Extracting the barbs from complement assays: identification and optimisation of a safe substitute for traditional buffers.

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Abbreviations: NHS, normal human serum; NMS, normal male mouse serum; NRS, normal rat serum; NRbS, normal rabbit serum; HU, haemolytic units; RT, room temperature; CFD, complement fixation diluent; VBS, veronal buffered saline; HBS, HEPES-buffered saline; Abs, absorbance; ShEA, antibody-sensitized sheep erythrocytes; ShE, sheep erythrocytes; GpE, guinea pig erythrocytes.

ABSTRACT

Complement assays have for many years utilised buffers based on barbitone (veronal) despite the well-recognised toxicity of this agent and the tight regulations on its use in most countries. The use of barbitone in complement assay buffers is steeped in history, from a time when no other suitable buffers were available. This is no longer the case, encouraging us to explore alternatives to barbitone for complement assays. We compared a simple, non-toxic HEPES buffer with commercially sourced complement fixation test diluent (CFD), the “gold standard” barbitone buffer, in several clinically relevant complement activity assays and across species.

In classical pathway haemolysis assays in human and non-human serum, there was no difference in haemolytic curves or calculated haemolytic activity (CH50) between CFD and an optimised HEPES buffer (HBS) supplemented with cations. Alternative pathway haemolysis assays in human serum were also identical in the two buffers. In a complement fixation test for anti-erythrocyte antibodies, complement consumption was identical for the two buffer systems.

The data demonstrate that barbitone-based buffers are unnecessary for assays of complement activity and can readily be replaced with safe and simple alternatives.

INTRODUCTION

With the increasing recognition of roles of complement activation in disease and the growing list of drugs that target complement, it is becoming ever more important to have simple and safe assays for complement activity that can be replicated across the globe (Morgan et al., 2015; Sim et al., 2016). Traditionally, assays of complement activity have involved measuring the haemolytic activity of a test sample for either antibody-sensitised sheep erythrocytes (ShEA) or unsensitised rabbit or guinea pig erythrocytes (RbE; GpE) to test the classical and alternative pathways respectively (Pillemer et al., 1943; Lachmann et al., 2006; Mayer et al., 1946; Morgan et al., 2000; Ghebrehiwet et al., 1997; Barnum et al., 2018). Complement fixation tests that have been widely used to measure antigen-antibody reactions also rely on quantification of residual haemolytic activity to assess the degree of fixation – actually, consumption – in the first incubation (Whillas et al., 1950; Breadstreet et al., 1962). These assays have, for more than 70 years, been performed in barbitone (5,5-diethyl barbituric acid) buffers, also called barbital or "veronal", at pH ~7.3 with physiological salt concentration and supplemented with Ca^{2+} and Mg^{2+} ions. These buffers were selected because they offered good buffering capacity at a pH optimal for complement activity and were non-chelating for Ca^{2+} and Mg^{2+} ions.

Barbitone is a long-acting barbiturate, the first to be made commercially available as a hypnotic drug and widely used for this purpose from the 1900s to the mid-1950s (Norn et al., 2015; Lopez – Munoz et al. 2005). "Veronal" was the trade name under which the agent was marketed by Bayer. Accidental (and deliberate) overdoses were common, often resulting in death, in part a consequence of its small therapeutic margin (Lopez – Munoz et al., 2005; Bateman et al., 1963). As a

consequence, barbitone and its sodium salt, also used in buffer preparation, are listed by the US Drug Enforcement Administration (DEA) as a schedule IV controlled substance and subject to strict control on transport, storage and record-keeping (http://www.controlleddrugs.org/Schedules.html). In many other countries, barbitone is subject to stringent restrictions on supply and in an increasing number it is banned completely. The risks and restrictions have resulted in the replacement of buffers based on barbitone for other applications, for example in immunoelectrophoresis, by safer and more readily accessible buffers (Monthony et al., 1978; Anani et al., 2015). In contrast, the use of barbitone-based buffers for complement assays has continued. Although there have been isolated publications that have utilised alternative buffers for complement assays (Moreno–Indias et al., 2012), there has been no formal testing and comparison of these with CFD or other barbitone buffers. Here we describe such a comparison. We show that a buffer based on HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), a zwitterionic buffer with excellent buffering capacity at pH7.4 and no significant toxicity, can be used in place of CFD and related barbitone buffers in classical and alternative pathway complement haemolytic assays and in complement fixation tests without any impact on test outcome.

