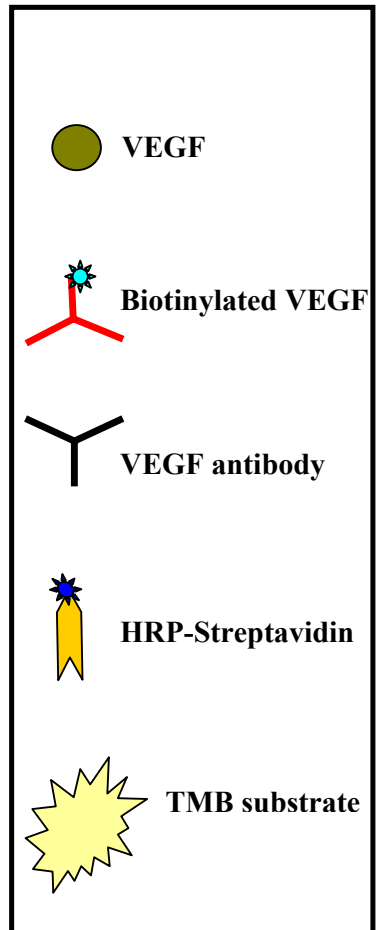
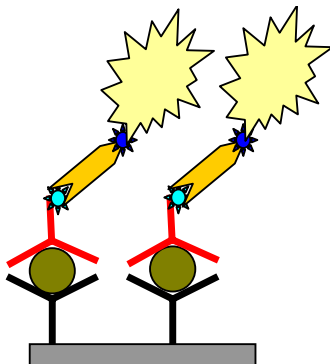
STEP 3

Add 100 μ L of HRP-Streptavidin to test well and incubate 30 minutes at Room Temperature



STEP 4

Add 50 μ L of TMB substrate to test well incubate 20 minutes at Room Temperature



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9. PROCEDURAL NOTES/LAB QUALITY CONTROL

- When not in use, kit components should be refrigerated. All reagents should be warmed to room temperature before use.
- 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 - 8°C to maintain plate integrity. Protect from humidity.
- Samples should be collected in pyrogen/endotoxin-free tubes.
- 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.
- When possible, avoid use of badly hemolyzed or lipemic sera. If large amounts of particulate matter are present, centrifuge or filter prior to analysis.
- It is recommended that all standards, controls and samples be run in duplicate.
- Samples that are >1000pg/mL should be diluted with *Sample Diluent*.
- When pipetting reagents, maintain a consistent order of addition from well-to-well. This ensures equal incubation times for all wells.
- Cover or cap all reagents when not in use.
- Do not use reagents after the kit expiration date.
- Read absorbencies within 20 minutes of assay completion.
- In-house controls should be run with every assay. If control values fall outside pre-established ranges, the accuracy of the assay is suspect.
- 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.
- Because *TMB Chromogen* is light sensitive, avoid prolonged exposure to light. Also avoid contact between *Stabilized Chromogen* and metal, or color may develop.

10. ASSAY PROCEDURE

1. Bring all reagents and samples to room temperature (18 - 25°C) before use. It is recommended that all standards and samples be run at least in duplicate. Leave some wells as a reagent blank (2 to 4 wells).

FIRST STEP: STANDARD, SAMPLES AND BLANK+ BIOTINYLATED ANTIBODY

2. Pipette 100 µl of sample and 100 µl of each diluted standard starting from 1000 pg/mL (see page 7) into appropriate wells. Pipette 100 µl of sample diluent to the wells which will be used as a blank. Incubate 3hrs at room temperature

SECOND STEP: BIOTINYLATED ANTIBODY

3. Wash 5x with 1x Wash Solution (300 µl each)

To wash: Empty plate contents. Use a multi-channel pipette to fill each well with 300 µl of wash buffer, then empty plate contents again. Repeat procedure 4 additional times for a total of FIVE washes. Gently blot plate onto paper towels or other absorbent material.

Note: For automated washing, aspirate all wells and wash 5 times with Wash Buffer. Blot plate onto paper towels or other absorbent material. Never let reaction wells dry. Continue to the next step without delay or interruption.

