Anti-Ficolin-2 and Anti-Ficolin-3 Autoantibody Detection by ELISA

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Abstract

Ficolins are recognition proteins of the lectin pathway of the complement system and also play an important role in innate immunity and in the maintenance of tissue homeostasis. They deserve special attention in the context of autoimmunity since they are involved in the uptake of dying cells. Because the monitoring of systemic lupus erythematosus (SLE) patients is particularly difficult, it is crucial to find new relevant serum biomarkers. The ability to detect autoantibodies in the patients'sera provides a diagnostic and prognostic advantage. We describe in this chapter quantitative enzyme linked immunosorbent assays (ELISA) to detect the presence of autoantibodies targeting ficolin-2 and ficolin-3 in human sera. Recombinant ficolins produced in a mammalian expression system are used as coating antigens. The described in-house ELISAs provide a valuable tool to efficiently quantify anti-ficolins autoantibodies in the sera of SLE patients.

Key words: ficolins, anti-ficolins autoantibodies, ELISA, systemic lupus erythematosus, lupus nephritis

1. Introduction

Ficolins (ficolin-1, -2, -3) are recognition proteins of the lectin pathway of the complement system and are also a part of the defense collagens family. They play an important role in innate immunity and in the maintenance of tissue homeostasis, thanks to its capacity to bind specific pathogen- and apoptotic cell–associated molecular patterns [1]. Ficolins deserve special attention in the context of autoimmunity since they are involved in the uptake of dying cells and could play a role in the prevention of autoimmune pathologies such as systemic lupus erythematosus (SLE).

An imbalance in complement, either by insufficient or excessive activity, can be associated with several pathological processes. In this context, the presence of autoantibodies targeting complement components has been described [2], as illustrated for anti-C1q autoantibodies initially quantified in the serum of patients with SLE, but also recently associated with autoimmune thyroid diseases and with anti-phospholipid syndrome. Moreover, autoantibodies against several complement recognition proteins, including C1q, have been reported to contribute to the development of SLE, supporting the hypothesis that the complement system is deeply involved in the pathogenesis of this disease [3].

Limited knowledge of the mechanisms of generation of autoantibodies in most autoimmune diseases presents a challenge for both diagnosing the disease and understanding the pathogenesis. However, the ability to detect autoantibodies in the patients'sera provides a diagnostic and prognostic advantage.

The course of SLE is unpredictable, thus making the monitoring of patients particularly difficult, especially regarding possible life-threatening organ involvement. Because the

associations between antibody profile and clinical symptoms are unclear, it is crucial to find new relevant diagnostic and/or prognostic biomarkers.

In a previous Japanese study, autoantibodies against ficolin-3 have been identified in the sera of some SLE patients with a low prevalence, using a non-quantitative technique [4]. We describe in this chapter quantitative enzyme linked immunosorbent assays (ELISA) to detect the presence of autoantibodies targeting against ficolin-2 and ficolin-3 in human sera. Recombinant ficolins produced in a mammalian expression system are used as coating antigens. ELISA is a powerful method for detecting and quantifying a specific protein (antigen or antibody) in a complex mixture, and is commonly used in medical laboratories. However the development and optimization of a specific ELISA always requires adjustments to set-up a reliable protocol.

The described in-house ELISAs provide a valuable tool to efficiently quantify anti-ficolins autoantibodies in the sera of patients with SLE but also with other autoimmune pathologies.

2. Materials

2.1 Ficolin production

1. Ficolin-2 and ficolin-3 plasmid expression vectors (*see* **Note 1**).
2. Mammalian expression plasmid: pcDNA3.1™(+) (ThermoFisher).
3. Chinese hamster ovary (CHO-K1) cells (American Type Culture Collection CCL-61™).
4. Cell culture equipment: laminar flow hood, CO_2 cell incubator, single-use flasks and pipettes.
5. Cell culture media: OptiMEM™ (ThermoFisher), D-MEM/F12 (1/1) with GlutaMAX™-I medium (ThermoFisher) supplemented with 10% heat-inactivated fetal calf serum (FCS) (*see* **Note 2**), CD CHO serum-free medium (ThermoFisher).

