

Supplementary Document 1

Human Follistatin Like Protein 3 (FSTL3) ELISA Kit

Catalogue Number: JYM2501Hu

- **For research use only.**
- **For the quantitative in vitro determination of FSTL3 concentrations in Human serum, plasma, culture media or any biological fluid.**
- **Expiration date: six months .**
- **Storage: 2-8c.**

Principle

This ELISA kit uses Sandwich-ELISA as the method. The Microelisa stripplate provided in this kit has been pre-coated with an antibody specific to FSTL3. Standards or samples are added to the appropriate Microelisa stripplate wells and combined to the specific antibody. Then a Horseradish Peroxidase (HRP)- conjugated antibody specific for FSTL3 is added to each Microelisa stripplate well and incubated. Free components are washed away. The TMB substrate solution is added to each well. Only those wells that contain FSTL3 and HRP conjugated FSTL3 antibody will appear blue in color and then turn yellow after the addition of the stop solution. The optical density (OD) is measured spectrophotometrically at a wavelength of 450 nm. The OD value is proportional to the concentration of FSTL3. You can calculate the concentration of FSTL3 in the samples by comparing the OD of the samples to the standard curve.

Materials provided with the kit

	Materials provided with the kit	48 determinations	96 determinations	Storage
1	User manual	1	1	R.T.
2	Closure plate membrane	2	2	R.T.
3	Sealed bags	1	1	R.T.
4	Microelisa stripplate	1	1	2-8c
5	Standard : 9000pg/ml	0.5mlx1 bottle	0.5mlx1 bottle	2-8c
6	Standard diluent	1.5mlx1 bottle	1.5mlx1 bottle	2-8c
7	HRP-Conjugate reagent	3mlx1 bottle	6mlx1 bottle	2-8c
8	Sample diluent	3mlx1 bottle	6mlx1 bottle	2-8c
9	Chromogen Solution A	3mlx1 bottle	6mlx1 bottle	2-8c
10	Chromogen Solution B	3mlx1 bottle	6mlx1 bottle	2-8c
11	Stop Solution	3mlx1 bottle	6mlx1 bottle	2-8c
12	wash solution	20ml(20X)X1bottle	20ml(30X)X1bottle	2-8c

Sample preparation

1. Serum preparation

After collection of the whole blood, allow the blood to clot by leaving it undisturbed at room temperature. This usually takes 10-20 minutes. Remove the clot by centrifuging at 2,000-3,000 rpm for 20 minutes. If precipitates appear during reservation, the sample should be centrifuged again.

2. Plasma preparation

Collect the whole blood into tubes with anticoagulant (EDTA or citrate). After incubated at room temperature for 10-20 minutes, tubes are centrifuged for 20 min at 2,000-3,000 rpm. Collect the supernatant carefully as plasma samples. If precipitates appear during reservation, the sample should be centrifuged again.

3. Urine samples

Collect urine into aseptic tubes. Collect the supernatant carefully after centrifuging for 20 min at 2,000-3,000 rpm. If precipitates appear during reservation, the sample should be centrifuged again. The preparation procedure of cerebrospinal fluid and pleuroperitoneal fluid is the same as that of urine sample.

4. Cell samples

If you want to detect the secretions of cells, collect culture supernatant into aseptic tubes.

Collect the supernatant carefully after centrifuging for 20 min at 2,000-3,000 rpm. If you want to detect intracellular components, dilute the cells to 1X100/ml with PBS (pH 7.2-7.4). The cells were destroyed to release intracellular components by repeated freezing and thawing. Collect the supernatant carefully after centrifuging for 20 min at 2,000-3,000 rpm. If precipitates appear during resuspension, the sample should be centrifuged again.

5. Tissue samples

Tissue samples are cut, weighed, frozen in liquid nitrogen and stored at -80 °C for future use. The tissue samples were homogenized after adding PBS (pH 7.4). Samples should be operated at 4°C. Collect the supernatant carefully after centrifuging for 20 min at 2,000-3,000 rpm. Aliquot the supernatant for ELISA assay and future use.

