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Quantification of porcine complement activation fragment C3a by a neoepitope based enzyme-linked immunosorbent assay

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Abstract

Enzyme-linked immunosorbent assay (ELISA) enables fast and simple quantification of analytes in the pico- to nanogram range in complex samples. Here, we describe an ELISA for the detection of porcine C3a as a marker for complement activation. Antibody specificity is critical for a robust assay. This assay is based on a pair of antibodies specific for the porcine C3a molecule and thus, does not react with native C3.

Key Words

Complement, Complement activation, C3a, Anaphylatoxin, Porcine, ELISA

1. Introduction

Pigs have a close relationship to humans regarding basic physiology and pathophysiological response in diseased conditions (1). However, reagents and assays available for pigs are largely missing, in particular for the detection of components of the complement system.

Activation of complement proceeds through three pathways merging at the level of C3. Upon C3 cleavage, the nine-kDa C3a anaphylatoxin is released into the fluid phase. In order to assess the level of complement activation, quantification of soluble markers originating from cleavage of the central C3 and C5 components are desirable. Antibodies targeting neoepitopes in the activated component, i.e., epitopes exposed only in the activated component and not in its native counterpart, must be employed. Here, we give a detailed description for quantification of porcine C3a by a sandwich enzyme-linked immunosorbent assay (ELISA).

In a sandwich ELISA, antigens in a sample are captured using a surface-bound capturing antibody and subsequently detected using a soluble detection antibody. The assay described here is based on C3a-neoepitope-specific antibodies, previously characterized in (2,3).

2. Materials

All reagents are recommended to be of analytical grade. All solutions should be prepared using ultrapure water (resistivity of 18.2 MΩ x cm at 25°C). Buffers can be stored for up to four weeks at 4°C unless otherwise stated. Carefully follow all local waste disposal recommendations and safety regulations.

2.1. Buffer preparation

1. 10x phosphate buffer (690 mM) with saline (770 mM), pH 7.2 (10x PBS). Prepare 1 L 10x stock solution by taking 89.5 g $Na_2HPO_4*2H_2O$ (MW: 177.99 g/mol) and 25.1 g KH_2PO (MW: 136.08 g/mol) and transfer to a 1 L Erlenmeyer flask. Add 900 mL of water and dissolve with a magnetic stir bar. Add 45.0 g NaCl (MW: 58.44 g/mol). When all the salts are fully dissolved, adjust the pH to 7.2 by adding either 1 M HCl or 1 M NaOH (*see* **Note 1**). Transfer to a 1 L volumetric flask and fill up with water. Store the stock solution at room temperature. Dilute the stock 1/10 in water to get the 1x PBS working solution.

2. 0.50 M EDTA solution, pH 7.4. Add 18.61 g EDTA (MW: 372.2 g/mol) to an Erlenmeyer flask. Add 80 mL of water and mix on a magnetic stirrer until the salt is dissolved (*see* **Note 2**). Adjust the pH with 5 M NaOH to pH 7.4, transfer to a 100 mL volumetric flask and fill up with water (*see* **Note 1**). Store the EDTA solution at room temperature.

3. 0.15 M acetate solution. Prepare 1 L 0.15 M sodium acetate solution by adding 20.4 g $C_2H_3NaO_2*3\ H_2O$ (MW: 136.08 g/mol) to a 1 L Erlenmeyer flask. Add

900 mL of water and mix on a magnetic stirrer until the salt is dissolved. Transfer to a 1 L volumetric flask and fill up with water.

4. 0.15 M acetic acid solution. Prepare 500 mL 0.15 M acetic acid solution by adding 4.1 mL acetic acid (glacial) 100% into 400 mL water in an Erlenmeyer flask. Transfer to a 500 mL volumetric flask and fill up with water.

5. 0.15 M acetate buffer pH 4.0. Prepare > 500 mL buffer by mixing the alkaline 0.15 M acetate solution with the acidic 0.15 M acetic acid solution to pH 4.0. As a rule use approximately 450 mL of the acidic solution for every 100 mL of the alkaline solution.

2.2. Working solutions

1. PBS with 0.1% Tween 20. Transfer 900 mL PBS into a 1 L volumetric flask. Add 1 mL Tween 20 (*see* **Note 3**) and fill up with PBS. Store the buffer in room temperature.