4. Promptly add 100 µl of Green colored Biotinylated VEGF detection antibody to all wells containing standards and samples. Tap the plate gently by hand to homogenize your mixture. Avoid touching to the reaction wells with pipet tip.

Incubate at room temperature for 1 hr without shaking.

THIRD STEP: HRP-CONJUGATED AVIDIN

5. Wash 5 times 5x as described in Step 2.

Add 100 µl of prepared HRP-conjugated avidin solution (Ready to use) to each well.
Incubate for 30 minutes at room temperature.

FOURTH STEP: TMB SUBSTRATE

6. Wash 5 times with 1x wash solution (300 µl each).

7. Using a multichannel pipet, promptly add 50 µl of TMB ready to use substrate reagent to each well.

Incubate for 20 minutes at room temperature in the dark.

9. Add 25 µl of Stop Solution to each well. Read at 450 nm within 15 minutes.

Correcting for optical imperfections in the microplates by subtracting $A_{630\text{ nm}}$ is recommended, but not an essential procedure.



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FIFTH STEP: READING AND CALCULATION

10. Calculate the mean of reagent blank absorbance values and subtract it from all test well values (standard and test samples). Mean reagent blank absorbance value at 450 nm should be less than 0.200.

11. Calculate your results against standard.

11. CALCULATION OF RESULTS

The standard curve must be determined individually for each experiment. Correct each absorbance value of all standards by subtracting from it the O.D. value of the reagent blank (Bl = only sample diluent). Calculate the mean absorbance value for each standard from the duplicates.

The standard curve is used to determine the amount of VEGF in an unknown sample. The standard curve is generated by plotting the average O.D. (450 nm) obtained for each of the standard concentrations on the vertical (Y) axis versus the corresponding VEGF concentration (pg/mL) on the horizontal (X) axis.

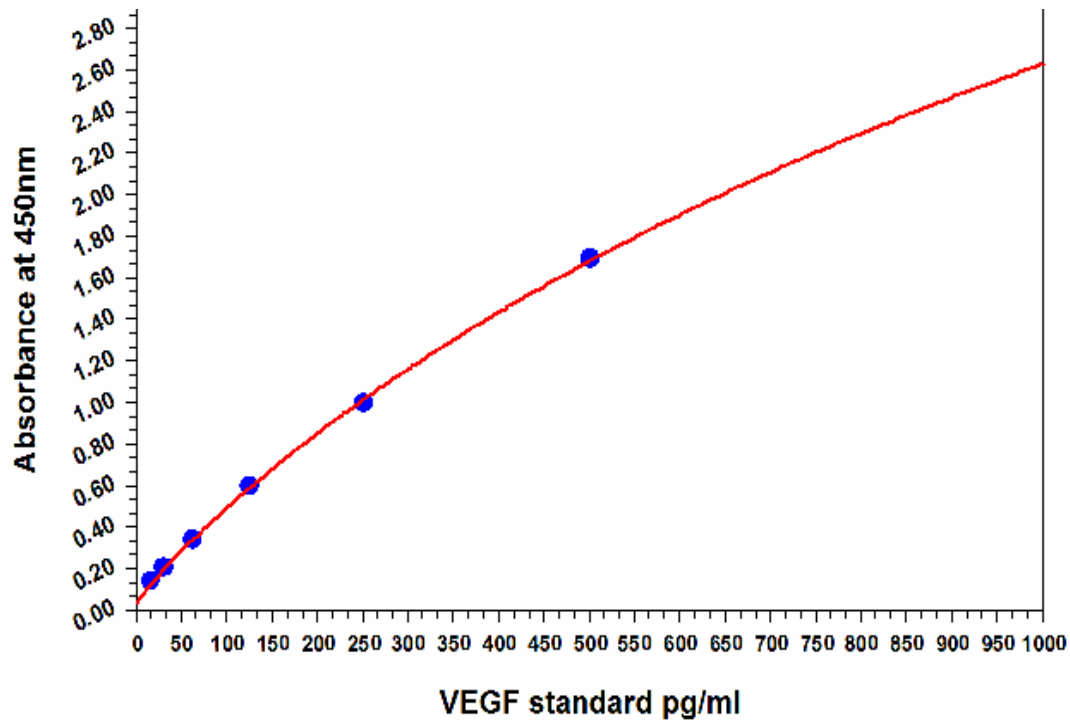
Construct the standard curve using graph paper or statistical software.