6. Transfection reagent: Lipofectamine™ 2000 (ThermoFisher).
7. Antibiotics and additives: Geneticin™ sulfate (G418), stock solution 50 mg/mL (ThermoFisher) ; glutamine (200 mM) (ThermoFisher) ; ascorbic acid (Sigma-Aldrich), stock solution 5 mg/mL in sterile H_2O.
8. HiSpeed Plasmid Midi Kit (Qiagen).
9. Affinity chromatography column for ficolin-2 purification: N-acetylcysteine (CysNAc)-Sepharose prepared by coupling 5 g CysNAc (Sigma-Aldrich) to 25 mL divinyl sulfone activated Sepharose CL-4B activated as described by Krarup et al [5].
10. Affinity chromatography column for ficolin-3 purification: acetylated BSA (AcBSA)-Sepharose prepared by coupling 125 mg acetylated BSA (Sigma-Aldrich) to 15 mL CNBr-activated Sepharose 4B (GE Healthcare), as described by Jacquet et al [6].
11. CysNAc- or AcBSA-Sepharose equilibration buffer and ficolin-2 or -3 storage buffer: 20 mM Tris-HCl, 145 mM NaCl, 5 mM $CaCl_2$, pH 7.4. For 1 L, weigh 2.42 g Tris base, 8.47 g NaCl, 0.735g calcium chloride dihydrate and dissolve in about 950 mL distilled water. Adjust the pH to 7.4 with HCl and complete to 1 L.
12. CysNAc-Sepharose elution buffer: 0.3 M N-acetylglucosamine. Dissolve 16.59 g in 250 mL equilibrium buffer:
13. AcBSA-Sepharose elution buffer: 1 M CH_3COONa and 10 mM EDTA (pH 7.4). For 250 mL, weigh 34 g sodium acetate trihydrate, add 6.25 ml EDTA (0.4 M solution, *see* **Note 3**) and dissolve in about 225 mL distilled water. Adjust the pH to 7.4 and complete to 250 mL.
14. CysNAc- or AcBSA-Sepharose regeneration buffer: 20 mM Tris-HCl, 1 M NaCl, 10 mM EDTA, pH 7.4. For 1 L, weigh 2.42 g Tris base, 58.44 g NaCl, add 25 mL EDTA (0.4 M solution,*see* **Note 3**) and dissolve in about 950 mL distilled water. Adjust the pH to 7.4 and complete to 1 L.

15. 10% (w/v) sodium azide (NaN_3) in distilled water (*see* **Note 4**).
16. Protein concentration: Amicon® Ultra-4 and -15 centrifugal filters (30K).

2.2 Anti-ficolin autoantibody ELISA

All solutions are prepared using distilled water. All reagents are stored at room temperature (unless otherwise indicated).

1. Nunc microtiter plates: flat-bottomed 96 microwells plates, Maxisorp (ThermoFisher) (*see* **Note 5**).
2. Absorbance-based microplate reader: Spectrophotometer (Biotek ELx800).
3. Coating buffer: 15 mM Na_2CO_3, 35 mM $NaHCO_3$ (pH 9.6).

   Weigh 0.159 g Na_2CO_3 and 0.293 g $NaHCO_3$ and transfer to a glass baker, add about 90 mL water, mix and adjust pH if necessary. Adjust the volume to 100 mL with distilled water.

   The coating buffer can be stored one week at 4°C.
4. Phosphate-Buffered Saline: PBS.

   Dissolve one PBS tablet (Gibco) in 500 mL distilled water.
5. Washing buffer: PBS-T.

   Phosphate-Buffered Saline (PBS) containing 0.1% TWEEN-20 (w/v) (Sigma-Aldrich).

   Weigh 0.5 g TWEEN-20, transfer to a glass beaker and add PBS to a volume of 500 mL.
6. Blocking solution: PBS-T containing 1% BSA (w/v) (Sigma-Aldrich).

   Weigh 1g BSA, transfer to a glass beaker and add PBS-T to a volume of 100 mL.
7. Sera and antibodies diluent buffer: PBS-T-BSA.

   PBS-T containing 0.1% BSA (w/v) and 0.1% TWEEN-20 (w/v).

   Weigh 0.1 g BSA, transfer to a glass beaker and add PBS-T to a volume of 100 mL.