Notes:

1. Sample extraction and ELISA assay should be performed as soon as possible after sample collection. The samples should be extracted according to the relevant literature. If ELISA assay can not be performed immediately, samples can be stored at -20 °C. Repeated freeze-thaw cycles should be avoided.
2. Our kits can not be used for samples with NaN₃ which can inhibit the activity of HRP.

Procedure

1. Dilution of Standards

Ten wells are set for standards in a Microelisa stripplate. In Well 1 and Well 2, 100µl Standard solution and 50µl Standard Dilution buffer are added and mixed well. In Well 3 and Well 4, 100µl solution from Well 1 and Well 2 are added respectively. Then 50µl Standard Dilution buffer are added and mixed well. 50µl solution is discarded from Well 3 and Well 4. In Well 5 and Well 6, 50µl solution from Well 3 and Well 4 are added respectively. Then 50µl Standard Dilution buffer are added and mixed well. In Well 7 and Well 8, 50µl solution from Well 5 and Well 6 are added respectively. Then 50µl Standard Dilution buffer are added and mixed well. In Well 9 and Well 10, 50µl solution from Well 7 and Well 8 are added respectively. Then 50µl Standard Dilution buffer are added and mixed well. 50µl solution is discarded from Well 9 and Well 10. After dilution, the total volume in all the wells are 50µl and the concentrations are 6000pg/ml, 4000pg/ml, 2000pg/ml, 1000pg/ml and 500pg/ml, respectively.



9000pg/ml 6000pg/ml 4000pg/ml 2000pg/ml 1000pg/ml 500pg/ml

2. In the Microelisa stripplate, leave a well empty as blank control. In sample wells, 40µl Sample dilution buffer and 10µl sample are added (dilution factor is 5). Samples should be loaded onto the bottom without touching the well wall. Mix well with gentle shaking.
3. Add 50 µl HRP-Conjugate reagent to each well except the blank control well.
4. Incubation: incubate 30 min at 37c after sealed with Closure plate membrane.
5. Dilution: dilute the concentrated washing buffer with distilled water (30 times for 96T and 20 times for 48T).
6. Washing: carefully peel off Closure plate membrane, aspirate and refill with the wash solution. Discard the wash solution after resting for 30 seconds. Repeat the washing procedure for 5 times.
7. Coloring: Add 50 µl Chromogen Solution A and 50 µl Chromogen Solution B to each well, mix with gently shaking and incubate at 37 c for 10 minutes. Please avoid light during coloring.
8. Termination: add 50 µl stop solution to each well to terminate the reaction. The color in the well should change from blue to yellow.
9. Read absorbance O.D. at 450nm using a Microtiter Plate Reader. The OD value of the blank control well is set as zero. Assay should be carried out within 15 minutes after adding stop solution.

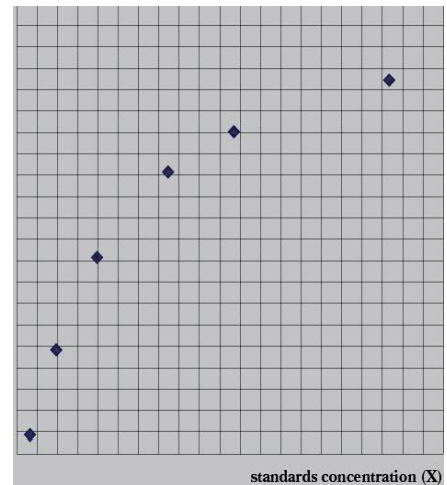
Notes:

1. Store the kit at 4 °C upon receipt. The kit should be equilibrated to room temperature before the assay. Remove any unneeded strips from Human FSTL3 Antibody-Coated plate, reseal them in zip-lock foil and keep at 4 °C.
2. Precipitates may appear in concentrated washing buffer. Please heat the buffer to dissolve all the precipitates, which will not affect the results.
3. Accurate pipette should be used to avoid experimental error. Samples should be added to the Microplate in less than 5 minutes. If a large number of samples are included, multiple channel pipette is recommended.
4. Standard curve should be included in every assay. Replicate wells are recommended. If the OD value of the sample is greater than the first well of standards, please dilute the sample (n times) before test. When calculating the original FSTL3 concentration, please multiply the total dilution factor (XnX5).
5. In order to avoid cross-contamination, Microplate sealers are for one-time use only.
6. Please keep Substrate away from light.
7. All the operation should be accordance with the manufacturer's instructions strictly. The results determined by the Microtiter Plate Reader.
8. All the samples, washing buffer and wastes should be treated as infectious agents.