2. PBS with 0.2% Tween 20. Transfer 900 mL PBS into a 1 L volumetric flask. Add 2 mL Tween 20 (*see* **Note 3**) and fill up with PBS. Store the buffer in room temperature.

3. PBS with 0.2% Tween 20 and 10 mM EDTA. Transfer 900 mL PBS into a 1 L volumetric flask. Add 2 mL Tween 20 (*see* **Note 3**), 20 mL 0.5 M EDTA pH 7.4 and fill up with PBS. This buffer shall be kept cold until use.

4. ABTS (2,2'-Azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt) substrate solution. Add 90 mg ABTS to an Erlenmeyer flask. Fill up with 450 mL 0.15 M Sodium-acetate buffer pH 4.0. Transfer to a 500 mL volumetric flask and fill up with 0.15 M sodium acetate buffer pH 4.0. Protect from light exposure. This buffer should be equilibrated to room temperature before use.

5. 1 M H_2SO_4 (optional).

6. H_2O_2 (3%). Dilute 30 % H_2O_2 1/10 in water. This solution must be stored dark and can then be kept for up to one week.

2.3. Antibodies

1. Mouse-IgG2bk anti-porcine C3a/C3a desArg (clone Z22/8, art. no: EGO008; Kerafast, Boston, MA).

2. Mouse-IgG1/k anti-porcine C3a/C3a desArg (clone K5/9, art. no: EGO009; Kerafast, Boston, MA).

3. Goat anti-mouse IgG1-HRP (art.no: 1070-05; Southern Biotech. Birmingham, AL).

2.4. Samples, standards, and controls

Plasma samples to detect complement activation products generated *in vivo,* should be obtained from EDTA-blood, and prepared and stored under strict conditions in order to avoid *in vitro* activation (4,5) (*see* **Note 4).** All handling of EDTA-blood tubes before centrifugation and plasma after centrifugation should be carried out on crushed ice (*see* **Note 5).** Samples should be stored at -70°C or colder (*see* **Note 6).**

The standard curve was created from recombinant porcine C3a (art. no: RP1132S, Kingfisher Biotech, Inc, Saint Paul, MN) (Fig. 1). An optimal curve was obtained by 2-fold dilution from 50 ng/mL to 0.39 ng/mL. Normal porcine plasma was found to have an optimal dilution factor of 1/20, giving a mean value for C3a of 37 mg/mL (as indicated by the arrow on Fig. 1).

A positive and negative control should be included in every assay (see Note **7).** The negative control should be a plasma sample with a low level of activation and the positive control should be a sample with a high level of activation, e.g. serum incubated with a complement activator (Table 1 and 2). Serum can be activated by addition of e.g., cobra venom factor or zymosan. Here, we incubated 20 units of cobra venom factor per mL serum for 90 minutes at 37°C to fully activate C3. A modest and medium level of C3-activation was achieved by activating complement serum with either zymosan (10 mg/mL) or *E. coli* (10^7/mL) for 60 minutes in two different sera. EDTA at a final concentration of 20 mM should be added to serum after activation to avoid *in vitro* activation of residual non-activated components.

2.5. Cross-reactivity, spiking and coefficients of variation.

The present assay was tested for cross-reactivity against human C3a (Fig. 2). Neither normal human plasma nor zymosan-activated human serum showed any reactivity in the assay, with values below the lower detection limit, comparable to the buffer blank (Fig 2, left panel). The lower detection limit was defined by the lowest standard (i.e. 7.8 ng/mL when corrected for a 1/20 dilution).

In order to control the precision of the assay, spiking of recombinant porcine C3a was performed in normal human plasma diluted 1/20, i.e. corresponding to the dilution of the porcine samples and thus with the same protein concentration, but with no activity in the assay as described above. Spiking with 250 and 500 ng/mL of C3a gave a recovery of 229 (92%) and 487 (97%) ng/mL, respectively.

Intra- and inter-assay coefficients of variation (CV) were calculated with samples containing different amounts of C3a as shown in table 1 and 2, respectively. The intra-assay CV was <10 (Table 1) and the inter-assay CV was <15 (Table 2).