8. Antibody: horseradish peroxidase (HRP)-conjugated goat polyclonal anti-human IgG (The Binding Site, dilution 1:15,000). Add 1 µL of antibody in 15 mL of PBS-T-BSA in a glass beaker.
9. Chromogenic HRP-microwell substrate: tetramethylbenzidine substrate (TMB) (Tebu-bio Laboratories). It is stored at 4°C and protected from light (*see* **Note 6**).
10. Stop solution: H_2SO_4 1N.

    Add 2.7 mL pure H_2SO_4 in 100 mL distilled water under a laminar flow hood.

3. Methods

3.1 Ficolins production

Ficolin-2 and ficolin-3 are expressed in stably transfected CHO-K1 cells and the recombinant proteins purified from the cell culture supernatants using a one-step affinity chromatography [6, 7]. The purification steps are performed at 4°C.

3.1.1 Production of recombinant ficolin-2

1. Clone the cDNA encoding full-length ficolin-2 into the multiple cloning site of the pcDNA3.1(+) expression vector.

2. Amplify the plasmid using a plasmid Midiprep DNA kit (recovery in 100 µL sterile water).

3. Cultivate CHO-K1 cells in D-MEM/F12 (1/1) with GlutaMAX I medium supplemented with 10% heat-inactivated FCS.

3. Mix the plasmid with Lipofectamine 2000 and OptiMEM medium and transfect CHO cells (24 µg plasmid for 3 x 10^6 cells in a 10-cm culture plate), according to the manufacturer's instructions. Change the medium 6 h after transfection.

4. Start selection of geneticin resistant cells 48 h after transfection by cultivating the cells in D-MEM/F12 (1/1) with GlutaMAX I medium supplemented with 10% heat-inactivated FCS in the presence of 600 µg/mL G418 and 50 µg/mL ascorbic acid (*see* **Note 7**).

5. After 3-4 weeks, subclone selected surviving colonies in 24-well tissue culture plates and assay supernatants for ficolin production by Western blot analysis [7].

6. Select the highest producer clone and expand it in 175-cm^2 flasks in MEM/F12 (1/1) with GlutaMAX I medium supplemented with 10% heat-inactivated FCS, 300 µg/mL G418 and 50 µg/mL ascorbic acid. Harvest the cell culture supernatant and replace with fresh medium every sixth day for 18 days.

7. Centrifuge the cell culture supernatant at 3,500 rpm (2250 g) for 10 min to eliminate cell debris (*see* **Note 8**).

8. Load the culture supernatant on the CysNAc-Sepharose column at a flow rate of 7-10 mL/min, wash with equilibrium buffer (10 column volumes). Elute bound ficolin-2 by applying the equilibrium buffer containing 0.3 M GlcNAc. Apply 3 column volumes of regeneration buffer, wash with the equilibration buffer and store at 4°C in the presence of 0.1% NaN_3.

9. Dialyze purified ficolin-2 against storage buffer and concentrate by ultrafiltration to 0.5-1 mg/mL and centrifuge (4°C, 10 min at 13,000 rpm (20,000 g)).

10. Measure the concentration using an absorbance coefficient ($A_{0.1\%,\ 1cm}$) at 280 nm of 1.76 [7]. About 370 µg of purified recombinant ficolin-2 are recovered from 600 mL of cell culture supernatant.

11. The quality of the purified protein is assessed by SDS-PAGE analysis. The protein migrates as a single band of MW 34,000 under reducing conditions and yields a band at 34,000 and a high molecular weight band under non-reducing conditions [7].

3.1.2. Production of recombinant ficolin-3

1. Use the protocol described for ficolin-2 in Subheading 3.1.1 (Steps 1-5).

2. Select the highest producer clone and expand it in 175-cm^2 flasks in MEM/F12 (1/1) with GlutaMAX I medium supplemented with 10% heat-inactivated FCS, 300 µg/mL G418 and 50 µg/mL ascorbic acid.

3. Once confluence is reached, replace the medium with CD CHO serum-free medium supplemented with 8 mM glutamine, 300 µg/mL G418, and 50 µg/mL ascorbic acid. Harvest the culture supernatant and replace it every sixth day for 18 days (*see* **Note 9**).

4. Centrifuge the cell culture supernatant at 3,500 rpm (2250 g) for 10 min to eliminate cell debris.

5. Load the culture supernatant on the AcBSA-Sepharose column at a flow rate of 8-10 mL/min, wash with equilibrium buffer (10 column volumes). Elute bound ficolin-3 by applying the elution buffer. Apply 3 column volumes of regeneration buffer, wash with equilibration buffer and store at 4°C in the presence of 0.1% NaN_3.