Materials and methods

All chemicals, except where otherwise stated, were obtained from either Fisher Scientific UK (Loughborough, Leicestershire, UK) or Sigma Aldrich (Gillingham, Dorset, UK) and were of analytical grade.

Human and animal sera were prepared in-house from freshly collected blood. For human, rabbit, rat and guinea pig, blood was clotted at room temperature for 1 hour, and then placed on ice for 2 hours for clot retraction prior to centrifugation and harvesting of serum. For mouse, blood was placed on ice immediately after harvest and clotted for 2 hours on ice prior to serum harvest. Sera were stored in aliquots at -80^{0}C. In some studies serum was heat-inactivated by incubation at 60^{0}C for 30 minutes.

Buffer preparation

HBS buffer was prepared with concentrations of 0.01 M HEPES, 0.15 M NaCl, 135 nM $CaCl_2$, 1 mM $MgCl_2$, at final pH 7.4.

CFD was prepared by dissolving one CFD buffer tablet (Oxoid, #BR0016) in 100ml deionised water; the resultant buffer contained 3 mM barbitone ($C_8H_{12}N_2O_3$), 900 nM sodium barbitone ($C_8H_{11}N_2NaO_3$), 0.15 M NaCl, 250 nM $CaCl_2$, 2 mM $MgCl_2$, at final pH 7.2 (Oxoid BT-SPEC 0026).

Alternative pathway buffer (APB) was prepared by adding EGTA (Ethyleneglycoltetraacetic Acid, from 0.5M EGTA stock) to a final concentration of 5 mM and additional $MgCl_2$ to 3mM final concentration into test buffer (HBS or CFD).

Haemolytic Assays

HEPES buffer was tested against CFD in haemolytic assays using antibody-sensitised sheep erythrocytes (ShE; sheep blood from TCS Bioscience,

Buckingham, UK) for classical pathway and guinea pig erythrocytes (GpE: blood from TCS) for alternative pathway. ShE were antibody-sensitised (ShEA) by incubation for 15 minutes at 37°C with a 1:2000 dilution in phosphate-buffered saline of rabbit anti-sheep erythrocyte antiserum (#ORLC25, Siemens Amboceptor; Cruinn Diagnostics Ltd., Dublin, UK) and washed into the test buffer at 2% final (vol:vol). For measurement of classical pathway complement activity in male mouse serum, ShEA were additionally incubated with mouse anti-rabbit IgG (#3123, at 20µg/ml, Invitrogen; Paisley, UK) for 30 minutes at 37°C prior to washing into test buffer. GpE were directly washed into the test buffer at 2% final. Cells in test buffer were aliquoted into wells of a 96-well round-bottomed plate (50 µl/well) followed by 50 µl/well of serum dilutions (in triplicate) in the same test buffer. A further 50µl/well of the test buffer was added to each well and plates sealed and incubated at 37°C for 30 minutes. Plates were then centrifuged and haemoglobin in the supernatant measured by absorbance at 415nm. Percentage lysis was calculated according to: % Lysis =100*(Absorbance (Abs) sample-Abs background)/(Abs max -Abs background). The 50% haemolytic complement activity (CH50 for classical, AH50 for alternative pathway) was calculated for each lytic curve (Morgan et al., 2000). In some assays, normal serum was mixed with heat-inactivated serum to create a low complement activity serum.