If samples generate values higher than the highest standard, dilute the samples with sample diluent and repeat the assay. Note that the concentration read from the standard curve must be multiplied by the dilution factor.

12. TYPICAL DATA

The following standard curve is obtained for various concentrations of VEGF₁₆₅ standard over the range of 0 to 1000 pg/mL.

Please note: The curve is provided for illustration only. A standard curve should be generated each time the assay is performed. Do not use these values in your calculations.



13. TEST PERFORMANCE

	VEGF
Assay range	15.62-1000 pg/ml
Standard curve points	500, 250, 125, 62.5, 31.25, 15.62 and 0 pg/ml.
Intra-Assay-Precision	≤6%
Inter-Assay-Precision	≤10%
Inter-Lot-Precision	≤12%
Cross-Reactivity	No cross-reactivity was observed with the following recombinant human proteins: IL-1 α , IL-1 β , IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12, IL-13, TNF α , TARC
Specificity	Recognizes both natural and recombinant human VEGF ₁₆₅ . Isoform VEGF ₁₂₁ cross-reacts 100% in the assay.
Sensitivity	<5 pg/ml.

14. REFERENCES

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4. Ferrara, N., Winer, J., and Burton, T. (1991). Aortic smooth muscle cells express and secrete vascular endothelial growth factor. *Growth Factors* 5, 141-148.
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7. Berse, B., Brown, L.F., Van de Water, L., Dvorak, H.F., and Senger, D.R. (1992). Vascular permeability factor (vascular endothelial growth factor) gene is expressed differentially in normal tissues, macrophages, and tumors. *Am. J. Pathol.* 3, 211-220.
8. Ladoux, A., and Frelin, C. (1993). Hypoxia is a strong inducer of vascular endothelial growth factor mRNA expression in the heart. *Biochem. Biophys. Res. Commun.* 195, 1005-1010.

LIABILITY

This kit is intended for research use only by personnel trained and qualified to carry out diagnostic or research activities.

If the recipient of this kit passes it on in any way to a third party, this instruction must be enclosed, and said recipient shall at own risk secure in favor of Orgenium Laboratories all limitations of liability herein.

Orgenium Laboratories shall not be responsible for any damages or loses due to using the kit in any way other than as expressly stated in these Instructions.

The liability of Orgenium Laboratories shall in no event exceed the commercial value of the kit.

Orgenium Laboratories shall under no circumstances be liable for indirect, special or consequential damages, including but not limited to loss of profit.



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15. TROUBLESHOOTING

Problem	Cause	Solution
Poor standard Curve	<ol style="list-style-type: none"> 1. Inaccurate pipetting or pipetting error 2. Improper standard dilution 	<p>Check pipettes and calibrate regularly.</p> <p>Vortex the stock before use and dilute carefully in an eppendorf tube.</p>
Low signal	<ol style="list-style-type: none"> 1. Shorter incubation than recommended 2. Inadequate reagent volumes or improper dilution or pipetting error 	<p>Ensure sufficient incubation time;</p> <p>Check pipettes and ensure correct performance.</p>
Large CV	Inaccurate pipetting and drying of wells during test procedure.	<p>Check pipettes</p> <p>Fill the wells promptly with wash buffer and reagents.</p>
High background	<ol style="list-style-type: none"> 1. Plate is washed insufficiently 2. Contaminated wash Buffer 3. Wash buffer volume is less than advised 	<p>Review the manual for proper wash. If using a plate washer, check that all ports are unobstructed and clean.</p> <p>Make a fresh wash buffer</p> <p>Use 300µl per well</p>
Low sensitivity	<ol style="list-style-type: none"> 1. Improper storage of the ELISA kit 2. Stop solution 3. Contamination of reagents 	<p>Store test kit components as advised in this user manual. Keep substrate solution protected from light.</p> <p>Stop solution should be added to each well before measure.</p> <p>Use clean sterile tips. Discard contaminated reagents.</p>