6. Dialyze purified ficolin-3 against storage buffer, concentrate by ultrafiltration to about 1 mg/mL and centrifuge (4°C, 10 min at 13,000 rpm (20,000 g)).

7. Measure the concentration using an absorbance coefficient ($A_{0.1\%, 1cm}$) at 280 nm of 1.94 [7]. About 1 mg of purified recombinant ficolin-3 is recovered from 600 mL of cell culture supernatant.

8. The quality of the purified protein is assessed by SDS-PAGE analysis. The protein migrates as a single band of MW 36000 under reducing conditions and yields a ladder-like pattern under non-reducing conditions [7].

3.2 Anti-ficolin autoantibody ELISA

The protocol of anti-ficolins autoantibody ELISA is illustrated in Fig. 1.

Carry out all procedures at room temperature unless otherwise specified. Allow all reagents to reach room temperature before use. Gently mix all liquid reagents prior to use.

3.2.1 Anti-ficolin-2 autoantibody ELISA

1. Coat microplate wells with 100 µL of recombinant ficolin-2 diluted at 4 µg/mL in coating buffer (*see* **Note 10**). We recommend using coated and non-coated control wells (*see* **Note 11**). Cover plate and incubate at 4°C overnight.
2. Discard the liquid and tap the plate upside down on a paper towel (*see* **Note 12**).
3. Wash wells 3 times with 250 µL of washing buffer.
4. Block wells by adding 250 µL of blocking solution. Cover plate and incubate 1 h at RT.
5. Repeat steps 2 and 3 three times.
6. Add 100 µL of serum samples, diluted at 1:100, in duplicate to the coated and non-coated wells (*see* **Note 13**). Cover the plate and incubate overnight at 4°C (*see* **Note 14**).
7. Empty wells as described in step 2.
8. Wash wells 4 times with 250 µL of washing buffer.
9. Add 100 µl of HRP-conjugated goat polyclonal anti-human IgGs to the wells. Cover plate and incubate 1 h at RT.
10. Empty wells and wash wells 4 times with 250 µL of washing buffer (*see* **Note 15**).
11. Add 100 µL of chromogenic substrate to each well.
12. Allow the enzymatic color reaction to develop at at RT in the dark for 15-30 min and observe the blue color development (*see* **Note 16**).
13. Add 50 µL of stop solution to each well. The solution color in the wells should change from blue to yellow.
14. Read the absorbance of each well at 450 nm using the microplate reader (*see* **Note 17**).

3.2.2 Anti-ficolin-3 autoantibody ELISA

Repeat all steps of Subheading 3.2.1 except that:

1. Wells are coated with 100 µL of recombinant ficolin-3 diluted at 2 µg/mL in coating buffer.
6. Hundred µL of serum samples, diluted at 1:50, are added in duplicate to the coated and non-coated wells.

3.3 Data analysis

The results are recorded as the absorbance at 450 nm in an ELISA reader, corresponding to average value of antigen wells minus average value of antigen-free wells. Since all samples are tested in duplicate, we average the absorbance obtained for each of our patient samples (*see* **Note 18**).

These absorbances are then converted into arbitrary units (AU) by being plotted against the absorbance of a positive control. The concentration of the autoantibody is defined as (sample absorbance / control absorbance) x 100 AU.

3.3.1. Anti-ficolin-2 autoantibody analysis

The threshold value of 95 AU is calculated using 98th percentile by testing 48 healthy blood donors. An autoantibody titer is considered positive when the concentration is above the threshold value.

Figure 2 illustrates the detection of anti-ficolin-2 autoantibodies by ELISA in sera of SLE patients and healthy subjects. They are found positive in 61/165 (37%) SLE patients. The presence of anti-ficolin-2 autoantibodies is significantly related only to renal involvement, with

a very high prevalence (86%) of anti-ficolin-2 autoantibodies in SLE patients with active LN [8].

3.3.2. Anti-ficolin-3 autoantibody analysis

The threshold value of 70 AU is calculated using 98th percentile by testing 48 healthy blood donors. An autoantibody titer is considered positive when the concentration was above the threshold value.