9. Reagents from different lots should not be mixed.

Calculation of Results

Known concentrations of Human FSTL3 Standard and its corresponding reading OD is plotted on the log scale (x-axis) and the log scale (y-axis) respectively. The concentration of Human FSTL3 in sample is determined by plotting the sample's O.D. on the Y-axis. The original concentration is calculated by multiplying the dilution factor.



Kit performance

1. Correlation coefficient (R) of linear regression of the samples is more than 0.92
2. The difference in intra-assay and inter-assay is less than 9% and 15% respectively.

Supplementary Document 2

Table 1. Disease-free survival

Characteristics	Total (N)	HR (95% CI) Univariate analysis	P-value Univariate analysis	HR (95% CI) Multivariate analysis	P-value Multivariate analysis
T stage (T3&T4 vs. T1&T2)	345	2.089 (1.192–3.660)	0.010	1.736 (0.952–3.167)	0.072
N stage (N1&N2&N3 vs. N0)	334	1.807 (1.075–3.036)	0.025	1.461 (0.843–2.532)	0.176
M stage (M1 vs. M0)	333	2.438 (1.221–4.870)	0.012	2.096 (1.030–4.263)	0.041
FSTL3 expression (High vs. Low)	349	1.502 (0.979–2.303)	0.062	1.515 (0.973–2.358)	0.066

HR, hazard ratio; CI, confidence interval

Table 2. Overall survival

Characteristics	Total (N)	HR (95% CI) Univariate analysis	P-value Univariate analysis	HR (95% CI) Multivariate analysis	P-value Multivariate analysis
T stage (T3&T4 vs. T1&T2)	362	1.719 (1.131–2.612)	0.011	1.450 (0.919–2.290)	0.111
N stage (N1&N2&N3 vs. N0)	352	1.925 (1.264–2.931)	0.002	1.661 (1.058–2.607)	0.028
M stage (M1 vs. M0)	352	2.254 (1.295–3.924)	0.004	1.866 (1.057–3.293)	0.031
FSTL3 expression (High vs. Low)	370	1.199 (0.862–1.667)	0.282		