2.6. Example of usage - detection of C3a in in vivo samples from porcine sepsis.

In order to exemplify the use of the assay, we examined *in vivo* formed C3a in a series of samples from a previous study performed with polymicrobial porcine sepsis (6). There, we showed a steady increase in the terminal sC5b-9 complement complex (TCC) during the course of the experiment. The same samples were here tested in the C3a assay, and the level of C3a was found to follow the curve of sC5b-9 (Fig. 3).

3. Methods

Coat wells in a 96-well microtiterplate (polystyrene, high binding, e.g. Nunc Maxisorp art.no 439454, Nunc A/S, Roskilde, Denmark) with anti-C3a clone Z22/8: Dilute the antibody 1/1000 in PBS pH 7.2. Add 100 µl of the diluted antibody per well, cover with sealing film and incubate for 12-24 hours at 4°C.

1. Prepare samples and controls: Dilute in PBS with 0.2% Tween 20 and 10 mM EDTA. As a guideline, samples are diluted 1/20. Samples with a high level of activation can be further diluted to a final 1/200-dilution (*see* **Note 8**). Samples, standards, and controls are preferably prepared in 1.5 mL low binding polypropylene tubes (*see* **Note 9**). Prepare a minimum of 250 µl of each sample and controls in order to be able to analyze them in duplicates.

2. Preparation of standard series: Prepare 600 µl of 50 ng/mL recombinant porcine C3a by diluting the stock solution in PBS with 0.2% Tween 20 and 10 mM EDTA. Dilute the 50 ng/mL-solution further in two-fold dilution steps seven times in PBS with 0.2% Tween 20 and 10 mM EDTA. Prepare in total eight concentrations of the recombinant C3a (50, 25, 12.5, 6.25, 3.13, 1.56, 0.78 and 0.39 ng/mL) and one blank containing only PBS with 0.2% Tween 20 and 10 mM EDTA.

3. Empty the wells of the microtiterplate and wash four times. For every wash cycle, add 300 µl of PBS with 0.1% Tween 20 per well and thereafter discard the washing solution by inverting the plate over the sink. After the last wash, tap dry on a layer of tissues (*see* **Note 10**).

4. Add 100 µl of sample, controls, and standards per well in minimum duplicates. Incubate for 60 minutes at 4°C.

5. Wash according to step 4 above.

6. Prepare the detecting antibody, clone K5/9, by diluting it 1/1000 in PBS with 0.2% Tween 20. Add 100 µl diluted detecting antibody per well and incubate for 45 minutes at room temperature (*see* **Note 11**).

7. Wash according to step 4 above.

8. Prepare the HRP-conjugated secondary goat anti-mouse IgG1 by diluting it 1/2000 in PBS with 0.2% Tween 20. Add 100 µl diluted secondary antibody per well and incubate for 30 minutes at room temperature.

9. Wash according to step 4 above.

10. Prepare the substrate solution by adding 20 µl 3% H_2O_2 to 12.5 mL ABTS-solution immediately prior addition to the plate. Add 100 µl substrate solution per well. Follow the color development by measuring OD continuously. Let the substrate incubate until the OD at wavelength 405 nm in the highest standard point reaches 1.0-1.2. Optional, stop the substrate development by adding 100 µl 1 M H_2SO_4 per well (*see* **Note 12**).

11. Measure OD in all wells using a microplate reader. The detection wavelength for ABTS is 405 nm with 490 nm as the reference wavelength.

12. Use the standard points to plot a sigmoidal four-parametric standard curve with OD as a function to the logarithmic C3a-concentration. Relate OD of the samples to the sigmoidal standard cure and correct with the dilution factor.

4. Notes

1. If the gap between the actual pH and the desired is large, the stronger 5 M HCl or 5 M NaOH can be used initially. Both HCl and NaOH are corrosive; the local safety regulations must be followed carefully.

2. EDTA can be difficult to dissolve but dropwise addition of 5 M NaOH and/or heat can help dissolvement. If heat is used, make sure that the solution is brought to room temperature before the pH is adjusted to pH 7.4.

3. Tween 20 is viscous and may be difficult to pipette accurately. Recommended is to use a positive displacement pipette instead of an air displacement pipette. If the latter is used, use the reverse pipetting technique, tilt the container and pipette slowly.