Figure 3 illustrates the detection by ELISA of anti-ficolin-3 autoantibodies in sera obtained from one SLE patient and one healthy blood donor. A dose-dependent binding of IgG to solid-phase ficolin-3 is demonstrated, while serum from a healthy blood donor does not show significant reactivity (Fig. 3A). These two sera are respectively used as positive and negative controls for anti-ficolin-3 ELISA.

Subsequently, anti-ficolin-3 autoantibodies are tested in serum samples from SLE patients and healthy controls. A highly significant difference is found in the anti-ficolin-3 levels between SLE patients and healthy subjects. Anti-ficolin-3 autoantibodies are detected as positive in 56 of 165 (34%) SLE patients (Fig. 3B) [9].

4. Notes

1. The cDNAs encoding ficolin-2 (also called L-ficolin/P35) and ficolin-3 (also called H-ficolin/Hakata antigen) may be purchased from the Origene Trueclone human collection (ficolin-2: SC303435; ficolin-3: SC 126138).
2. Fetal bovine serum is inactivated by heating at 56°C for 30 min. We do not freeze again heat-inactivated fetal calf serum, but keep it at 4°C in sterile 50 mL Falcon tubes.

3. EDTA is difficult to dissolve in H_2O when the pH is below 7. To ensure fast dissolution, we mix 58.44 g of EDTA and 25 g of NaOH pellets in 500 mL of H_2O, which yields a 0.4 M solution with a pH of approximately 7.8.
4. NaN_3 is extremely toxic, and the stock solution (10% or 1.54 M) should be prepared under a ventilated hood. Alternatively, a ready-to-use solution can be purchased (0.1 M, Sigma-Aldrich) and used at a 15.4 mM final concentration.
5. Nunc Maxisorp plates are recommended for use in capturing immunoglobulins in ELISA. They have high protein-binding capacities.
6. TMB is sensitive to contamination from a variety of oxidizing agents. To avoid contamination, never pipette directly from the bottle. TMB may cause sensitization by skin contact. Wear gloves.
7. Ascorbic acid (vitamin C) is important for the production of ficolins, which have collagen-like domains that require ascorbic acid-dependent hydroxylation (of proline and lysine amino acid residues) for proper folding and stability.
8. Proceed further with recombinant protein purification or store the supernatants -80°C until use.
9. Multimeric ficolin-3 can be successfully produced in serum-free medium whereas ficolin-2 shows an oligomerization defect when expressed in CHO-K1 cells in the absence of FCS. However, ficolin-2 in FCS containing medium can be satisfactorily purified by affinity chromatography on CysNAc-Sepharose. Fractionation of FCS on CysNAc-Sepharose showed no detectable bovine ficolins.
10. Ficolins are directly attached to the plate by passive adsorption, using a carbonate/bicarbonate buffer at pH >9. It should be noticed that most but not all proteins bind tightly to the polystyrene surface of microplates in alkaline conditions.

11. It is recommended to use coated and non-coated wells in order to evaluate the specificity of antibodies-protein binding. The subtraction of sera absorbance of non-coated wells allows to take into account only the specific antibodies-protein binding.
12. It is important to tap conscientiously and vigorously the plate upside down on the paper towel to get rid of any remaining liquid between each step.
13. All sera are stored at -70 °C until use, and repeated freeze/thaw cycles are avoided. Frozen sera should always be thawed on ice before making dilutions.
14. It should be kept in mind that incubation time and temperature affect signal generation.
15. While performing the last washing step, prepare the plate reader for absorbance reading. Set up the program for the wells to be read at 450 nm.
16. For the color development in the wells, color change in 15-30 min is desirable. It mainly depends on the coated protein concentration and autoantibodies (sera dilution).
17. The reading of the absorbance of the wells has to be done quickly after addition of the stop solution.
18. Because recombinant ficolins used for the coating step are home-made proteins, without standardization, it is recommended to optimize the conditions of ficolin/anti-ficolin autoantibody interaction. Therefore, several conditions of protein concentrations and sera dilutions have to be tested concomitantly (Fig. 4).