To test the impact of the buffers on screening for complement deficiencies, sera depleted of C5 or deficient in C6 or C8 were diluted 1 in 10 in the appropriate buffer and aliquoted in to wells of a 96 well plate as above. Purified terminal pathway proteins (C5, C6, C7, C8, C9; physiological levels for each) were added in triplicate to separate sets of wells for each of the depleted/deficient sera. ShEA were added to each well, incubated and absorbance read as above.

Complement Fixation Test (CFT)

Human erythrocytes from a blood group A-positive donor (HuEA+) were washed, suspended to 2% in test buffer (HBS or CFD) and 500 µl aliquots placed in four tubes. Serum from a blood group 0-positive donor (1:10 dilution in test buffer, 500 µl/tube) was added and pairs of tubes incubated either at 37^0C or on ice for 30 minutes. As an additional control, A-positive erythrocytes were incubated with A-positive serum as above. Tubes were centrifuged at 4^0C, supernatant removed, and a serial dilution series prepared, 50 µl/well in triplicate in the test buffer. ShEA at 1% in test buffer were added (100 µl/well), plates were sealed and incubated at 37^oC for 30 minutes, centrifuged and haemoglobin in the supernatant measured by absorbance at 415nm. Percentage lysis was calculated according to: % Lysis =100*(Absorbance (Abs) sample - Abs background)/(Abs max - Abs background)%. CH50 was calculated for each lytic curve (Morgan et. al, 2000).

Stability Testing

To test the effect of the test buffers on erythrocyte stability we utilised the protocol described by Oxoid (BT-SPEC-0026). Fresh sheep blood (1ml) was added to 10ml of test buffer (CFD or HBS) and incubated at 37°C for 20 hours; 0.5ml samples were removed at time points; 0.3, 3, 6 and 20 hours, aliquoted 150 µl/well in triplicate wells of a 96-well round-bottomed plate, centrifuged and haemoglobin in the supernatants measured by absorbance at 415nm. Controls included blood incubated with deionised water or 0.1% Tween 20 in deionised water. Percentage

lysis was calculated according to: % Lysis =100*(Absorbance (Abs) sample-Abs background)/(Abs max -Abs background) %.

RESULTS

HBS is indistinguishable from barbitone-based buffer in haemolytic assays

For assessment of classical pathway activity, HBS containing Ca^{2+} and Mg^{2+} was compared with commercially sourced CFD containing Ca^{2+} and Mg^{2+}. ShEA in the relevant buffer were incubated with a dilution series of human or non-human (rabbit, guinea pig, rat and mouse) sera. With each of the sera tested, the lytic curves obtained in the two buffers were superimposed, and the calculated CH50 values for each serum in the two buffers were the same (Figure 1 A-E). For assessment of alternative pathway activity, HBS was supplemented with Mg^{2+} and EGTA to generate an alternative pathway-specific buffer (no Ca^{2+}); this was compared to CFD similarly supplemented with Mg^{2+} and EGTA (alternative pathway buffer; APB). Only human serum was tested in this assay. The lytic curves obtained in the two buffers were superimposed and the calculated AH50 values for the serum in the two buffers were the same (Figure 1 F).

To test the capacity of the buffers to measure low levels of haemolytic activity, NHS was diluted with heat-inactivated NHS (1:3) and tested as above; lytic curves and calculated CH50 values were identical in the two buffers (Figure 2A).

To test the utility of the HBS buffer for identifying complement component deficiencies, add-back studies to deficient or depleted sera were performed. For each serum tested, HBS and CFD showed identical results and correctly identified the missing component (Figure 2 B-D).