4. The baseline complement activation level, including C3a, is higher in serum compared to plasma. This is explained partly from that the whole blood is kept without EDTA when blood is coagulated, traditionally at room temperature for 60 minutes. In addition, the clotting process and platelet activation can potentiate complement activation. Serum samples should therefore never be used in order to evaluate activation in whole blood samples (*in vivo* or *ex vivo*) but serum may be used as the media to perform complement activation studies.

5. Proper sample handling is critical in order to avoid *in vitro* complement activation and to achieve a reliable measurement. Low temperature slows

down the activity of the C3-convertase and EDTA chelates the Mg^{2+}-ions, essential for convertase activity.

6. Avoid measuring C3a on samples after repeated freeze-thawing. If multiple markers will be measured, freeze in several aliquots. C3a can be measured on samples stored for several years if stored properly.

7. Prepare a large volume of negative and positive controls. Divide the controls in multiple aliquots and thaw a new aliquot for every single run. Register the absolute values of positive and negative controls over time in order to keep control of the inter-assay coefficient of variation (Table 2).

8. If plasma or serum samples are to be diluted >100 times, dilution in two steps is recommended. If possible, avoid pipetting lower plasma- and serum-volumes than 5 µl. Pipetting small volumes of plasma and serum is a source of error.

9. Proteins may adhere to surfaces, therefore, use tubes and tips that provide low protein adherence, typically tubes of polypropylene.

10. This step can be performed either manually by an 8- or 12-multichannel automatic pipette or in a standard ELISA-washing machine. Avoid letting the wells stand dry for longer times without buffer. If this is unavoidable, wait with tapping the plate dry.

11. Room temperature is a non-precise definition, and usually refers to 20-25°C. If the appropriate temperature cannot be assured, an incubator may be considered.

12. If the OD-measurement cannot be performed immediately, stop the substrate development with 1 M H_2SO_4. In other cases, the OD can be measured continuously as the color develops and the final measurement can be performed when OD in the highest standard has reached 1.0-1.2. Stop solution is however recommended for development times below five minutes in order to ensure equal substrate developing time.

5. References

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Figure legends

Figure 1. A representative standard curve of recombinant porcine C3a. C3a was diluted to 50 ng/mL and further in twofold dilutions. Absorbance values at 405 nm are plotted against the C3a-concentration on a logarithmic x-axis. A sigmoidal four-parametric standard curve was fitted to the plotted standard values. The standard points were analyzed in duplicates, standard deviation is represented by the error bars. The arrow indicates the point on the standard curve corresponding to the value of the normal porcine plasma diluted 1/20.

Figure 2. Porcine specificity of the C3a ELISA versus human and spiking of porcine C3a. *Panel A:* Cross-reactivity towards human C3a in the assay was tested by comparing normal porcine plasma (NPP), normal human plasma (NHP), and zymosan-activated human serum (ZAHS), all diluted 1/20. Reactivity towards human C3a was lower than the limit of detection (LOD) even in ZAHS. *Panel B:* Precision of the assay was examined by spiking NHP with 0, 250 and 500 ng/mL recombinant porcine C3a (rC3a). Recovery was 92% and 97%, respectively. Data are shown as mean with 95% confidence interval.

Figure 3. Porcine *in vivo* complement activation evaluated by quantification of plasma C3a and sC5b-9. EDTA-plasma samples from eight anesthetized pigs developing polymicrobial sepsis were collected over time until death. Complement activation was evaluated by quantification of C3a by the assay described here. The sC5b-9 data are from the original study and described in detail in (6). Data are expressed as mean values. CAU = complement arbitrary units.