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Figures legends

Fig. 1 ELISA protocol for detection of anti-ficolin autoantibodies

Fig. 2 Serum anti-ficolin-2 autoantibody titers in patients with SLE. (**A**) Anti-ficolin-2 titers in healthy controls (n = 48) and SLE patients (n = 165). (**B**) Anti-ficolin-2 titers in SLE patients with high disease activity disease (SLEDAI > 4) (n = 77) and SLE patients with low disease activity (SLEDAI ≤ 4) (n = 88). (**C**) Anti-ficolin-2 titers in SLE patients with high disease activity (SLEDAI > 4) (n = 77) with renal involvement (n = 36) or without renal involvement (n = 41). Horizontal lines in each group indicate the median values. Statistical analyses were performed by Mann-Whitney tests. This figure is reproduced with permission from John Wiley and Sons as published by Colliard et al [8].

Fig. 3 Detection of anti-ficolin-3 autoantibodies in patients with SLE. (**A**) Binding of anti-ficolin-3 autoantibodies to immobilized ficolin-3. Sera from SLE patients and healthy controls were added in serial dilutions. Results represent the means +/- standard deviation. (**B**) Anti-ficolin-3 autoantibodies in serum samples. Anti-ficolin-3 autoantibodies were measured in 48 samples from healthy controls and in 165 samples from patients with SLE. Horizontal lines in each group indicate the median values. Statistical analyses were performed by Mann-Whitney test. A, absorbance; AU, Arbitrary units. Reproduced from Plawecki et al [9].

Fig. 4 Optimization of anti-ficolin-2 autoantibody ELISA. Coating with several ficolin-2 concentrations (from 0 to 8 µg/mL) and sera dilutions of positive and negative controls (anti-ficolin-2 autoantibodies) (1:100, 1:200, 1:400) were tested concomitantly. The selected concentration for the coating was 4 µg/mL and the sera dilution was 1:100.