HBS is indistinguishable from barbitone-based buffer in a complement fixation test

To compare the buffers in a complement fixation test, A-positive HuE were incubated in the relevant buffer with group O serum containing anti-A antibodies, either on ice to inhibit fixation or at 37°C to facilitate fixation (Petz et al., 1974). After this incubation, the serum was tested in a classical pathway assay in the same buffer to measure residual complement activity. Incubation on ice in either buffer did not reduce lytic activity of the serum, whereas incubation at 37°C resulted in a ~90% reduction in classical pathway activity in each buffer (CH50; samples incubated at 37°C in HBS average HU= 20.7, in CFD = 22.4; samples incubated on ice in HBS average HU= 111.2, in CFD = 100.5) (Figure 3 A, B). Incubation of A-positive HuE with A-positive serum caused no reduction in complement activity at either temperature and in either buffer, confirming that consumption was dependent on antigen-antibody complexes. (Figure 3 C, D).

Erythrocytes are stable on storage in HBS

To compare the stability of ShE in CFD and HBS, sheep blood was diluted in HBS or CFD and incubated at 37°C for up to 20 hours. In either buffer ShE displayed no lysis across the time course. In control incubations with deionised water or 0.1% tween 20, all cells were lysed within 30 minutes. The data demonstrate that HBS is compatible with and can be used for storage of target erythrocytes.

Discussion

Over the last twenty years, interest in complement, particularly its roles in disease and suitability as a therapeutic target has exploded (Morgan et al., 2015, Ricklin et al., 2013). There is thus an increased need for robust, reliable and generalisable assays of complement activation and activity (Harboe et al., 2011; Bergseth et al., 2013). Studies on characterisation of complement activity and its interactions require reliable tools. A major stumbling block has been the dependence on barbitone-containing buffers both for measuring complement activity and complement fixation. Commercial barbitone-based complement fixation test diluent (CFD) tablets and concentrates have been widely marketed for decades and became the gold standard for complement haemolytic assays; however, they are increasingly difficult to access, impossible in many countries. Their continued use for complement assays is a historical artefact, dating back more than 70 years to a time when the choice of available buffers was much smaller and barbitone-based buffers offered excellent buffering in the optimal pH range for complement activity and no interference with complement activation (Levine et al., 1953; Heidelberger et al., 1941; Eagle et al., 1929).

The use of barbitone-based buffers for complement assays is an anachronism. Given the disadvantages inherent in the use of a buffer that is toxic and heavily regulated, it is surprising that this practice has survived into the 21st century. We here demonstrate that barbitone has no place in complement assays; it can readily be replaced by easily available, safe and relatively inexpensive alternatives that do not impact assay performance.

We chose to test a HEPES-based buffer system based upon: 1. published BiaCore evidence that complement convertases formed efficiently in the recommended BiaCore HBS-P buffer (0.01 M HEPES pH 7.4, 0.15 M NaCl, 0.005% v/v Surfactant

P20) supplemented with excess Ca^{2+} and Mg^{2+} (Harris et al., 2007; Paixao – Cavalcante et al., 2012); 2. the fact that HEPES is a strong buffer in the optimal pH range for complement assays; 3. that it does not chelate divalent cations essential for complement activity and 4. that it is non-toxic. Other equally suitable buffer systems undoubtedly exist.

Our optimal HEPES-buffered saline (HBS; 0.01 M HEPES pH 7.4, 0.15 M NaCl, supplemented with Ca^{2+} and Mg^{2+}) was directly compared with commercial barbitone buffer (CFD) in haemolysis assays in human and non-human serum and serum modified to have low haemolytic activity, in a complementation study to define terminal pathway component deficiencies and in a complement fixation test. In all cases, haemolysis was obtained in HBS and the lytic curves for HBS and CFD were superimposed. Calculated CH50 and AH50 values for the sera tested were essentially identical in HBS and CFD assays (AH50; HBS, 22.4 HU, CFD, 20.7 HU). CH50 values were also identical when serum modified to have ~30% of normal haemolytic activity was tested. In the complementation study, HBS and CFD showed the same results in identifying terminal pathway deficiencies. In the complement fixation test, consumption in the first incubation was the same in HBS and CFD as shown by identical measures of residual lytic activity in the second incubation (haemolysis).

We comprehensively demonstrate that barbitone buffers are not necessary for assays requiring efficient complement activation; a simple HEPES-based buffer yields identical results. We hope that these findings will help laboratories establish robust assays of complement activity and activation that will have broad applicability for diagnosis of disease and monitoring of therapy.