Table 1. Intra-assay variation

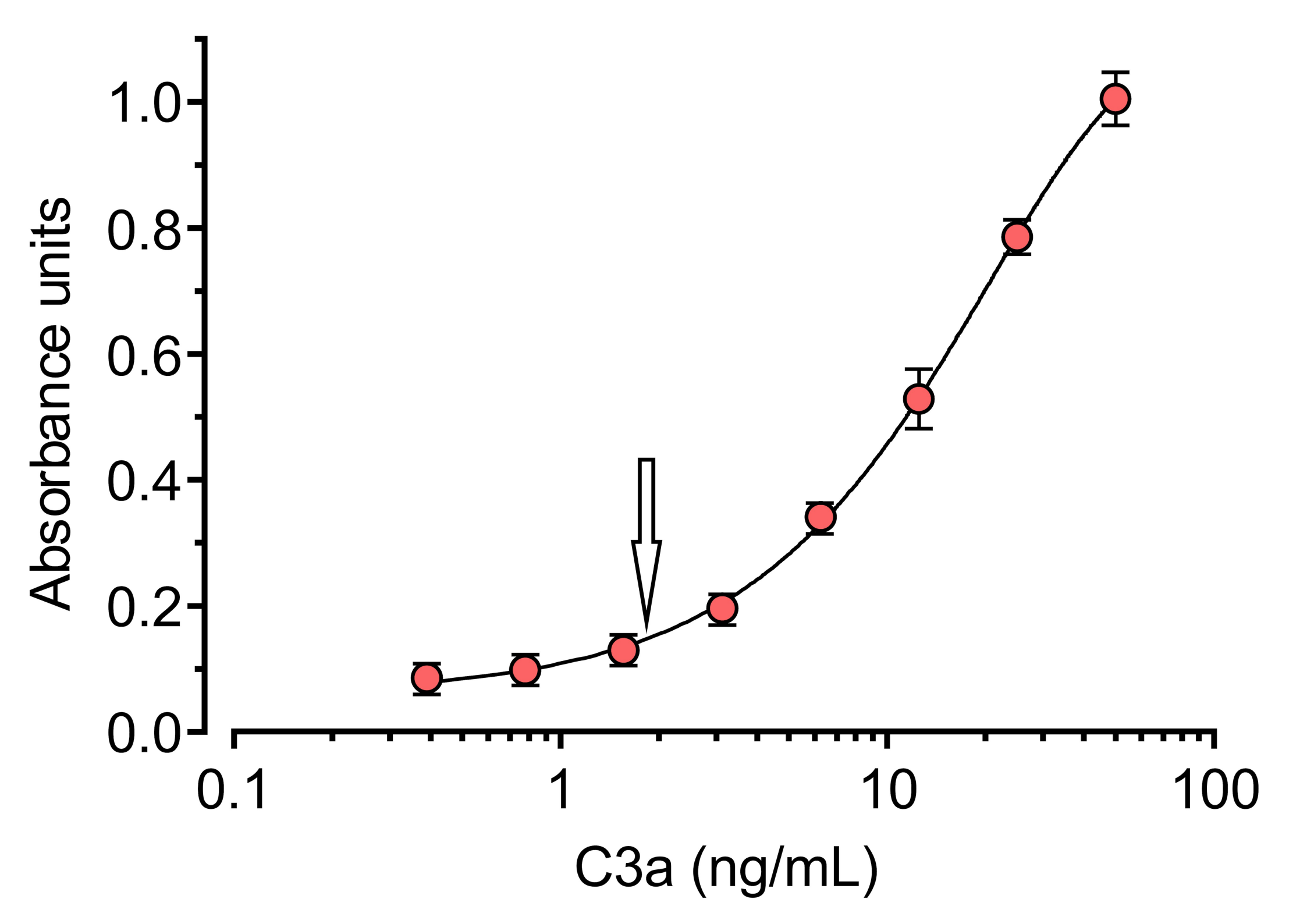
Sample	Sample activation [1]	n [2]	Dilution factor	Mean C3a (ng/mL) [3]	CV [4] (%
NPP [5]	No	20	20	37	7.1
*E.c*APS [6]	Yes	9	200	2224	3.7

[1] *In vitro* sample activation
[2] Number of observations
[3] C3a after dilution factor correction
[4] Coefficient of variation
[5] Normal porcine plasma
[6] *E. coli*-activated porcine serum

Table 2. Inter-assay variation

Sample	Sample activation [1]	n [2]	Dilution factor	Mean C3a (ng/mL) [3]	CV [4] (%)
NPP [5]	No	9	20	37	14
ZAPS [6]	Yes	7	800	1452	10
CAPS [7]	Yes	7	12800	27020	9.2