HR, hazard ratio; CI, confidence interval

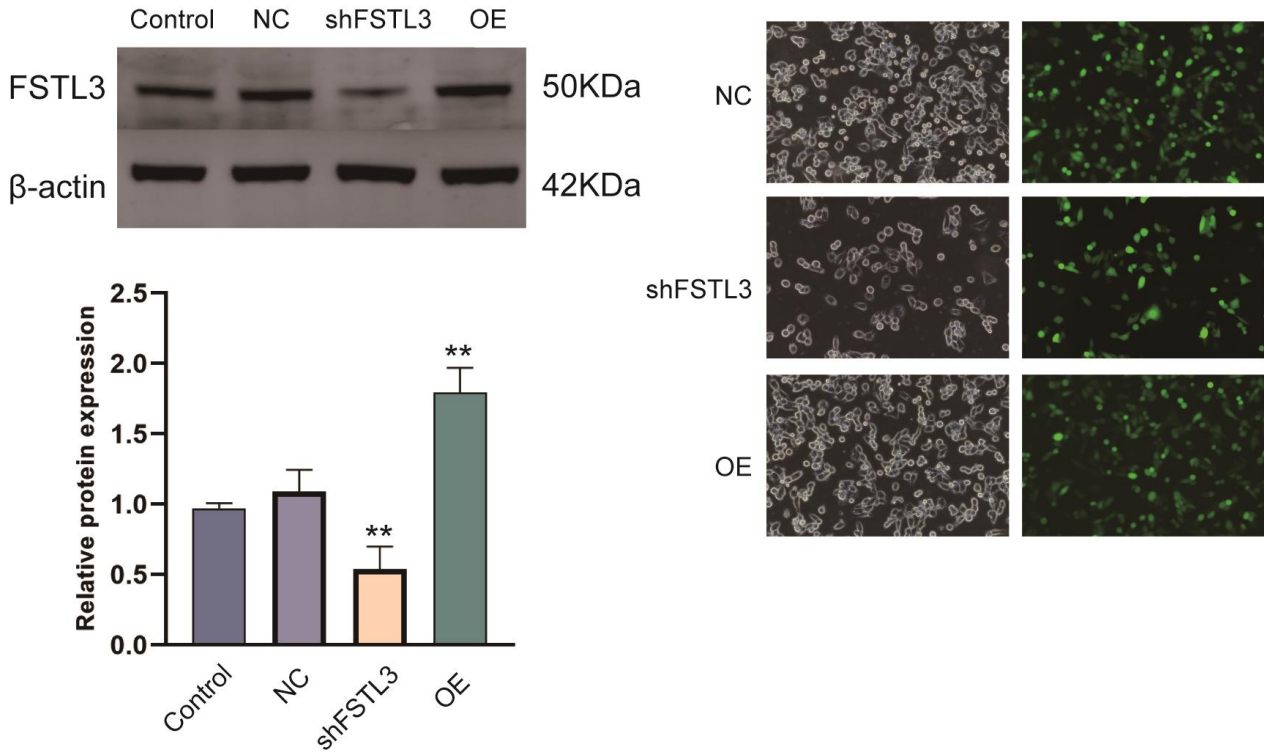
Supplementary Document 2

Table 3. Disease-free interval

Characteristics	Total (N)	HR (95% CI) Univariate analysis	P-value Univariate analysis	HR (95% CI) Multivariate analysis	P-value Multivariate analysis
T stage (T3&T4 vs. T1&T2)	364	1.705 (1.095–2.654)	0.018	1.382 (0.863–2.213)	0.178
N stage (N1&N2&N3 vs. N0)	354	1.640 (1.075–2.501)	0.022	1.422 (0.912–2.218)	0.121
M stage (M1 vs. M0)	353	2.224 (1.194–4.144)	0.012	1.922 (1.017–3.632)	0.044
FSTL3 expression (High vs. Low)	372	1.486 (1.036–2.130)	0.031	1.507 (1.041–2.183)	0.030

HR, hazard ratio; CI, confidence interval

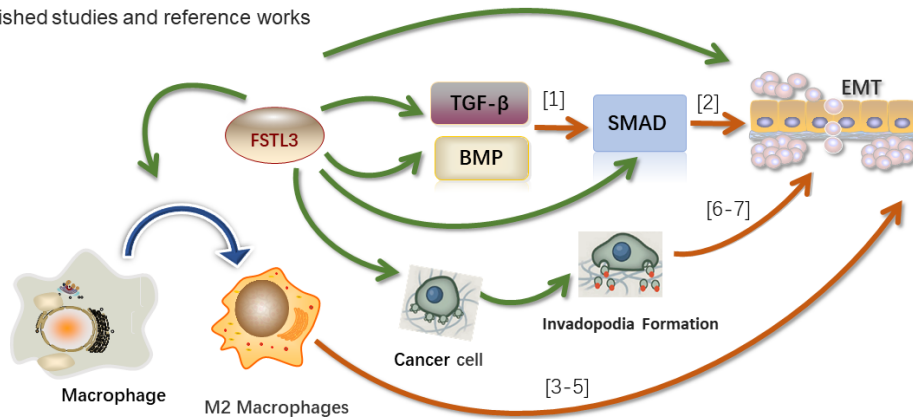
Supplementary Document 3



Supplementary Document 4

→ The content of this study

→ Published studies and reference works



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