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CONFLICT OF INTEREST

BPM provided advice on complement to Roche. BPM is a consultant to GlaxoSmithKline, all fees are paid to Cardiff University. CLH is a past-employee of GlaxoSmithKline and provides consultancy or advice to a number of external companies; all consultancy income is donated to the University for research. Other

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AUTHOR CONTRIBUTIONS

WMZ performed all the laboratory analyses and wrote the first draft of the manuscript, CLH provided critical expertise on the development of the modified HBS buffer, BPM conceived and planned the study and oversaw the data handling and manuscript preparation, All authors contributed to and have approved the final manuscript.

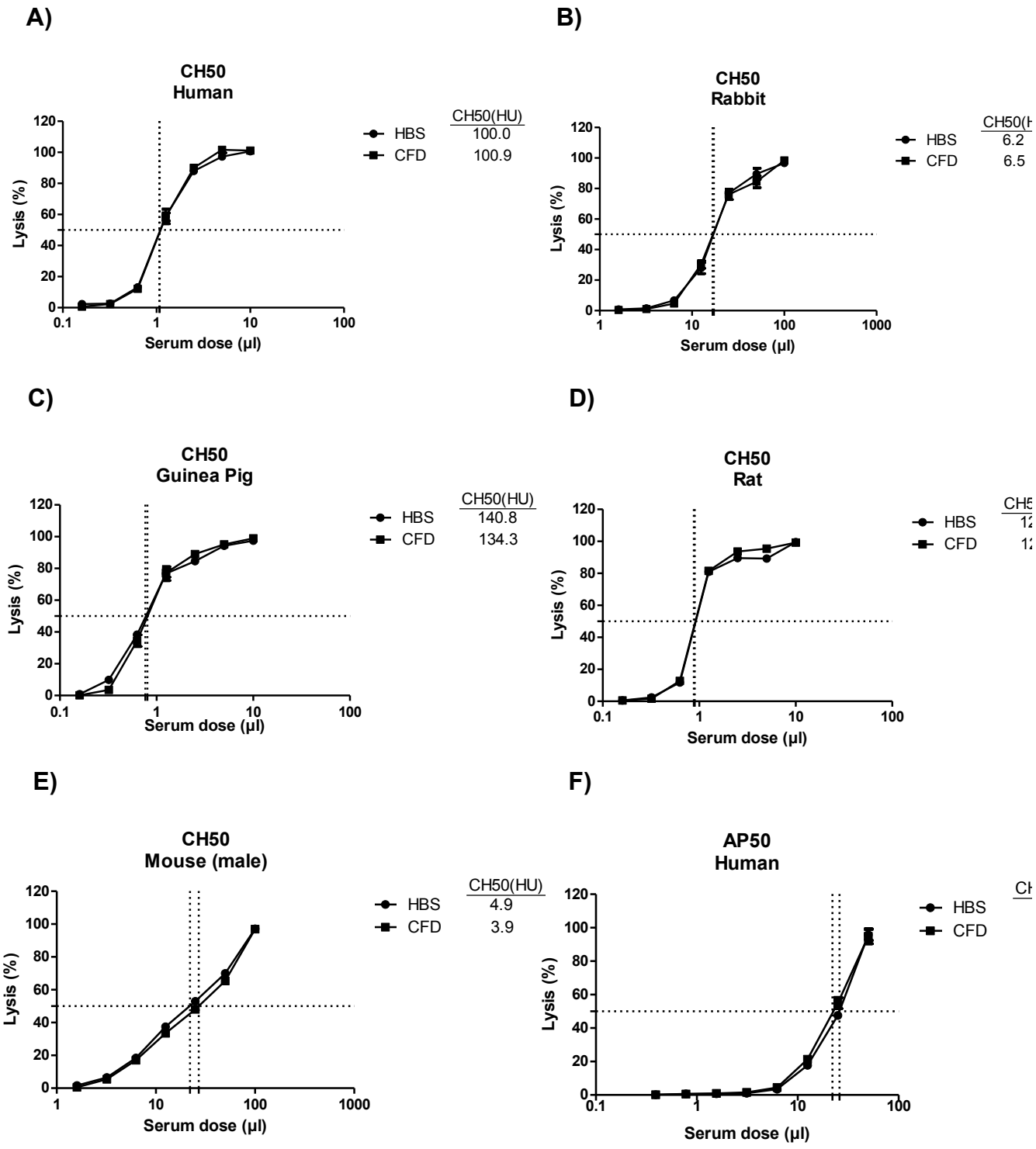
FIGURE LEGENDS

Figure 1. HBS tested in haemolytic assay (CH50, AH50) in comparison to CFD buffer. ShEA in the relevant buffer were incubated with a dilution series of human or animal (rabbit, guinea pig, rat and mouse) sera. With each of the sera tested, the lytic curves obtained in the two buffers were superimposed, and the calculated CH50 values for each serum in the two buffers were the same (A-E). For assessment of alternative pathway activity, HBS supplemented with Mg^{2+} and EGTA was compared to CFD supplemented with Mg^{2+} and EGTA (alternative pathway buffer; APB). Only human serum was tested in this assay (AH50). The lytic curves obtained in the two buffers were superimposed and the calculated AH50 values for the serum in the two buffers were the same (F). CFD; complement fixation diluent, APB; alternative pathway buffer, HBS; HEPES buffer. The assays were performed a minimum of three times with the same outcome.