[1] *In vitro* sample activation
[2] Number of observations
[3] C3a after dilution factor correction
[4] Coefficient of variation
[5] Normal porcine plasma
[6] Zymosan-activated porcine serum
[7] Cobra venom factor-activated porcine serum


Absorbance units
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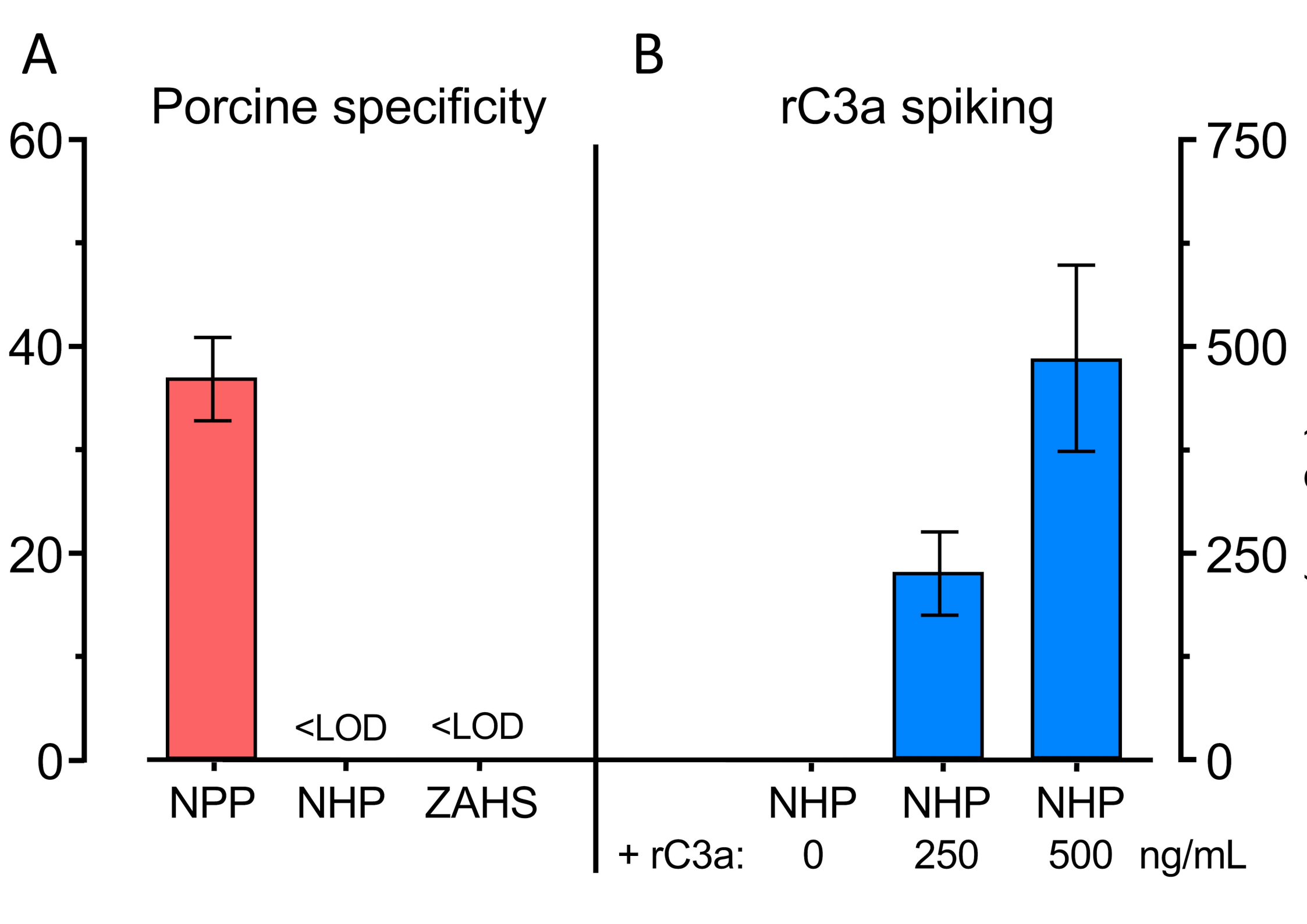
0.8
0.6
0.4
0.2
0.0
0.1
1
10
100
C3a (ng/mL)