Fig.1

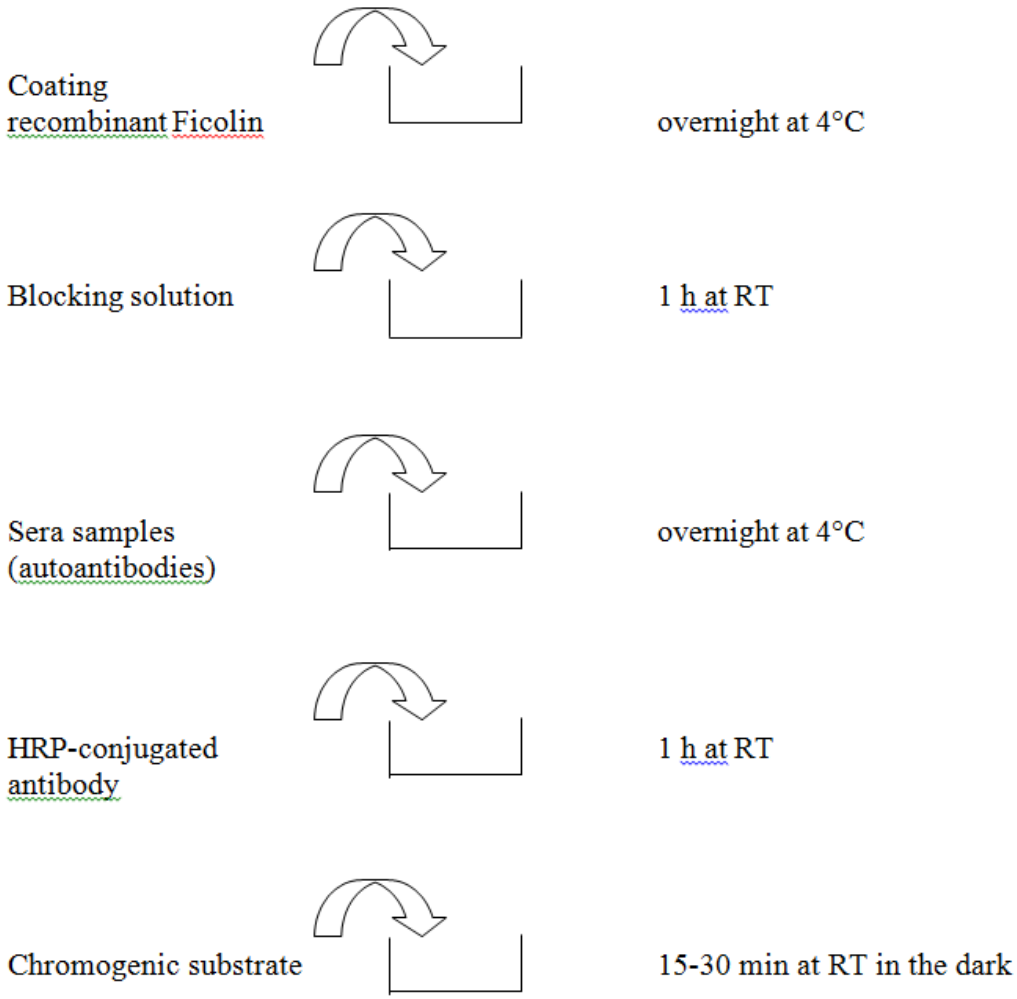

Coating
recombinant Ficolin
overnight at 4°C
Blocking solution
1 h at RT
Sera samples
(autoantibodies)
overnight at 4°C
HRP-conjugated
antibody
1 h at RT
Chromogenic substrate
15-30 min at RT in the dark
Reading of absorbance at 450 nm.

Fig.2

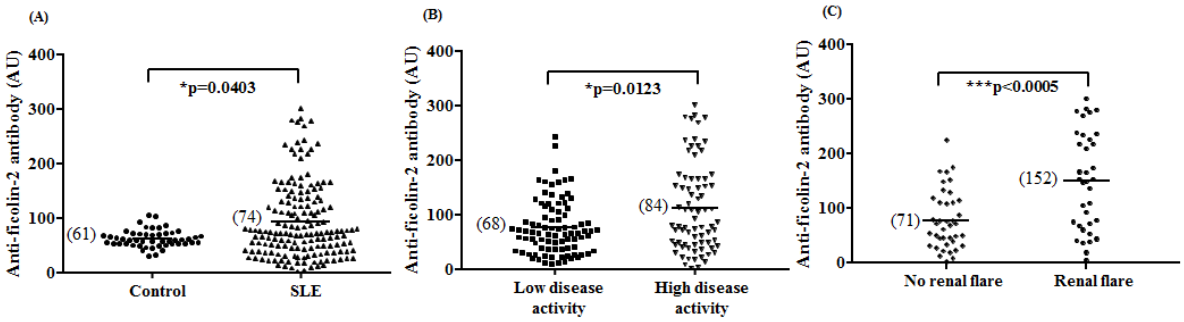

(A)
Anti-ficolin-2 antibody (AU)
400
300
200
100
0
*p=0.0403
(61)
(74)
Control
SLE
(B)
Anti-ficolin-2 antibody (AU)
400
300
200
100
0
*p=0.0123
(68)
(84)
Low disease activity
High disease activity
(C)
Anti-ficolin-2 antibody (AU)
400
300
200
100
0
***p<0.0005
(71)
(152)
No renal flare
Renal flare