Figure 2. Compering HBS and CFD under conditions of low complement activity and for confirmation of component deficiency. A) Normal human serum (NHS) or NHS mixed 1:3 with heat inactivated NHS was tested in haemolysis assays diluted in CFD or HBS. Haemolysis was identical in the two buffers under both conditions. B) Sera depleted of C5 (C5D) or deficient in C6 (C6D) or C8 (C8D) were diluted 1:10 in either HBS or CFD. The terminal pathway proteins C5, C6, C7, C8 and C9 were added in physiological amounts to triplicate sets of wells and incubated. The percentage lysis was calculated for each condition and shown as means of triplicates (+/-SE). 100% lysis (NHS and 0.1% Tween20) and 0% (deficient serum alone and no serum) controls were included.

Figure 3. Complement fixation test (CFT) results. To compare the buffers in a complement fixation test, A-positive human erythrocytes (HuE) were incubated in the relevant buffer with group O serum containing anti-A antibodies, either on ice to inhibit fixation or at 37°C. After this incubation, the serum was tested in a classical pathway assay (CH50) in the same buffer to measure residual complement activity, replicate assays are shown (A, B). Incubation on ice in either buffer did not reduce lytic activity of the serum, whereas incubation at 37°C resulted in a ~90% reduction in classical pathway activity in each buffer (CH50; samples incubated at 37°C in HBS average HU= 20.7, in CFD = 22.4; samples incubated on ice in HBS average HU= 111.2, in CFD = 100.5). Controls were subject to the same incubations but with group A serum replacing group O (C, D); no consumption was seen.