A
Porcine specificity
B
rC3a spiking
60
40
20
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750
500
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0
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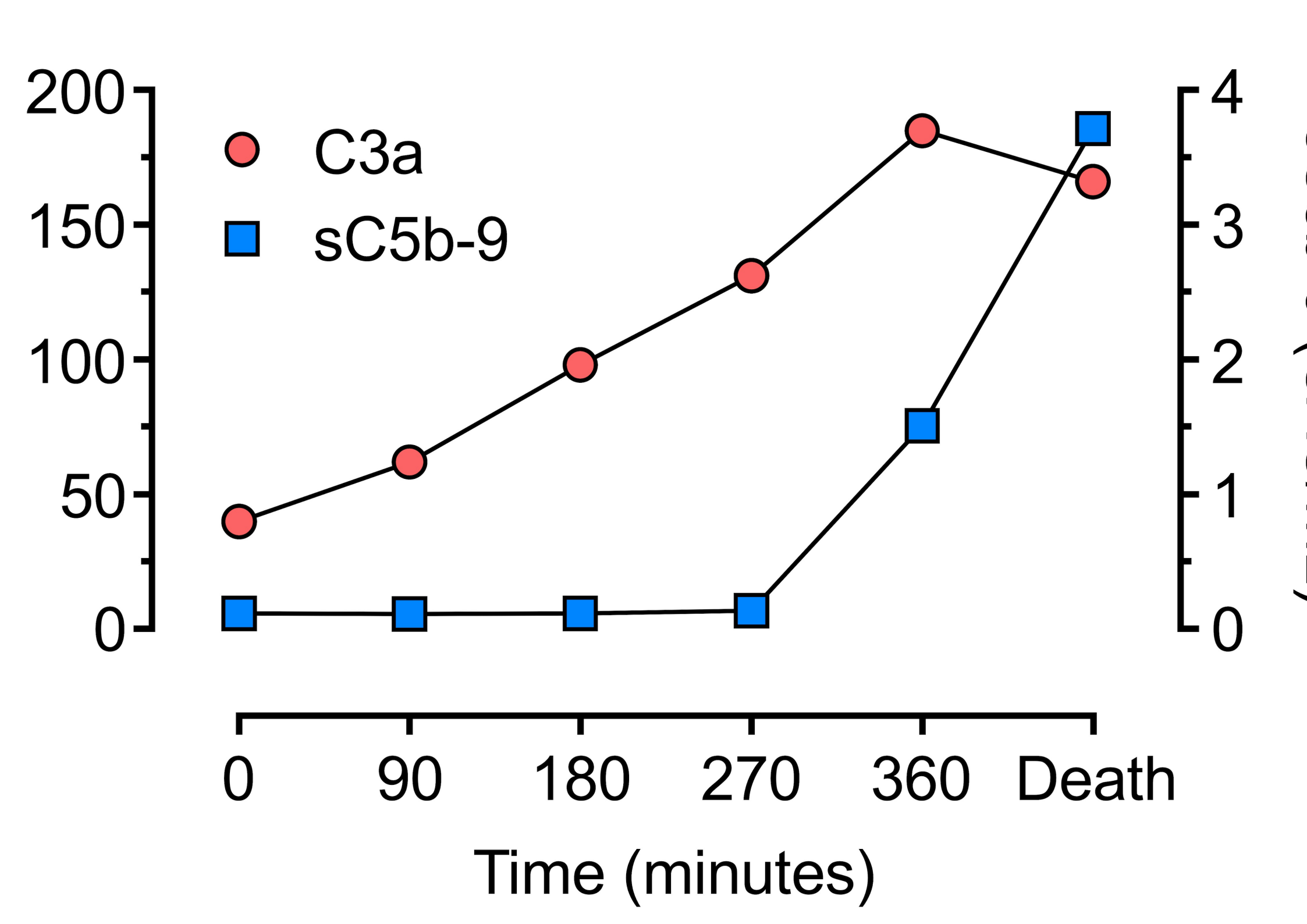
200
150
100
50
0
C3a
sC5b-9
4
3
2
1
0
0
90
180
270
360
Death
Time (minutes)