(FOR RESEARCH USE ONLY. DO NOT USE IT IN CLINICAL DIAGNOSTICS !)

Mouse VEGFR3/Flt4 (Vascular Endothelial Cell Growth Factor Receptor 3) CLIA Kit
Catalog No : E-CL-M0681
96T

This manual must be read attentively and completely before using this product.

If you have any problems, please contact our Technical Service Center for help (info in the header of each page).

Phone: 240-252-7368(USA) 240-252-7376(USA)
Email: techsupport@elabscience.com
Website: www.elabscience.com

Please refer to specific expiry date from label on the side of box.

Please kindly provide us with the lot number (on the outside of the box) of the kit for more efficient service.

Intended use
This CLIA kit applies to the in vitro quantitative determination of Mouse VEGFR3/Flt4 concentrations in serum, plasma and other biological fluids.

Specification

●Sensitivity: 9.38 pg/mL
●Detection Range: 15.63-1000 pg/mL
●Specificity: This kit recognizes Mouse VEGFR3/Flt4 in samples. No significant cross-reactivity or interference between Mouse VEGFR3/Flt4 and analogues was observed.
●Repeatability: Coefficient of variation is < 15%.

Test principle
This CLIA kit uses the Sandwich-CLIA principle. The micro CLIA plate provided in this kit has been pre-coated with an antibody specific to Mouse VEGFR3/Flt4. Standards or samples are added to the micro CLIA plate wells and combined with the specific antibody. Then a biotinylated detection antibody specific for Mouse VEGFR3/Flt4 and Avidin-Horseradish Peroxidase (HRP) conjugate are added successively to each micro plate well and incubated. Free components are washed away. The substrate solution is added to each well. Only those wells that contain Mouse VEGFR3/Flt4, biotinylated detection antibody and Avidin-HRP conjugate will appear fluorescence. The Relative light unit (RLU) value is measured by the Chemiluminescence immunoassay analyzer. The RLU value is positively associated with the concentration of Mouse VEGFR3/Flt4. You can calculate the concentration of Mouse VEGFR3/Flt4 in the samples by comparing the RLU value of the samples to the standard curve.

Kit components & Storage

An unopened kit can be stored at 2-8℃ for 1 month. If the kit is not used within 1 month, store the items separately according to the following conditions once the kit is received.

Item	Specifications	Storage
Micro CLIA Plate (Dismountable)	8 wells ×12 strips	-20℃, 6 months
Reference Standard	2 vials	
Concentrated Biotinylated Detection Ab (100×)	1 vial, 120 μL	
Concentrated HRP Conjugate (100×)	1 vial, 120 μL	-20°C(shading light), 6 months
Reference Standard & Sample Diluent	1 vial, 20 mL	4°C, 6 months
Biotinylated Detection Ab Diluent	1 vial, 14 mL	
HRP Conjugate Diluent	1 vial, 14 mL	
Concentrated Wash Buffer (25×)	1 vial, 30 mL	
Substrate Reagent A	1 vial, 5 mL	4°C(shading light)
Substrate Reagent B	1 vial, 5 mL	4°C(shading light)
Plate Sealer	5 pieces	
Product Description	1 copy	
Certificate of Analysis	1 copy	

Note: All reagent bottle caps must be tightened to prevent evaporation and microbial pollution.
The volume of reagents in partial shipments is a little more than the volume marked on the label, please use accurate measuring equipment instead of directly pouring into the vial(s).

Other supplies required

Chemiluminescence immunoassay analyzer

High-precision transfer pipette, EP tubes and disposable pipette tips

Incubator capable of maintaining 37℃

Deionized or distilled water

Absorbent paper

Loading slot for Wash Buffer

Note

1. Please wear lab coats, eye protection and latex gloves for protection. Please perform the experiment following the national security protocols of biological laboratories, especially when detecting blood samples or other bodily fluids.
2. A freshly opened CLIA Plate may appear to have a water-like substance, which is normal and will not have any impact on the experimental results.
3. Do not reuse the reconstituted standard, biotinylated detection Ab working solution, concentrated HRP conjugate working solution. The unspent undiluted concentrated biotinylated detection Ab (100×) and other stock solutions should be stored according to the storage conditions in the above table.
4. Do not mix or use components from other lots.
5. Change pipette tips in between adding standards, in between sample additions, and in between reagent additions. Also, use separate reservoirs for each reagent.

Sample collection

Serum: Allow samples to clot for 2 hours at room temperature or overnight at 2-8℃ before centrifugation for 15 min at 1000×g at 2-8℃. Collect the supernatant to carry out the assay. Blood collection tubes should be disposable and be non-endotoxin.

Plasma: Collect plasma using EDTA or heparin as an anticoagulant. Centrifuge samples for 15 min at 1000×g at 2-8℃ within 30 min of collection. Collect the supernatant to carry out the assay. Hemolysed samples are not suitable for CLIA assay!