Figure 1


A)
CH50
Human
Lysis (%)
Serum dose (µl)
HBS
CFD
CH50(HU)
100.0
100.9
B)
CH50
Rabbit
Lysis (%)
Serum dose (µl)
HBS
CFD
6.2
6.5
C)
CH50
Guinea Pig
Lysis (%)
Serum dose (µl)
HBS
CFD
CH50(HU)
140.8
134.3
D)
CH50
Rat
Lysis (%)
Serum dose (µl)
HBS
CFD
E)
CH50
Mouse (male)
Lysis (%)
Serum dose (µl)
HBS
CFD
CH50(HU)
4.9
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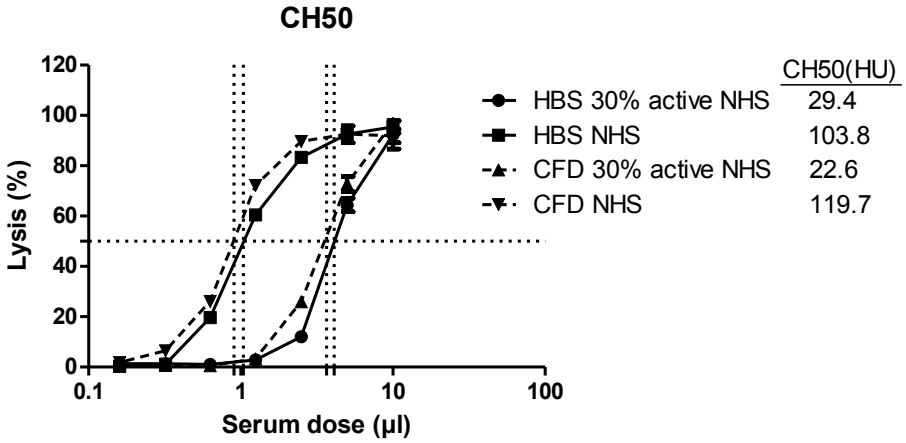
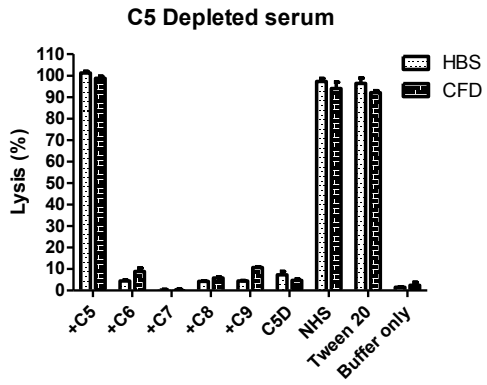
F)
AP50
Human
Lysis (%)
Serum dose (µl)
HBS
CFD

Figure 2

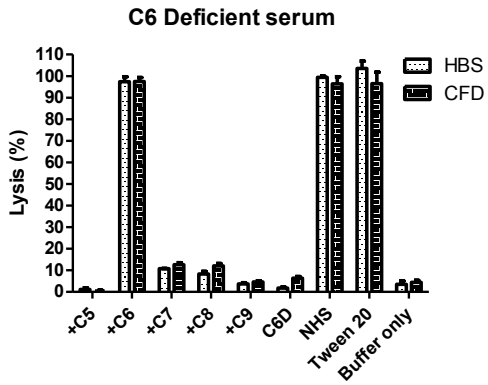
A)



B)



C)