Fig.3

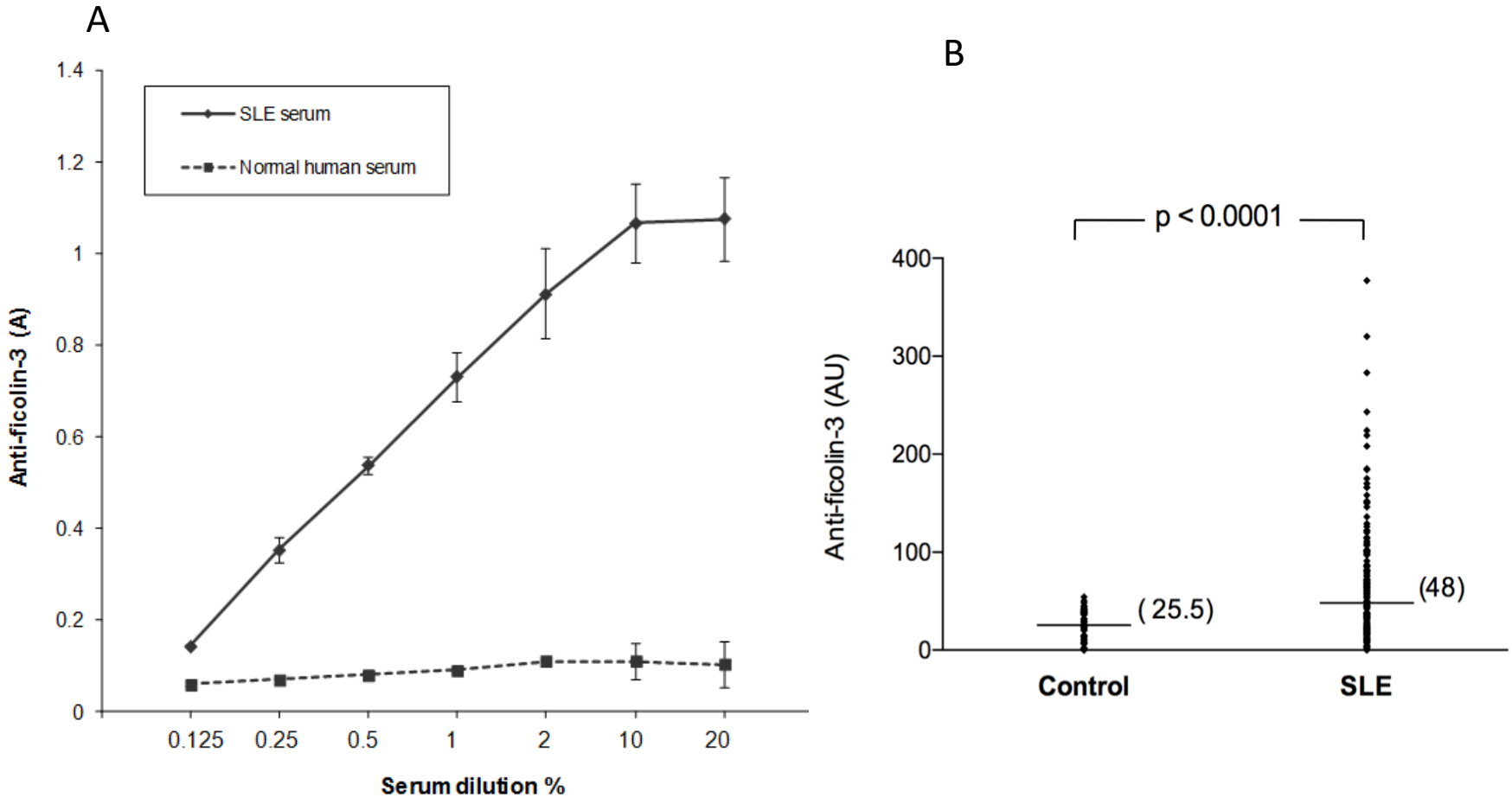
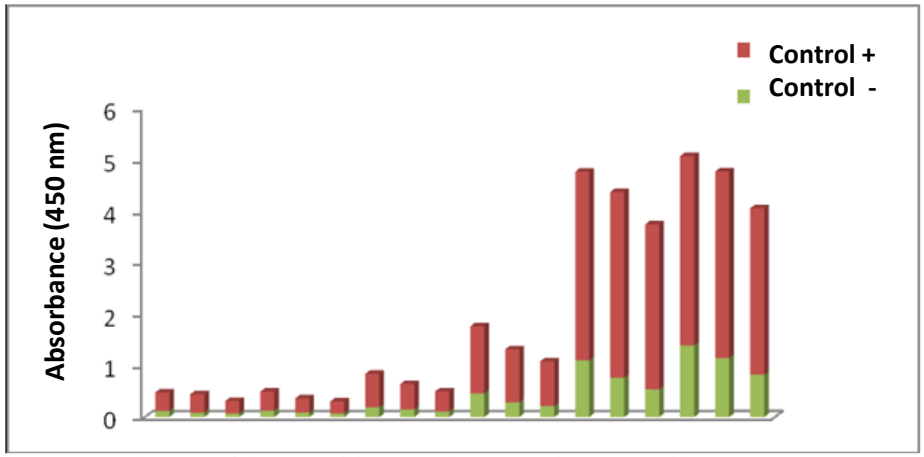

A
SLE serum
Normal human serum
Anti-ficolin-3 (A)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0.125
0.25
0.5
1
2
10
20
Serum dilution %
B
p < 0.0001
Anti-ficolin-3 (AU)
400
300
200
100
0
(25.5)
(48)
Control
SLE

Fig.4


Control +
Control -
Absorbance (450 nm)
6
5
4
3
2
1
0
0
0.5
1
2
4
8
Ficolin-2 coating
(µg/ml)
Sera dilutions at 1:100, 1:200, 1:400