Cell lysates: For adherent cells, gently wash the cells with moderate amount of pre-cooled PBS and dissociate the cells using trypsin. Collect the cell suspension into a centrifuge tube and centrifuge for 5 min at 1000×g. Discard the medium and wash the cells 3 times with pre-cooled PBS. For each 1×10^6 cells, add 150-250 μL of pre-cooled PBS to keep the cells suspended. Repeat the freeze-thaw process several times until the cells are fully lysed. Centrifuge for 10min at 1500×g at 2-8℃. Remove the cell fragments, collect the supernatant to carry out the assay. Avoid repeated freeze-thaw cycles.

Tissue homogenates: It is recommended to get detailed references from the literature before analyzing different tissue types. For general information, hemolysed blood may affect the results, so the tissues should be minced into small pieces and rinsed in ice-cold PBS (0.01M, pH=7.4) to remove excess blood thoroughly. Tissue pieces should be weighed and then homogenized in PBS (tissue weight (g): PBS (mL) volume=1:9) with a glass homogenizer on ice. To further break down the cells, you can sonicate the suspension with an ultrasonic cell disrupter or subject it to freeze-thaw cycles. The homogenates are then centrifuged for 5 min at 5000×g to get the supernatant.

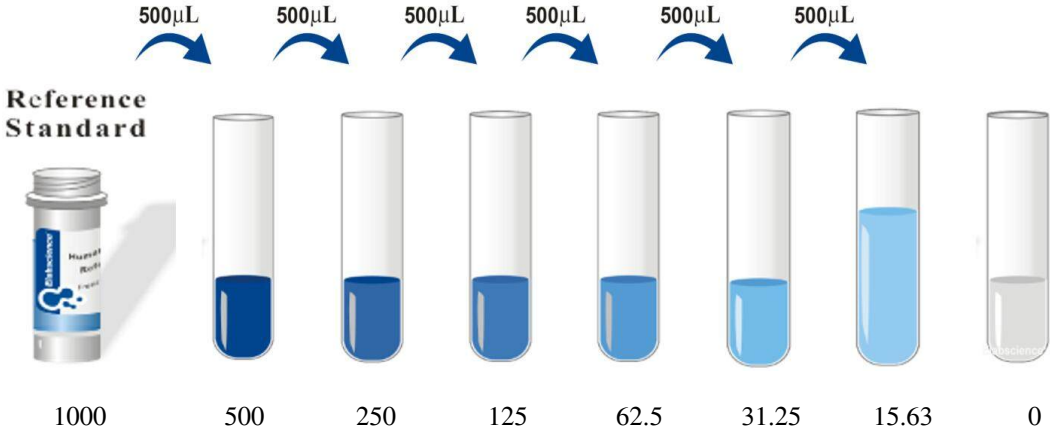
Cell culture supernatant or other biological fluids: Centrifuge samples for 20 min at 1000×g at 2-8℃. Collect the supernatant to carry out the assay.

Note for sample:

1. Samples should be assayed within 7 days when stored at 2-8℃, otherwise samples must be divided up and stored at -20℃ (≤1 month) or -80℃ (≤3 months). Avoid repeated freeze-thaw cycles.
2. Please predict the concentration before assaying. If the sample concentration is not within the range of the standard curve, users must determine the optimal sample dilutions for their particular experiments.
3. If the sample type is not included in the manual, a preliminary experiment is suggested to verify the validity.
4. If a lysis buffer is used to prepare tissue homogenates or cell culture supernatant, there is a possibility of causing a deviation due to the introduced chemical substance.
5. Some recombinant protein may be detected due to a mismatching with the coated antibody or detection antibody.

Reagent preparation

1. Bring all reagents to room temperature (18~25℃) before use.
2. **Wash Buffer:** Dilute 30 mL of Concentrated Wash Buffer with 720 mL of deionized or distilled water to prepare 750 mL of Wash Buffer. Note: if crystals have formed in the concentrate, warm it in a 40℃ water bath and mix it gently until the crystals have completely dissolved.
3. **Standard working solution:** Centrifuge the standard at 10,000×g for 1 min. Add 1.0 mL of Reference Standard &Sample Diluent, let it stand for 10 min and invert it gently several times. After it dissolves fully, mix it thoroughly with a pipette. This reconstitution produces a working solution of 1000 pg/mL. Then make serial dilutions as needed. The recommended dilution gradient is as follows: 1000, 500, 250, 125, 62.5, 31.25, 15.63, 0 pg/mL.
   Dilution method: Take 7 EP tubes, add 500uL of Reference Standard & Sample Diluent to each tube. Pipette 500uL of the 1000 pg/mL working solution to the first tube and mix up to produce a 500 pg/mL working solution. Pipette 500uL of the solution from the former tube into the latter one according to these steps. The illustration below is for reference. Note: the last tube is regarded as a blank. Don't pipette solution into it from the former tube.