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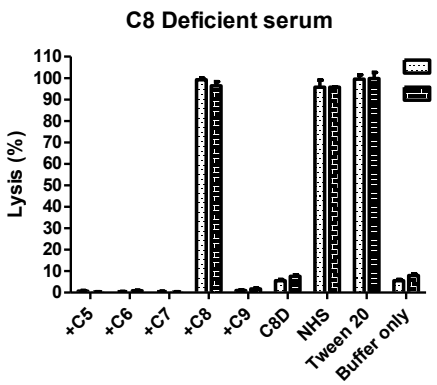
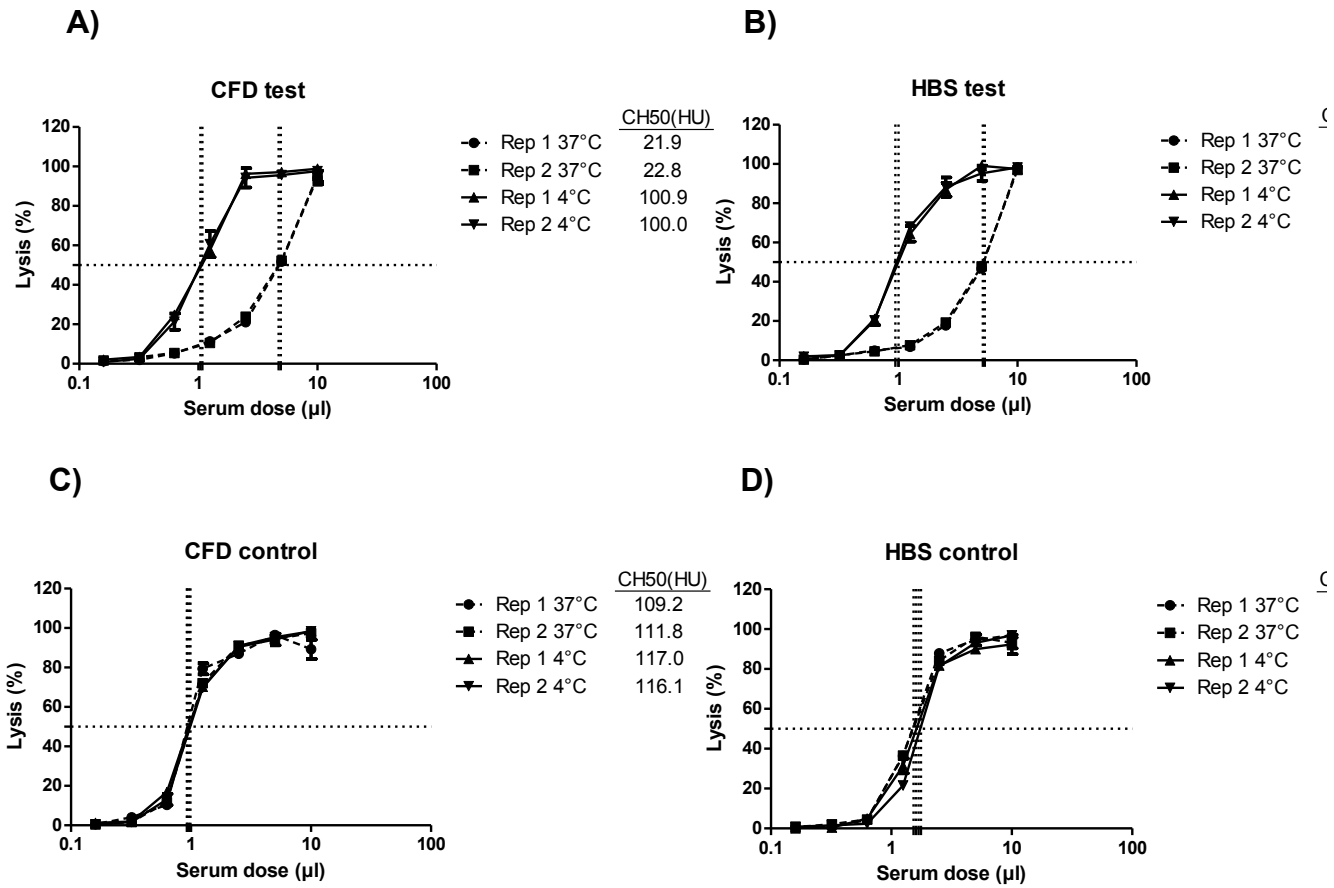


Figure 3


A)
CFD test
Lysis (%)
Serum dose (µl)
CH50(HU)
Rep 1 37°C 21.9
Rep 2 37°C 22.8
Rep 1 4°C 100.9
Rep 2 4°C 100.0
B)
HBS test
Lysis (%)
Serum dose (µl)
Rep 1 37°C
Rep 2 37°C
Rep 1 4°C
Rep 2 4°C
C)
CFD control
Lysis (%)
Serum dose (µl)
CH50(HU)
Rep 1 37°C 109.2
Rep 2 37°C 111.8
Rep 1 4°C 117.0
Rep 2 4°C 116.1
D)
HBS control
Lysis (%)
Serum dose (µl)
Rep 1 37°C
Rep 2 37°C
Rep 1 4°C
Rep 2 4°C