4. **Biotinylated Detection Ab working solution:** Calculate the required amount before the experiment (100 μL/well). In preparation, slightly more than calculated should be prepared. Centrifuge the stock tube before use, dilute the 100× Concentrated Biotinylated Detection Ab to 1×working solution with Biotinylated Detection Ab Diluent.
5. **Concentrated HRP Conjugate working solution:** Calculate the required amount before the experiment (100 μL/well). In preparation, slightly more than calculated should be prepared. Dilute the 100× Concentrated HRP Conjugate to 1× working solution with Concentrated HRP Conjugate Diluent.
6. **Substrate Mixture Solution:** Calculate the required amount before the experiment (100μL/well). In preparation, slightly more than calculated should be prepared. Mix the Substrate Reagent A and B with equal volumes before use. Note: don't open the vial until you need it.

Assay procedure (A brief assay procedure is on the 11th page)

1. Add the **Standard working solution** to the first two columns: Each concentration of the solution is added in duplicate, to one well each, side by side (100 uL for each well). Add the samples to the other wells (100 uL for each well). Cover the plate with the sealer provided in the kit. Incubate for 90 min at 37℃. Note: solutions should be added to the bottom of the micro CLIA plate well, avoid touching the inside wall and causing foaming as much as possible.
2. Remove the liquid out of each well, do not wash. Immediately add 100 μL of **Biotinylated Detection Ab working solution** to each well. Cover with the Plate sealer. Gently mix up. Incubate for 1 hour at 37°C.
3. Aspirate or decant the solution from each well, add 350 uL of **wash buffer** to each well. Soak for 1~2 min and aspirate or decant the solution from each well and pat it dry against clean absorbent paper. Repeat this wash step 3 times. Note: a microplate washer can be used in this step and other wash steps.
4. Add 100 μL of **HRP Conjugate working solution** to each well. Cover with the Plate sealer. Incubate for 30 min at 37°C.
5. Aspirate or decant the solution from each well, repeat the wash process for five times as conducted in step 3.
6. Add 100 μL of **Substrate Mixture Solution** to each well. Cover with a new plate sealer. Incubate for not more than 5 minutes at 37°C. Protect the plate from light.
7. Determine the RLU value of each well at once.

Calculation of results

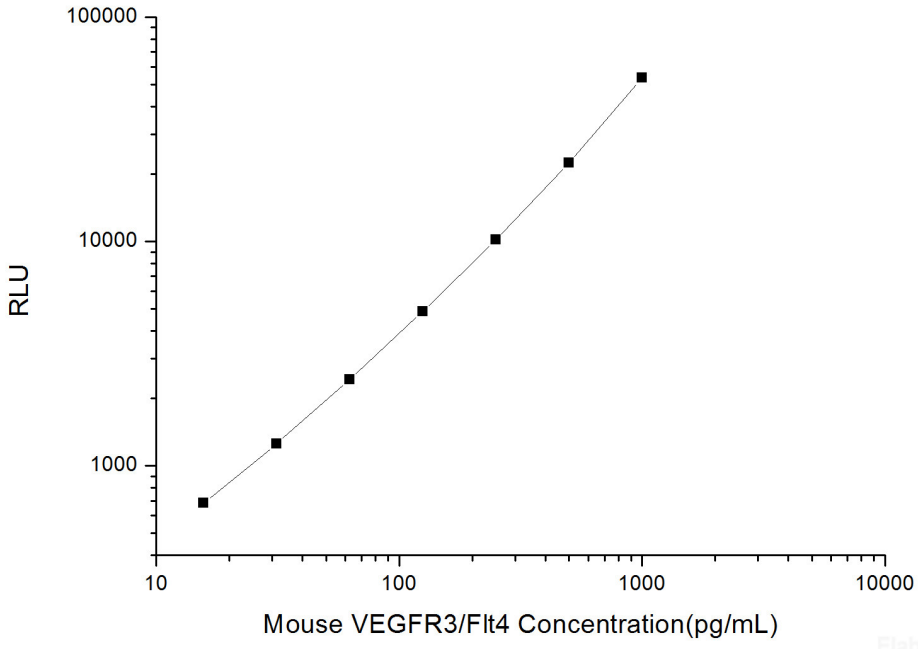
Average the duplicate readings for each standard and samples, then subtract the average zero standard RLU. Plot a four-parameter logistic curve on log-log graph paper, with standard concentration on the x-axis and RLU values on the y-axis.

If the samples have been diluted, the concentration calculated from the standard curve must be multiplied by the dilution factor. If the RLU of the sample surpasses the upper limit of the standard curve, you should re-test it with an appropriate dilution. The actual concentration is the calculated concentration multiplied by the dilution factor.

Typical data

As the RLU values of the standard curve may vary according to the conditions of the actual assay performance (e.g. operator, pipetting technique, washing technique or temperature effects), the operator should establish a standard curve for each test. Typical standard curve and data is provided below for reference only.

Concentration(pg/mL)	1000	500	250	125	62.5	31.25	15.63	0
RLU 1	51187	21711	10668	4596	2483	1344	661	24
RLU 2	56163	23267	9754	5206	2423	1218	755	26
Average RLU	53675	22489	10211	4901	2453	1281	708	25
Corrected RLU	53650	22464	10186	4876	2428	1256	683	--



Precision

Intra-assay Precision (Precision within an assay): 3 samples with low, mid range and high level Mouse VEGFR3/Flt4 were tested 20 times on one plate, respectively.

Inter-assay Precision (Precision between assays): 3 samples with low, mid range and high level Mouse VEGFR3/Flt4 were tested on 3 different plates, 20 replicates in each plate.

	Intra-assay Precision			Inter-assay Precision		
Sample	1	2	3	1	2	3
n	20	20	20	20	20	20
Mean(pg/mL)	47.54	153.41	358.82	43.42	151.94	390.32
Standard deviation	4.30	14.41	32.33	4.33	17.17	27.21
CV (%)	9.05	9.39	9.01	9.97	11.30	6.97

Recovery

The recovery of Mouse VEGFR3/Flt4 spiked at three different levels in samples throughout the range of the assay was evaluated in various matrices.

Sample Type	Range (%)	Average Recovery (%)
Serum (n=5)	84-95	90
EDTA plasma (n=5)	94-108	102
Cell culture media (n=5)	94-108	101

Linearity

Samples were spiked with high concentrations of Mouse VEGFR3/Flt4 and diluted with Reference Standard & Sample Diluent to produce samples with values within the range of the assay.

		Serum (n=5)	EDTA plasma(n=5)	Cell culture media(n=5)
1:2	Range (%)	96-112	90-104	98-112
	Average (%)	102	97	106
1:4	Range (%)	93-105	101-118	102-114
	Average (%)	99	109	108
1:8	Range (%)	88-100	98-115	95-107
	Average (%)	95	105	102
1:16	Range (%)	85-96	98-114	93-109
	Average (%)	90	104	100

Troubleshooting

Problem	Causes	Solutions
Poor standard curve	Inaccurate pipetting	Check pipettes.
	Improper standard dilution	Ensure briefly spin the vial of standard and dissolve the powder thoroughly by a gentle mix.
	Wells are not completely aspirated	Completely aspirate wells between steps.
Low fluorescence	Insufficient incubation time	Ensure sufficient incubation time.
	Incorrect assay temperature	Use recommended incubation temperature. Bring substrate to room temperature before use.
	Inadequate reagent volumes	Check pipettes and ensure correct preparation.
	Improper dilution	
Large CV	Inaccurate pipetting	Check pipettes.
High background	Concentration of target protein is too high	Use recommended dilution factor.
	Plate is insufficiently washed	Review the manual for proper wash. If using a plate washer, check that all ports are unobstructed.
	Contaminated wash buffer	Prepare fresh wash buffer.
Low sensitivity	Improper storage of the CLIA kit	All the reagents should be stored according to the instructions.
	Too long incubation time	Ensure precise incubation time.

SUMMARY

1. Add 100 μL standard or sample to each well. Incubate for 90 min at 37°C.
2. Remove the liquid. Add 100 μL Biotinylated Detection Ab. Incubate for 1 hour at 37°C.
3. Aspirate and wash 3 times.
4. Add 100 μL HRP Conjugate. Incubate for 30 min at 37°C.
5. Aspirate and wash 5 times.
6. Add 100 μL Substrate Mixture Solution. Incubate 5 minutes at 37℃.
7. Determine the RLU value.
8. Calculation of results.

Declaration

1. Limited by current conditions and scientific technology, we can't conduct comprehensive identification and analysis on all the raw material provided. So there might be some qualitative and technical risks for users using the kit.
2. The final experimental results will be closely related to the validity of products, operational skills of the operators and the experimental environments. Please make sure that sufficient samples are available.
3. To get the best results, please only use the reagents supplied by the manufacturer and strictly comply with the instructions!
4. Incorrect results may occur because of incorrect operations during the reagents preparation and loading, as well as incorrect parameter settings of the Chemiluminescence immunoassay analyzer. Please read the instructions carefully and adjust the instrument prior to the experiment.
5. Even the same operator might get different results in two separate experiments. In order to get reproducible results, the operation of every step in the assay should be controlled.
6. Every kit has strictly passed QC test. However, results from end users might be inconsistent with our data due to some variables such as transportation conditions, different lab equipments, and so on. Intra-assay variance among kits from different batches might arise from the above reasons, too.